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THE NATURE OF THE BACTERIAL SURFACE

A Symposium of
The Society for General Microbiology
April, 1949

Edited by
A. A. MILES and N. W. PIRIE

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PREFACE

SOMETHING of the nature of the bacterial cell has been known for as long as bacteria themselves have been studied. Gross characters like spores, capsules, granules and flagella were recognized, but it is only in recent years that the finer structure and still more the extraordinary enzymic activity of these minute bodies has attracted the general attention of microbiologists.

The fact that bacteria are so small makes the surface phenomena of particularly great importance. On April 20, 1949, the Society for General Microbiology accordingly held a symposium in London at which chemists, physicists and biologists expressed their views on certain aspects of 'The Nature of the Bacterial Surface'.

The Society took the opportunity of inviting certain distinguished workers from France, Holland, South Africa and the United States of America and is grateful to these scientists for the help they gave in making the symposium a success.

The proceedings are set forth in this volume and, though it is not a complete account of everything that happens on the bacterial surface, some of the more important problems are discussed.

It is hoped that the reader will derive some benefit or get some new idea by a study of the various views expressed.

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THE NATURE OF THE BACTERIAL SURFACE

I

INTRODUCTION

by N. W. PIRIE

THE organizers of this symposium on 'The Nature of the Bacterial Surface' hoped to be able to include papers about every line of evidence that bears on the problem. This has unfortunately not proved possible and it may therefore be permissible in these introductory remarks, by one whose connection with the subject is obviously slender, to mention some themes which might otherwise be neglected in the discussion. It is no longer necessary to apologize for the intrusion of biochemists into what used to be a purely bacteriological field. There is now general recognition that staining reactions, the antigen-antibody reaction, phagocytosis and the phenomena of lysis are all biochemical processes and can only be described fully in biochemical terms. The biochemist needs vigorous biological control if over-confident simplifications, based on ignorance and *naïveté*, are not to lead him astray. The biologist is well aware of this need, but is often less well aware of his reciprocal need if he is to avoid the pitfalls of assuming that because a defined chemical structure gives a manifestation—e.g. maximum absorption at a certain light wave length, fixation of a certain dye or destruction by a certain enzyme preparation—every appearance of the manifestation can be taken as evidence for the presence of the structure. Simple logical fallacies, that in any other field would shock their perpetrators, abound in this field.

In the title the broad word 'surface' was chosen deliberately so as to avoid a probably fruitless attempt to define the precise

differences between the various superficial structures. Any part of the organism that is likely to make direct contact with components of the environment is relevant to our discussion. Discussion must however be limited, except in so far as analogies are used, to bacteria. More is probably known about the surface of erythrocytes or sea-urchin eggs than about any single micro-organism, and the problems involved in all these fields may often be similar. An extension of scope would certainly be logical but it would take us so far afield as to make discussion impracticable.

The surface must be envisaged as a series of shells that merge into one another at more or less clearly defined levels. The outermost layer is an ionic atmosphere held in the neighbourhood by corresponding charges on the cell. Inside this there may be a capsule and this also is a dynamic thing; it is built up of material that is gradually going into solution, and its presence and condition depend on the balance that is struck between its rate of production by the bacterium and its rate of dispersal in the suspending medium. The latter is controlled by many factors among which may be mentioned intrinsic viscosity and the degree of entanglement or cross linkage that exists between the individual particles of the main capsular material or between this material and other components of the capsule. Any process of purification is likely to disrupt the fundamental particles at the same time that it separates them from their chemically dissimilar companions. A purified capsular material is therefore likely to be to some extent degraded. This is not a special peculiarity of the capsule, it is a difficulty that arises whenever large molecules are being separated (Pirie 1940), but it becomes unusually serious when the material that is being handled is as viscous as the capsular materials seem to be.

Anatomical capsules are dispensable parts of organisms. Not only are there non-capsulated variants but the amount of capsule produced by a strain is greatly influenced by the medium on which it is grown. The enzymic removal of a pneumococcus capsule without killing the organism is well known; a comparable removal of what appears to be a dispensable lipid film from *Staphylococcus aureus* has recently been described (Dyar 1948). The next layers are more intimately associated with the viability of the organism, and it is for this reason that they have been distin-

guished from capsules. Outside the cell wall proper—i.e. the structure that is responsible for the mechanical integrity of the cell—there is often a layer that seems to be removable only by a process involving the death of the organism. The lipid layers associated with acid-fastness are well known, but there are mechanically comparable structures in other organisms. Thus killed Gram-positive pneumococci are rendered Gram-negative, without disintegration of the cell structure, apparently by enzymic digestion of ribonucleic acid on the surface (Dubos and Thompson 1938) and *Brucella melitensis* liberates a lipo-polysaccharide from its surface when killed by dilute phenol or chloroform (Miles and Pirie 1939). Structures of this type can be recognized when on the organism if they have characteristic staining reactions, or they can be recovered from the circumambient fluid after liberation from the cell. They differ from capsules mainly in that there is too little to form a layer thick enough to see by the ordinary cytological methods.

The mechanical properties of some bacteria may be due simply to a surface film formed on the interface between cell and medium and formally similar to the film surrounding an oil droplet in water. It is clear, however, that most bacteria have a robust membrane that can be handled by microdissection (Wamoscher 1930). It can be seen separated from the cytoplasm of the bacterium in electron micrograms of intact organisms (Mudd, Polevitzky, Anderson and Kast 1942, van Iterson 1947), and can also be seen as crumpled or curled up fragments in some electron micrograms of organisms undergoing lysis by bacterial viruses (Wyckoff 1948a). The interpretation of electron micrograms is notoriously difficult. Not only are they of necessity pictures of dried preparations, but the processes of separating from the original fluid the material to be investigated, and of mounting it for study, introduce the risk that some of the structures seen will be artefacts. Nevertheless, even if the division from the cytoplasm of the cell is an artefact, it is clear that there is a layer with mechanical properties different from those of the material inside it. Such a layer is not however invariably present. It does not, for example, show on electron micrograms of *Cytophaga myxococcoides* (van Iterson 1947).

This classification of the surface layers under four headings is

obviously neither comprehensive nor rigid, but it may suffice for a consideration of the different types of evidence that can be got. The outermost layer, which is in contact with the ion atmosphere, is a base exchange system and its behaviour with different ions is not only of primary importance in controlling what the ultimate fate of a nutrient, dye or antibiotic will be, but it should also throw light on the nature of the charged groups. Changes in the electrical properties of the surface have been studied in media with different salt contents (cf. McCalla 1940) or after association (Bradbury and Jordan 1942) or combination (Cohen 1945) with more complex agents. Such observations will ultimately lead to a recognition of the nature of the surface groupings involved and they are of the greatest value. Agglutination phenomena, whether brought about by ions or antisera, are also manifestations of an action on the outermost layer. For many years it has been clear that microscopically recognizable structures such as flagella can give specific antisera which are antiflagellar and not anti-somatic, but little progress has been made in identifying the types of chemical grouping involved in these actions. Information on this point would not only shed light on the nature of the surface, but also on the controversial question of the origin and connections of the flagella.

Lysis is the most obvious index that a change has been brought about in the cell wall. The diversity of agents that can bring it about suggests that in different organisms a wide variety of materials are used in cell walls; that in the same organisms there may be independent methods of bringing about lysis; and that the external agent is often initiating an autolytic enzyme action. In the last case the chemical properties of the lytic agent would not throw any direct light on the chemical nature of the linkage that is being affected. The interpretation of the phenomena associated with lysis is also made difficult by the chemical versatility of the more effective lytic agents. Thus detergents such as sodium dodecyl sulphate are often thought to be lytic because they produce surface tension changes, and can affect lipid linkages. This may well be the mechanism, but it does not necessarily follow. Dodecyl sulphate affects lipoproteins, but it will also combine with, or disrupt materials as diverse as simple proteins, nucleoproteins, starch, and chlorophyll. Lysis may be the result

of a linear split in the limiting membrane; it may therefore be caused by the opening of such a small number of links that the change would not be detectable analytically. This factor may explain the small amount of material that sometimes seems to be involved. In other cases the lytic action takes place over the whole surface of the cell, for it is preceded by a general increase in transparency (Boasson 1938, Doermann 1948). Lysozyme is probably the best understood of the lytic agents, it digests an insoluble amino-polysaccharide, which appears to be present in all organisms susceptible to the lytic action of the enzyme (Epstein and Chain 1940) and in other organisms killed without lysis. It is unlikely that there is only one lysozyme or that the substrate in all susceptible organisms is identical, but the conclusion seems to be inescapable that there is a group of mucopolysaccharides whose presence in certain organisms is essential for the stability of the cell membrane, and that these can be destroyed enzymically. Specific enzyme preparations are tools that will be of the greatest value in identifying structures in the capsule or cell wall of bacteria because their field of action is more circumscribed than that of other lytic agents such as detergents, glycine (Maculla and Cowles 1948) or urea. It must be remembered, however, that specificity is a negative property of an enzyme preparation and that it cannot be demonstrated. All that can be demonstrated is the failure of the preparation to attack specified substrates and the possibility always remains open, that the action of an enzyme preparation on a bacterial structure is due to an unsuspected activity. Too much emphasis cannot be laid on the fact that the crystallinity of the enzyme preparation is irrelevant.

Bacterial viruses have already given some information about the bacterial surface and more is coming quickly from this rapidly developing field. Three phases can be distinguished in the action of a virus on a cell; adsorption, a local quasi-enzymic action that may lead to penetration of the cell wall, and multiplication of the virus. It is well known that the first and second process can proceed without the third, and it is these processes that are obviously involved with the surface.

It has long been thought that polysaccharides on the cell surface play a part in the attachment of viruses and positive

evidence, with both bacterial and animal viruses, has been accumulating during recent years. Thus incubation of infected *Bacillus megatherium* (Wollman and Wollman 1936) or *Vibrio cholerae* (White 1937) with lysozyme from egg white liberates the virus, and preliminary incubation of *B. megatherium* (Pirie 1940) with the same enzyme prevents adsorption of virus on the bacterium. The linkage can also be affected by what is apparently competition between polysaccharides; in the early work (e.g. Burnet and Freeman 1937) union was inhibited by a bacterial polysaccharide and recently (Maurer and Woolley 1948) virus has been released from infected *Bacterium coli* by apple pectin without lysis of the bacterium. All this evidence is indirect, but it is in agreement with what evidence is available about the union between viruses and animal and plant tissues, and erythrocytes.

The recognition of enzyme activities in virus preparations has an intrinsic interest to the virus worker; the lysis or other change that such an enzyme brings about in an infected bacterium is relevant to our discussion. Very few enzyme activities have been found associated with purified virus preparations. If an apparently enzymic action is brought about by a virus, the probability will therefore be enhanced that the effect is in fact due to whatever enzyme may be identified in the virus. Lysis is by no means a simple criterion of enzyme action. It may be due either to a virus enzyme or to the activation by the virus of lytic enzymes which are known to be present in many bacterial cells; in no case has there been a clear differentiation between these possibilities. A lysin acting on *Bact. coli* can be split off from a virus by ultrasonic or ultra-violet irradiation and this will work on killed cells (Anderson 1945) in a manner comparable to lysozyme. If an enzyme is responsible for this action it has a different range of specificity from lysozyme for it fails to lyse *Micrococcus lysodeikticus*; the recognition of its substrate would be of the greatest interest.

Studies of virus multiplication have not so far given any information about the nature of the bacterial surface. We have already seen that lysis can take place without virus multiplication; there is also evidence which suggests that virus multiplication can take place without lysis (e.g. Maurer and Woolley 1948, Price 1948). Under these conditions the multiplication itself has

presumably taken place on the surface, for it would be unreasonable, in the absence of compelling evidence, to assume two passages by particles as large as viruses through cell membranes which are known to be able to retain the smaller metabolites. It is tempting to wonder whether all virus multiplication takes place on the surface and no evidence seems positively to exclude this possibility, but there is a suggestion (Wyckoff 1948 b) in some electron micrograms that multiplication takes place in the bacterial protoplasm released from the cell on lysis. In these experiments the partly lysed bacteria were formolized and washed repeatedly on the centrifuge; it may well be that the virus particles and the protoplasm became associated secondarily during these operations. The possibility of multiplication on the surface has been emphasized, not only because it suggests a new direction from which our subject can be approached, but also because the mechanism of virus multiplication is more amenable to experiment outside than it is inside the cell. If external multiplication can be conclusively demonstrated it will have the most far-reaching consequences, for it will become reasonable, both in unicellular and in multicellular organisms, to keep the cell wall in mind as a possible site for the multiplication of other viruses and for the synthesis of those macromolecules, such as antibodies, that are later dispersed throughout the tissues of a complex organism.

The cell was originally conceived as a bag with a wall that had mechanical, or at most osmotic, duties. Other capacities, offensive, defensive and synthetic are slowly being attributed to it. Our physiological knowledge develops along the same lines as our engineering practice. At first each system or structure is independent, and simply attached to the others; later there is an amalgamation. Thus part of an aeroplane wing becomes the petrol tank, and the wheels tuck into another part. The more successful organisms have been surviving by virtue of this telescoping technique for rather a long time and it will not be surprising if they have achieved some remarkable feats of condensation.

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II

THE SURFACE STRUCTURE OF *SHIGELLA SHIGÆ* AS REVEALED BY ANTIGENIC ANALYSIS

by W. T. J. MORGAN

A STUDY of the communications which are to form the basis of this symposium on 'The Nature of the Bacterial Surface' shows clearly that there are many lines of approach to the problem. This communication deals with one rather narrow aspect, and is concerned with the identification of surface molecules by means of specific serological reactions. The use of this technique and the conclusions which may be drawn from it are strictly limited.

Bacteria, although unicellular organisms, are complex structurally and are composed of many different chemical constituents. A study of the nature and position of certain of these constituents is greatly facilitated by their specific immunological properties which allow them to be detected and their concentration measured in the presence of the other substances which are components of such complex biological systems. There is little doubt that in the intact bacterial cell the different cellular materials bear a spatial morphological relationship to each other and, therefore, by selecting an antigenic component of the cell surface, it is possible to gain an insight into the nature of at least a part of that surface. It might reasonably be expected that the antigenic constituents normally components of the cell surface would, when inoculated into animals in the form of living organisms or intact cells, induce the formation of antibodies more readily than other antigenic material residing deep in the bacterial cytoplasm. Indeed, it is most probably for this reason and because these substances are better antigens than other constituents of the cell, that the formation of antibody essentially specific for an organism occurs so readily when animals are immunized with it and why the well-known methods of serological analysis, based on antisera pro-

duced in this manner, can be applied for the differentiation and classification of organisms within, for example, the numerous and complex *Salmonella* and *Shigella* species. In choosing an immunochemical approach to our problem, however, it is evident that a great deal is to be gained by selecting for study an organism which possesses a single dominant antigen that appears to behave as a surface antigen and whose serological characters are simple and fairly well understood. Fortunately the Gram-negative bacillus *Shigella shigæ* falls into this category and has already been studied in considerable detail. Although the results of these investigations have arisen largely from an experimental approach not primarily designed to contribute to the problem under discussion to-day, nevertheless they can be used for this purpose and offer a solid basis on which to plan further work more specially designed to contribute to our knowledge of the surface structure of the bacterial cell.

It will be the purpose of this communication to present evidence for the view that material characteristic of *Sh. shigæ* and known as the *O* antigen, is a component of the bacterial surface. Observations will then be considered which indicate that the method used to isolate the *O* antigen from the cell surface causes no significant change in its dominant immunological properties, that the material recovered is essentially *O* antigen, and that it is a relatively stable polymolecular complex composed of polysaccharide, phospholipin and conjugated protein residues. Finally, some evidence for believing that the antigen is specifically orientated at the cell surface will be presented. Passing reference will be made to similar studies involving other Gram-negative organisms belonging to the family Enterobacteriaceæ when evidence derived from these organisms contributes directly to the discussion.

Arkwright (1920, 1921) first observed that strains of *Sh. shigæ* gave rise to two different colonial forms when grown on solid media and that the organisms comprising the colonies behaved differently when suspended in saline or in the ordinary broth media. One type of colony was smooth, glistening, round with an even edge and on transferring to saline or broth gave rise to stable suspensions. These organisms are the Smooth forms. If a small amount of this smooth culture is inoculated into rabbits

or horses, a specific antiserum capable of agglutinating the suspension or a suspension derived from the Smooth form of another strain of *Sh. shigæ*, is readily produced. The other type of colony grows in a form which is larger, flatter, irregular in shape and possesses a granular surface. The organisms derived from these so-called Rough colonies, though possessing many of the characteristic properties of the original Smooth strain such as fermentation reactions, toxin production, etc., fail completely to induce the formation in experimental animals of the agglutinins specific for the Smooth form of *Sh. shigæ* and they give unstable suspensions in saline or broth. It is evident, therefore, that the Smooth-Rough change which has taken place is accompanied by loss or inactivation of a specific antigenic component normally associated with the Smooth form. There appears to be a strict correlation between the Smooth form and ability to synthesize and accumulate the specific *O* antigenic material, for extraction of the Smooth and Rough organisms with hot dilute acetic acid reveals at once that the *O* specific and reactive component of the antigenic complex of the Smooth bacilli, the specific polysaccharide hapten, is absent from the Rough organism; it is not merely present in an inactive form.

The results of studies of specific serum agglutination contribute reasonably trustworthy evidence that some antigens are surface components of the bacterial cell. Direct microscopic examination of the agglutination of the *O* forms of certain of the Gram-negative members of the coli-typhoid-dysentery group of organisms by homologous sera shows that the organisms are held together by attractions acting through polar contacts (Pijper 1938). A mutual attraction of this kind is observed with living cultures of the Smooth or *O* antigen-containing form of *Sh. shigæ* in the presence of homologous serum and it appears that the antibody is reacting specifically with its antigen which it picks out as a surface component of the intact cell. No agglutination takes place when the corresponding Rough strain, which is without the specific *O* antigen, is used. The characteristic polar attractions observed in *O* agglutination might be considered to indicate that these areas of the surface carry the major part of the *O* antigen.

Further evidence in support of the belief that the *O* antigen of *Sh. shigæ* is a component of the cell surface arises from observa-

tions made during the isolation of the antigen from the intact organism. The various techniques that have been introduced to isolate the antigenic material differ greatly. Some, such as treatment of the organisms with trypsin (Douglas and Fleming 1921, Raistrick and Topley 1934) involve almost complete solution of the organism and are therefore quite unsuitable for surface studies. Other methods employed, such as treatment of the organism with trichloroacetic acid (Boivin and Mesrobeanu 1933), give rise to complete or partial destruction of the structure of the cell and extract much unspecific somatic material together with the specific *O* antigen. Probably the method of choice is one which utilizes a neutral solvent that will extract only the *O* antigen and leave behind all other constituents of the bacterial surface, the cell cytoplasm and the intracellular structures. Morgan (1937) observed that the specific polysaccharide of *Sh. shigæ* which had been isolated earlier (Morgan 1931, 1936) was soluble in a number of anhydrous polyhydroxy organic solvents of which ethyleneglycol, diethyleneglycol, trimethyleneglycol, propyleneglycol and glycerol were satisfactory reagents and could be used to fractionate the polysaccharide by precipitation with acetone or ethanol. Further experiments showed that other polysaccharides were soluble in these solvents and that a number of proteins, such as horse serum protein, edestin, egg-albumin and casein, were insoluble. The knowledge that the specific polysaccharide haptens were major components of the *O* antigens of many Gram-negative bacteria (Boivin and Mesrobeanu 1933, Raistrick and Topley 1934, Mesrobeanu 1936) suggested that the complete antigenic complex might also be soluble to some extent in one or other of these solvents. The results of a few simple experiments showed that this was true and indicated that of the solvents examined anhydrous diethyleneglycol was the most suitable for the isolation of the *O* antigen of *Sh. shigæ*. This solvent possesses a number of properties, other than its capacity to dissolve the *O* antigenic material, which are very desirable qualities in a reagent that is to be used for the removal of a labile antigenic complex. Diethyleneglycol is neutral in reaction, miscible in water, alcohol and acetone in all proportions, free from nitrogen, readily eliminated by dialysis and can be used at low temperatures. These properties made it possible to develop

a method which employed diethyleneglycol for the isolation of the *O* antigen and avoided the use of an acid or alkaline medium. The process could be carried out at 0° or lower temperatures and would thus minimize the risk of changing the antigenic complex during its isolation. Furthermore, the use of this anhydrous organic solvent with dry preparations of *Sh. shigæ* and low temperatures reduced or eliminated completely the activity of enzymic processes which might otherwise destroy or modify the antigenic complex. If it is assumed that the bacterial enzymes causing autolysis and destruction of the *O* antigen are of protein nature then it is probable that diethyleneglycol will fail to dissolve many of these enzymes even if they are situated at the cell surface.

Extraction experiments with this solvent showed that, after the removal of the bacterial bodies by high speed centrifugation and passage of the diethyleneglycol extract through a Berkefeld filter candle, about 10 per cent of the weight of the dry *Sh. shigæ* culture employed passed into solution. Three successive treatments of the dry organisms with the solvent removed approximately 60, 25 and 12 per cent respectively of the total antigen extractable under the experimental conditions used. The results of other experiments which re-extracted the diethyleneglycol-treated organisms with less selective solvents, such as 90 per cent phenol or 50 per cent aqueous pyridine showed that a few per cent only of additional antigen was recovered. Five to seven per cent by weight of the dry bacteria is recovered as purified material and it would appear that this figure represents approximately the amount of material in the organisms extractable with diethyleneglycol. A control extraction process must be included, however, if these figures are to have significance in the present discussion. The control must be one which will reveal the amount of material extractable under identical conditions by the solvent from a preparation of the rough variant of *Sh. shigæ*, i.e. from Shiga bacilli which lack the specific *O* antigenic complex. This has been determined and the quantity of material recovered is quite small; it represents about 0·2 per cent of the dry weight of the organisms used. It is, therefore, apparent that whatever the nature of the material that is extracted from the Smooth organism it is almost completely absent from the cells of the Rough form. The cells of the Rough *Sh. shigæ* are, as far as can be ascertained, similar in

general chemical make-up to the Smooth form except for the presence of the *O* antigen complex.

After thorough extraction the bacilli, examined microscopically, are morphologically indistinguishable from the unextracted organisms. The staining characteristics are likewise largely unchanged. These facts indicate that the cell has not been so grossly damaged as to involve disintegration and from the weight of the cells remaining after the extraction is complete it is possible to state that there has been no extensive liberation of the cell contents and that therefore the main bulk of the cytoplasmic material remains after the specific antigen has been removed by the extraction process.

The staining techniques used do not reveal the presence of capsules in *Sh. shigæ* but this is not surprising for if the capsule was composed of the *O* antigenic complex alone the quantity present, almost certainly less than 10 per cent of the dry weight of the organism, would be insufficient to give a capsule of detectable thickness when seen under the microscope. For the purposes of detecting the presence of the *O* antigen as a surface component the so-called 'capsule swelling reaction' of Neufeld (1902) might be employed as this renders the capsular material more dense and more readily visible owing to its increased staining capacity without, however, necessarily increasing its size.

The next step in our inquiry is to show that the material extracted by diethyleneglycol from the Smooth form of *Sh. shigæ* is the *O* antigen. This leads to a consideration of any changes which might occur in the antigenic material during its isolation from the living or killed but intact organism. It is well known, but all too frequently ignored, that bacterial antigens are labile complexes and that methods for their isolation should whenever possible involve procedures which avoid exposure of the material to degrees of acidity or alkalinity which are far removed from neutrality. It is probable that any changes in structure of the *O* antigen arising during the isolation process would lead to the development of modified immunological characters which would be detected in the subsequent immunological examination of the material. The active immunization of rabbits by the injection intravenously of amounts of the diethyleneglycol extracted material of the order of 5–10μg causes an immediate and powerful

response of specific *Sh. shigæ* agglutinins and precipitins (Morgan 1937) and protective antibody (Steabben 1943). The subcutaneous injection of two doses of 100μg of the material into man likewise induces the formation of 'Shiga' agglutinins and the specific antiserum so obtained protects mice against infection with a fully toxigenic strain of *Sh. shigæ* (Morgan and Schütze 1943). The material injected into mice (Schütze 1943) and horses (Morgan 1937) also gives rise to the formation of specific agglutinins. The *O* antibody induced by the inoculation of experimental animals with living or killed *Sh. shigæ* bacilli can be completely removed from the serum as a specific antigen-antibody complex by the material extracted from the homologous organism by diethyleneglycol. The specific *Sh. shigæ* agglutinin induced in the rabbit by the diethyleneglycol extract is likewise completely absorbed by treatment with a suspension of the homologous Shiga bacilli. There seems little doubt, therefore, that the material extracted by diethyleneglycol from the Smooth form of *Sh. shigæ* is a powerful and specific antigen and gives rise to an antibody indistinguishable from the *O* antibody induced by the Smooth form of the living organism. We may conclude, therefore, that the immunological properties of the *O* antigen have not been significantly altered by the process of isolation.

We now turn to a more intimate consideration of the material obtained by diethyleneglycol extraction. It seems probable that large labile complexes of this type will form weak associations with quite unrelated tissue components co-existing in the common environment of the cell. If this association takes place, and there is strong evidence from other fields of study that it does, there arises the problem of deciding at what stage in the purification procedure the *O* antigenic complex is free from these additional substances and can be termed homogeneous. There is no doubt that some loosely bound material, especially substances of relatively low molecular weight, can be removed by simple procedures which appear to have no deleterious effect on the specificity and antigenicity of the material. There comes a point, however, at which the removal of a material causes the loss of antigenic power and one must conclude that the substance so removed is an essential part of the antigenic complex. In view of our limited knowledge of the behaviour of these exceedingly

complex biological materials it is perhaps more profitable at the present time to study the detailed chemical structure of the simplest antigenic unit and to leave for the moment the problem of the nature of the material in its native state.

In any event, after simple fractionation from aqueous solution by cold organic solvents or inorganic salts, it is found that the major part of the material is of fairly constant composition, and solubility studies, using several grams of the material, indicate that it is not grossly heterogeneous. Gentle hydrolysis (pH 3·5) of the antigenic complex shows that it is composed of residues of polysaccharide, phospholipin and conjugated protein, the prosthetic group of which has not been identified. The hydrolysis products are no longer antigenic, but the polysaccharide retains the dominant specificity of the intact antigen and is entirely responsible for the specific serological character of the whole antigenic complex and of the intact *Sh. shigæ* organisms (Morgan 1937, Morgan and Partridge 1940).

The amounts of each of the constituents recovered after acid hydrolysis, when added together, make up 80–90 per cent of the original material and indicate that other components, if they exist, must be quite small in amount. The polysaccharide component ($[\alpha]_{5461}+110°$; N, 1·7 per cent) represents 50–60 per cent of the material, contains N-acetylglucosamine, D-galactose and L-rhamnose; other sugars may be present. The phospholipin ($[\alpha]_{5461}+12°$; N, 1·8 per cent; P, 3·9 per cent) is present to the extent of 10 per cent or so of the antigen and contains palmitic acid, oleic acid and α-glycerophosphoric acid. The nitrogenous constituent of the phospholipin has not been examined. The phospholipin component is not removed by thorough extraction of the unhydrolysed antigenic complex with alcohol-ether mixture, acetone, pentane or chloroform. The protein-like component is an acidic material which contains about 11·5 per cent N and 1·0 per cent P and is *lævo*-rotatory, $[\alpha]_{5461}-48°$.

The degradation of the antigenic complex by more gentle means can be accomplished by the use of cold anhydrous formamide (Morgan and Partridge 1939). In solution in this neutral, highly polar solvent, the bonds which hold the component residues together break, dissociation of the complex occurs and, as a first step, the separation of phospholipin from the original

antigenic material is brought about. This process can be accomplished more readily by treatment of the antigenic material at 0° with an alcohol-ether mixture which contains 0·5 per cent HCl (Miles and Pirie 1939). Material can be obtained in this way which is not sedimented under a force of 140,000*g* for 90 minutes, is composed of polysaccharide and protein molecules and is a powerful antigen in doses of 10μg. One may conclude, therefore, that the phospholipin is not essential for the manifestation of antigenicity. More thorough treatment with formamide gives rise to complete dissociation of the phospholipin-free, conjugated polysaccharide-protein moiety and it is possible by this process to obtain the specific polysaccharide in a form that is probably closely similar to, or identical with, the material as it occurs in the native antigen. The isolated undegraded polysaccharide and conjugated protein components are not antigenic when measured by their ability to induce in the rabbit the formation of the specific *O* agglutinins for *Sh. shigæ* and, so far as is known, the polysaccharide-conjugated protein complex is the simplest unit which will act as an antigen and engender specific *O* agglutinins, precipitins and protective antibodies in all the usual experimental animals. The antigenic properties of the material are not destroyed on exposure to the action of crystalline lysozyme or ribonuclease. The action of trypsin on the original antigenic complex, however, has made it possible to isolate the phospholipin-polysaccharide moiety and to demonstrate that this complex is without significant antigenicity. Other procedures, which need not receive more than a passing reference, such as treatment of the diethyleneglycol extracted material with 90 per cent phenol or cold dilute alkali (Morgan and Partridge 1941) are likewise able to bring about the separation of the residues present in the antigenic complex.

The results obtained from this experimental approach have helped us to build up a mental picture of the *O* antigenic complex of *Sh. shigæ* and to consider the possibility that the antigenic material as it exists on the cell surface is not a rigid chemical structure of fixed composition, but consists of a labile molecular aggregate which possesses an essential component—the specific polysaccharide—of definite chemical structure and of fixed composition, which determines the dominant immunological

specificity of the antigen, together with other loosely bound constituents. Of these the conjugated protein material is indispensable if an antigenic complex is to result and presumably always accompanies the polysaccharide component when the latter is present as the specific antigen. The phospholipin and other components undoubtedly present in relatively small amounts in the native antigenic complex are not essential for antigenicity. It is of considerable interest to find that this surface antigen, after removal of those residues which are loosely associated with it, contains no nucleic acid or material which absorbs light in the ultra-violet region 210–280 mμ. It would appear, therefore, that nucleic acid, pyrimidines or purines are absent and are not necessary for the manifestation of antigenic properties characteristic of the *O* antigenic complex of *Sh. shigæ*. One cannot conclude from these observations that nucleic acid is not a component of the cell surface, unless it is known that the *O* antigen covers the whole of the bacterial cell.

Having brought forward some reasons for believing that the bacillary surface of the Smooth form of *Sh. shigæ* carries the *O* antigen which, as we have seen, is a complex of polysaccharide, protein and phospholipin, it is expedient to consider if there is evidence for a specific orientation of this functional aggregate when it is present at the cell surface. From what we know concerning the spatial configuration of the components of artificial antigens (see Landsteiner 1945), which are themselves responsible for the dominant specificity of these substances, it seems probable that the determinant group, or an essential portion of it, must protrude from the general contour of the antigenic complex. The polysaccharide component of the *O* antigen of *Sh. shigæ* is, on the basis of a great deal of immunological evidence, some of which has been discussed earlier, responsible for the characteristic serological specificity of the antigen and we must conclude therefore that it is the structural orientation of this residue when fixed *in situ* at the cell surface, rather than that of the other residues in the antigenic complex, that is responsible for its functioning as a determinant structure. As such the whole polysaccharide, or the serologically important part of it, is almost certainly the most prominent feature of the surface structure of the intact organism.

Incidentally, an interesting observation, pertinent to the present discussion, was made many years ago by White (1929) who showed that Smooth cultures of certain of the coli-typhoid-dysentery group of organisms give an immediate coloration with Molisch's reagent whereas the Rough form fails to react or reacts poorly. On the basis of the results of these simple qualitative chemical tests, involving as they do the relative behaviour of the two forms of the organism, it would seem that a polysaccharide substance is an important component of the cell surface of the Smooth form, i.e. of a form which contains the *O* antigen.

When considering, especially in relation to specific serological reactivity, the forms which a complex molecule can assume, it is necessary to consider the effect produced by the surrounding structures on the particular configuration normally taken up by the molecule when it is fixed at the cell surface. In this instance one may inquire if there is any evidence to indicate the formation of an altered polysaccharide structure and, in consequence, of the development of a modified serological specificity due to changed local conditions of this kind. The serological data available contain no evidence that the polysaccharide is serologically modified and one may assume that this residue possesses the same structure whether it is a component of the bacterial surface or is a free unit in solution.

Are the protein and phospholipin components of the *O* antigen reactive as surface structures or are these substances so arranged at the cell surface that their molecules are covered by the polysaccharide residues? It is known that the conjugated protein component of the *O* antigen is itself antigenic and gives rise to the formation of homologous precipitins which are, however, devoid of Shiga specificity. Antibodies produced against the whole organism are practically free from precipitins against the conjugated protein and fail to fix complement in its presence. Guinea-pigs passively immunized with these sera show no sign of anaphylaxis when given conjugated protein intravenously. It must be assumed, therefore, that this component is not an actively antigenic structure when present as a constituent of the *O* antigen. Furthermore, anticonjugated-protein rabbit serum fails to agglutinate *Sh. shigæ* to a significant titre and, therefore,

it would again appear that the conjugated protein is not a prominent surface structure of the intact organism.

White (1929) observed that suspensions of Smooth bacilli of the coli-typhoid-dysentery group of organisms develop, on treatment with Millon's reagent, a yellowish tint only, whereas the corresponding Rough suspensions quickly assume the deep red-pink coloration of the positive test. Smooth bacilli from which the specific polysaccharide has been removed by extraction likewise behave as do the Rough organisms. These observations may be considered to indicate that the conjugated protein of the *O* antigen or, for that matter, any other protein constituent of the cell, is not a major component of the surface of the Smooth organism.

There appears to be no immunochemical evidence that will help us to decide whether or not the phospholipin component of the *O* antigen is a true surface structure of the Smooth organisms. The *spontaneous* agglutination of the Rough form of *Sh. shigæ* when suspended in broth or saline is most probably due to a lipoid substance at the bacterial surface, but there is no evidence that this material is the same as the phospholipin component of the *O* antigen. The material can be removed by extracting the organisms with alcohol (White 1927), after which treatment stable suspensions of the bacilli are obtained. A similar material is present in the Smooth organisms but here it does not appear to be a dominant surface component for it fails to determine the character of the cell surface. Stable suspensions of the Smooth organisms are formed in spite of the presence of the lipoid material, which can be readily recovered by extracting the bacilli with alcohol. It seems probable that although this lipoid material is a surface component of both forms of *Sh. shigæ* its capacity to function as an agglutinating agent for the Smooth organism is inhibited by the presence of the spatially prominent polysaccharide component which prevents the lipoid areas of the cell surface from coming into sufficiently close contact to give rise to the attraction which brings about spontaneous agglutination. Such neighbouring polysaccharide structures would presumably not influence the removal of the lipoid with alcohol.

Schütze (1943) showed that the agglutinability of Shiga strains by specific anti-serum varies considerably and that suspen-

sions which are resistant to specific agglutination can be rendered agglutinable by heating at 100° for 30 minutes. The results of more recent work by Stuart, Feinberg and Feinberg (1948) indicate that 'thread' agglutination is a manifestation of *O* inagglutinability and that the substance responsible for the inhibition of *O* agglutination is thermostable, can be easily dissociated from the cell and is most probably a phospholipin constituent of the cell surface. This material appears to be a common component of many members of the family Enterobacteriaceae irrespective of the antigenic character of the organisms within the family. Its presence must therefore be recognized when considering the nature of the components of the cell surface. The fact that the less agglutinable strains of *Sh. shigæ* possess a larger content of *O* antigen than do the more agglutinable forms suggests that the phospholipin associated with the specific antigen might play some part in the phenomenon of *O* inagglutinability.

No attempt has been made to treat the problem under discussion in general terms, but it should be noted that materials similar in type and function have been obtained from *Brucella melitensis* (Miles and Pirie 1939), *Br. abortus* (Paterson, Pirie and Stableforth 1947), *Salmonella typhi murium* and *S. typhi* (Topley, Raistrick, Wilson, Stacey, Challinor and Clark 1937, Henderson and Morgan 1938, Freeman, Challinor and Wilson 1940, Freeman and Anderson 1941, Freeman 1942) and from the most important types of *Sh. flexneri* by Goebel, Binkley and Perlman (1945). It would appear, therefore, that within the Gram-negative group of organisms the so-called *O* specific antigens are composed of polymolecular aggregates built up from residues of polysaccharide, phospholipin and protein and although the component residues are only loosely bound together, these important functional aggregates possess sufficient stability under ordinary conditions to behave as independent molecular species and exist as such on the bacterial surface. It seems probable that the facts disclosed by these and other investigations (Boivin 1946) could be used to establish the position and nature of the dominant antigenic component of the selected organism in the same manner as I have attempted to do for *Sh. shigæ*.

It might appear that evidence as to the nature of a major

constituent of the bacterial surface could have been obtained more readily from a study of the Pneumococcus (Type III). The virulent form of this organism has a well-developed capsule which is of such a size as to be readily visible under the microscope. The capsular material, which covers the organism completely and to a considerable depth, was isolated and its chemical nature established many years ago (Heidelberger and Goebel 1927). The material is a polymer of aldobionic acid (glycuronic acid 1 : 4 glucose) units joined together by glycoside linkages which involve the reducing group of the glucose component and the hydroxyl group attached to the third carbon atom in the glucuronic acid molecule (Reaves and Goebel 1941). The capsular material can be removed from the living organism by means of a specific enzyme and the virulent type-specific organism changed to a living avirulent form which is devoid of a capsule, a changed condition which lasts only so long as the enzyme is present. Transferred to a suitable medium devoid of enzyme, the cocci rapidly again develop a capsule which is composed of the polysaccharide characteristic of the Type III pneumococcus (Dubos 1932, 1939, Dubos and Avery 1931). It seems clear, therefore, that with the pneumococcus Type III at least the cell surface is completely covered with a polysaccharide of known structure. The reason I have not considered this organism to be a particularly suitable one from which to obtain the information needed for to-day's discussion is because in this instance it is difficult to determine to what extent the capsular material is truly a component of the cell surface or, indeed, if it is not entirely a simple secretory product which accumulates at the surface by virtue of its viscous nature. Thorough washing with neutral aqueous solvents reduces considerably the size of the capsules without killing the cocci and it is to be noted that, whereas the isolated and apparently homogenous polysaccharide material is without antigenicity when tested in rabbits, the living encapsulated organisms, after thorough washing to remove most of the non-antigenic capsular material, continue to induce the formation of type specific agglutinins and precipitins in this animal. These results indicate, therefore, that some of the polysaccharide is combined with another component of the cell and one may conclude that this antigenic complex is a component of

the true surface of the cell. Little is known about the nature of this additional material for the complete Pneumococcus (Type III) specific antigen has not been isolated and its component residues examined. It is known, however, that during autolysis (Dubos 1937) this antigen is destroyed, but the polysaccharide hapten remains and retains completely its original serological characters. In any event there seems little doubt that a polysaccharide composed entirely of aldobionic acid units covers the surface of the virulent form of this organism. Hershey (1940) found that the amount of antibody actually absorbed by an encapsulated cell is considerably in excess of that required to form a single surface layer of closely packed molecules. He suggests that the reason for the great excess of antibody taken up may be the permeation of the viscous polysaccharide mass by the antibody molecules. This would certainly arise if the surface of the capsular material is composed of projecting fibrous structures of the specific polysaccharide to which the antibody molecules become attached.

To summarize the conclusions reached concerning the nature of the surface of *Sh. shigæ* it would probably be fair to say that the Smooth form of the organism has at least a part of its surface made up of a material composed of polysaccharide, protein and phospholipin residues. This polymolecular aggregate has sufficient stability to exist as a functional unit and is identified as the specific *O* antigen of *Sh. shigæ*. The immunological properties of the intact cell, the isolated *O* antigen and its component residues indicate that the polysaccharide is a major component, constitutes the outermost molecular layer and probably protrudes from and dominates the general surface configuration. The protein moiety of the *O* antigen, judging from its immunochemical behaviour, is most probably not a surface molecule. Similarly, the phospholipin component of the *O* antigen and the other fatty constituents external to the cell wall cannot be considered as prominent structures of the molecular surface.

The experimental observations which have been considered are in many respects inadequate and, therefore, conclusions based on them must be accepted with caution. Nevertheless, it is hoped that this immunochemical approach to the problem will stimulate others to isolate and examine the main chemical and immuno-

logical features of substances considered to be surface components on the basis of the reactivity of the living cell with specific immunological reagents. The understanding we seek of the bacterial surface is of the composition, the configuration and the spatial interrelationships of the components at a molecular level. It is certain that a clearer perception of the surface structure, which must surely arise from a full discussion of the subject, will be helpful in understanding the function and behaviour of cell surfaces.

Discussion

Prof. M. Stacey asked whether Dr. Morgan thought that some parts of the *O* antigen were more deeply buried than others and so were masked, and whether it was in this way that a complex molecule was able to act as a specific polysaccharide antigen.

Dr. Morgan replied that the term *O* antigen was of bacteriological origin and was introduced to differentiate organisms in terms of their antigens, including the flagellar or *H* antigen. On the basis of immunological evidence it appeared that the most prominent surface structure is the specific polysaccharide. There was, however, no evidence that the whole of the cell surface is covered with polysaccharide and it might be that the other components of the antigen are surface structures but that they are immunologically impotent owing to the overwhelming physical dominance of the polysaccharide residues.

Dr. C. M. Chu asked whether the very low antigenicity of the conjugated protein component, rather than its burial in the depths of the organism, might be responsible for its failure to evoke antibody production when whole organisms were injected.

Dr. Morgan replied that injection of the protein residue into an animal gave a definite immune response whereas injection of whole organisms did not give a significant response specific for this material. He did not think the protein residue was *deep* in the organism. Rather, it was part of the surface, external to the cell wall, and its reactivity was masked by its close association with, and the physical prominence of, the polysaccharide component.

Dr. C. P. STEWART asked what the difference was between the *O*. antigen which is boiled and the *O* antigen which is extracted, and whether any antigenic difference between them could be shown up by the antiserum produced in the animal.

Dr. MORGAN replied that, according to Shelubsky and Olitzki (1947), there is a labile antigen present in *Sh. shigæ*, but he had made no immunization experiments using heated preparations of the isolated and essentially homogeneous antigen which would enable him to decide whether this labile antigen was in some way associated with the *O* antigen or was a separate substance.

Mr. P. MITCHELL referred to Dr. Morgan's statement that the staining techniques used did not reveal the presence of capsules in *Sh. shigæ*, but that this was not surprising, because if the capsule were composed of the *O* antigenic complex alone, the quantity present, almost certainly less than 10 per cent of the dry weight of the organism, would be insufficient to give a capsule of detectable thickness when seen under the microscope. He said that such a layer would be something like 10 or 20 mμ thick on the surface; that would mean that microscopically one could not determine how thick it was; it would not mean that one would not see it. A layer only 5 mμ thick or less, provided that there is material in it which will absorb a good deal of light, will be readily visible when stained although one would not be able to say from normal microscopy what the thickness was.

Dr. MORGAN said his statement was based on the fact that neutral polysaccharides stain poorly and that a layer 5 mμ or so thick, the probable thickness of the polysaccharide layer in this instance, would, after the usual staining procedures, absorb so little light that it would remain invisible.

Mr. MITCHELL said it would be interesting to inquire what kept this particular substance from receiving the stain. He would expect that if staining had gone there, one would see it.

Dr. MORGAN replied that capsulated organisms treated with homologous antiserum frequently became considerably thickened and dense and, therefore, one might expect that such a structure would become visible after this treatment, especially after staining.

Dr. EMMY KLIENEBERGER-NOBEL said she had examined stained preparations of the Smooth and Rough forms of Dr. Morgan's *Sh. shigæ* strains both before and after treatment with homologous antiserum but had seen little evidence for the presence of a distinct capsular layer characteristic of the Smooth form.

Dr. A. FELIX asked whether Dr. Morgan, in dealing with the material extracted from *Sh. shigæ*, had ever come across other antigens which might also be called somatic antigens of *Sh. shigæ*, and which, by the usual serological techniques, have been quite clearly demonstrated. There could be little doubt from the work of Olitzki and of Braun that there exists in Shiga bacilli also a so-called heat-labile antigen which has very similar properties and which inhibits the reaction between the *O* antigen and the *O* antibody; and it was rather surprising that so far the methods of chemical analysis of Shiga antigens have not given an indication of the presence of these additional factors.

There were at least two other well defined antigenic components. One had received the label *I*, and inhibits the *O* reaction, and the other the label *N* because it has a specific neurotoxic effect. Both were labile in many respects as compared with the *O* antigen, and it was rather surprising that chemically no lead has yet been given in this direction.

Dr. MORGAN agreed with the immunological facts given by Dr. Felix. There was no knowledge of these antigens simply because the immunochemical studies on *Sh. shigæ* were carried out some years before these additional antigenic factors were discovered. Further studies must be undertaken in order to establish their chemical nature.

Dr. CHU suggested, on the basis of his own work on anthrax, that bacteria should be examined after extraction and should be compared with normal bacteria and with the extracted antigens.

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III

THE NATURE OF THE SURFACE OF GRAM-POSITIVE BACTERIA

by M. STACEY

FACED with the task of writing on this topic, I am tempted to say that 'anyone's guess is as good as mine'. However, on reviewing our investigations of certain Gram-positive micro-organisms, it does appear that most of our work has dealt with surface components. Consequently, it is my purpose to summarize our results which are gained by essentially chemical methods and then to examine the impacts they may have on the work of other investigators.

The problem is of the highest importance to all concerned with the activities of micro-organisms especially those involving the action of antibodies, chemotherapeutic agents and antigens.

Our experience has been limited to a few typical Gram-positive organisms. Bacteria show a sharp individuality and each species, although undoubtedly possessing many components and modes of behaviour in common with others, ought to be considered strictly on its own merits. Arguments valid for a rod-shaped bacterium may not hold for a coccus, etc., and this limitation must be imposed on the considerations outlined below.

We decided to investigate the nature of the surface of Gram-positive bacteria because of the differences between Gram-positive and Gram-negative bacteria, especially in their susceptibility to antibiotics, chemotherapeutics and in immunological properties.

Our own contribution to the chemistry of the Gram complex (Henry and Stacey 1946) consists in the stripping from typical Gram-positive bacteria by means of a suitable wetting agent such as bile salt, of an essential part of the Gram dye-retaining complex, thereby leaving a residual Gram-negative 'cytoskeleton' which still retains the shape and cytoplasmic contents of the original cell.

The material soluble in bile salt contains mainly polysaccharides

and the magnesium salt of ribonucleic acid. Further, provided that the Gram-negative cytoskeleton has been maintained during extraction in a suitably reduced state, the magnesium ribonucleate component from the cell or from any other source, can be 're-plated' on to the cytoskeleton to restore in a large measure the Gram-positive condition. By disruption of the whole cellular structure (Henry, Stacey and Teece 1945) the Gram-positive dye-retaining material was isolated in cell-free state and was shown to consist of a magnesium ribonucleoprotein, the protein component of which contains strongly basic amino acids such as arginine. The Gram complex can be dissociated into Gram-negative ribonucleic acid and Gram-negative protein and then, under reducing conditions, recoupled without much difficulty to restore the dye-retaining property.

It is to be noted that in the presence of a reducing agent such as formalin, the disruption of the Gram complex by a wetting agent can only be effected with the greatest difficulty.

The method of stripping is to suspend normal washed cells of, for example, *Clostridium welchii* from an eighteen-hour culture in a fairly large volume of 2 per cent sodium cholate and to shake frequently at 60°. The course of the stripping of the cells can be followed by periodic removal of small samples which are washed two or three times in saline and stained by the Gram method.

This procedure readily shows the progressive extraction of what must be essentially surface material, for contact with the bile salt for a few hours causes the cells to become perceptibly thinner and less uniform, even though they still remain Gram-positive. After periods of up to twelve hours some of the cells become completely Gram-negative, leaving cytoskeletons, and, though a few do remain Gram-positive, others stain in patches and have a stippled appearance. In some cases the stripping produces a beaded effect with Gram-positive residual deposits along the edges of the bacillus. The removal of the surface material can be shown by the loss in weight of the cell, the Gram-positives being readily thrown down while the Gram-negative cytoskeletons are extremely difficult to spin down at all. Upwards of one-fifth of the total dry weight of the cells can be removed from the *welchii* bacillus by this method. Centrifugation for varying times is a practicable method of separation.

We have shown repeatedly that the extract contains mainly magnesium ribonucleate, small amounts of deoxyribonucleic acid, polysaccharides and some fatty material and traces only of protein, all of which can in a measure by separated by precipitation from water by various solvents.

Provided that the cell cytoskeletons have not become irreversibly oxidized—e.g. if they are kept in formalin, ascorbic acid, etc. —a good deal of the magnesium ribonucleate can be plated back on to a receptive protein on the cell skeleton. The coupling is frequently almost immediate. This phenomenon and the information it gives about the cell surface is discussed below along with the general significance of this stripping process. We have frequently observed that Rough forms of Gram-positive cells can be stripped more readily than the Smooth and that less material comes off into solution.

The complex carbohydrates removed along with the magnesium ribonucleate could not be replated, though crude unfractionated ribonucleic acid, containing polysaccharides possibly bound firmly to the nucleic acid, gave a more intense and complete replating as determined by the Gram reaction than did highly purified ribonucleic acid.

Negative results were obtained with attempts to couple deoxyribonucleic acids and ribonucleic acid degradation products. It is possible with carefully prepared non-fibrous forms of deoxyribonucleic acid to get a recoupling on some occasions. Other metals such as sodium, calcium, zinc, etc., could not satisfactorily replace magnesium in this process.

Qualitative differences were apparent between various reactive cells though the process could be demonstrated with yeasts, *Cl. welchii*, with anærobic bacteria such as *Cl. sporogenes* (Pl. 1, figs. 1 and 2), with ærobic sporers such as *Bacillus anthracis* and with certain strains of pneumococci, streptococci and sarcinæ.

With some cocci—e.g. staphylococci—the complete extraction process is relatively difficult, for with each extraction the cell gradually shrinks without becoming completely Gram-negative and some dye-retaining complex is apparently diffused throughout the whole of the cell. Yeast cells present a special case, for they stain intensely blue-black by Gram's method and the stripping process can be shown to occur in stages. The replating process

is of special interest and presents several analogies to the phenomena obtained with bacteria. However, it may not be considered correct to discuss the special case of yeast in this symposium so that my arguments will be largely confined to the case of *Cl. welchii*. The absence of deoxyribonucleic acid from the yeast Gram complex leads us to believe that it plays no essential staining role in bacterial Gram complexes. Pollen grains and mould spores behave in many ways like yeast.

Cl. welchii *cells*. The cell of this organism consists of an inner somatic region, the protoplast or cytoplasm, which contains the group polysaccharide, mucolipids and chromatin, etc., with other components that at least initiate and take part in cell division. Encasing the cytoplasm is the cytoplasmic membrane and outside that the cell wall upon which under certain conditions and in certain cases the capsule may be built. This capsule—a variable component according to conditions—deserves special consideration and will be discussed separately.

We consider the cytoplasmic membrane to be mainly lipoprotein in nature and to arise from the ends of chains of the protein in the cell wall in combination with a lipid from the cytoplasm. It may be regarded as the boundary between the cell wall and the cytoplasm, and the Gram-negative cytoskeleton may be considered as the cytoplasm encased in the cytoplasmic membrane and with a very large part of the cell wall stripped off. We have found that methods for staining the cell wall work just as well on the thinner residual cell wall of the cytoskeleton as on the intact bacillus.

It appears from the effect of stripping that the Gram dye-retaining complex is distributed evenly on the surface of the cytoplasmic membrane. In photographs in ultra-violet light, the absorbing material, mainly nucleic acids, appears as innumerable tightly packed though discrete minute granules. Once the packing of these is upset by the action of the bile salt, the nucleoprotein tends to become dislodged and aggregated. As it is removed the granules frequently are arranged as a string round the perimeter of the cell giving the previously-mentioned beaded effect, an appearance which is very striking and which in itself clearly demonstrates that the Gram material is on the cell wall surface. This same beading occurs when a solution of a lithium

salt or preferably a lanthanum salt, reagents which precipitate nucleic acid, is added to a suspension of killed welchii cells.

There is evidence that part of the Gram material, mainly the nucleic acid component, is relatively mobile on the surface of the cell wall, i.e. it can be dislodged from one site and coupled on to another.

In the Gram complex there is a certain proportion (up to 10 per cent) which varies between different organisms, of the deoxyribose type of nucleic acid, a substance which in ultra-violet light cannot be distinguished from the ribose type and ultra-violet photographs show light-absorbing granules throughout the whole of the *welchii* cell. The precise relationships between the two types of nucleoprotein are not clear, but they each contain numerous enzyme systems.

The obvious thinness of the cells after extraction appears to be due to loss of surface material. In electron micrographs the cell wall of the *welchii* cytoskeleton appears shrunken and usually sags quite obviously—an effect partly caused by diffusion of cytoplasmic fluids. After replating with magnesium ribonucleate the cell wall becomes more rigid again.

Evidence on the nature of the Gram complex from a study of lytic procedures

In considering the mode of action, usually at 60° C., of the bile salt series and of other detergents in removing the nucleic acids of the Gram-positive complex, we have reached the conclusion that the action is purely physical. Under the influence of the detergent, particularly in the presence of oxygen, there is a disruption both of electrovalent bonds and of covalent bonds uniting the basic proteins in the cell wall and the nucleic acid. The linkages are much stronger and the dissociation is much more difficult to effect in presence of reducing agents and probably -S-S- types are involved. Those components at the surface—the polysaccharides—can clearly be removed first.

We consider that there is no undue stimulation of the normal lytic processes of the cell under the conditions of the reactions with bile salts. It has been clearly demonstrated by ourselves, and by Avery, Goebel, Dubos (see Dubos 1945) and others, that

spontaneous autolysis, in the pneumococcus group particularly, goes in stages—the first stage being the conversion of Gram-positive to Gram-negative forms. We find that this conversion to Gram-negative forms by the action of autolytic enzymes only goes well under reducing conditions—an observation adding weight to the belief that the similar bile salts effect is a physical one.

Preparations of non-specific cell-free autolytic enzymes bring about changes indistinguishable from those of spontaneous autolysis. The similarity of the effect of the bacterial enzyme and of pancreatic ribonucleinase and leucocyte ribonucleinase inspired us to examine the action of various bacterial ribonucleinases. We concluded that ribonucleinases destroy ribonucleic acid at the cell surface of Gram-positive bacteria, liberating polysaccharides, fats and occasionally traces of protein, and leaving residual intact Gram-negative cytoskeletons. These cytoskeletons produced by enzyme stripping could not be replated with magnesium ribonucleate and this points to the destruction or oxidation of some coupling component of the basic protein in the cell wall. The bacterial ribonucleinases were species and frequently strain specific and we found the reason for this specificity in the fact that before the nucleinase could act, another enzyme, which we termed a 'polysaccharide releasing' enzyme, must split linkages between the surface polysaccharides and the ribonucleic acid of the Gram complex. This effect can be also brought about by the less specific mucinase, lysozyme (Stacey and Webb 1948).

This finding is in accordance with our previous discovery that the magnesium ribonucleate must be attached to the basic protein on the cell wall if the Gram dye is to be retained and with our recent finding that the polysaccharides of the capsular type can be isolated as macromolecules in firm combination with a nucleic acid both of the ribose and deoxyribose types. We have recently examined polysaccharides obtained by complete autolysis from Types II and V pneumococcus and find that they contained as much as 5 per cent of a ribonucleic acid constituent in firm combination.

Further evidence of the basic protein of the Gram complex in the cell wall arises from the fact that by an autolytic procedure

the Gram complex itself can be obtained in the form of a magnesium ribonucleoprotein bound by covalent linkages. The autolysis procedure necessarily entails the destruction of the whole cell including the cytoskeleton.

That the cytoskeleton cell wall material is essentially protein is evident from our work on its complete autolysis, which can readily be effected by proteolytic enzymes, particularly those elaborated by the whole cells themselves. Thus when a suspension of cytoskeletons from killed Gram-positive bacteria—made by removal of the surface polysaccharide and nucleic acids by bile salts of ribonucleinase—is adjusted to pH 8, complete lysis follows rapidly. We have isolated from Gram-negative cytoskeletons made by autolysis under reducing conditions at pH 6 a casein-hydrolysing enzyme and a peptone-hydrolysing enzyme, and we have further shown that the initial disintegration of the cytoskeleton is brought about by the casein-hydrolysing enzyme while further degradation of the released protein fragments is caused by the peptone-hydrolysing enzyme. The casein-hydrolysing enzyme will not only dissolve the cytoskeletons of all Gram-positive, but also will render soluble heat-killed true Gram-negative bacteria. Further, the enzymes have no action on living or killed Gram-positive bacteria and this is taken as further evidence that there is no closely related protein material exposed in the surface of Gram-positive bacteria.

We consider that the lytic system comprising the successive action of polysaccharide-releasing enzyme, ribonucleinase and a two-stage proteolytic enzyme system is common to Gram-positive bacteria and that closely-related systems also occur in moulds, actinomycetes and pseudomonads, etc.; that the surface material of Gram-positive bacteria consists mainly of polysaccharides in partial combination with nucleic acids, mainly of the ribonucleic acid type; and that except in special cases where proteins may form capsules, relatively small amounts of proteins or fats are present. It is clear that the ribonucleic acid of the Gram complex is in a large measure protected from enzyme action by the presence of a closely-bound polysaccharide, but that it is in no way protected by its combined basic protein.

Capsular Material

Capsular material can be composed of complex polysaccharides or, in a few cases, of unusual proteins. Capsule formation in most micro-organisms depends sharply upon correct cultural conditions and we find that a suitable magnesium supply and often the presence of specific sugars are critical.

The function of the magnesium we regard as being specific in co-ordinating into the correct spacing the purine and pyrimidine bases of the nucleic acids so that they fit precisely on to the protein chain in the cell wall to form various nucleoproteins such as those of the Gram complex.

When formed, these nucleoproteins can act as self-duplicating molecules and play some general role in the conversion and the transference of nucleotides from the cytoplasmic cell wall through the membrane to the nuclear constituents in the cytoplasm. A proportion only of the magnesium in Gram-positive bacteria is ionic.

A major function of the deoxyribonucleoprotein and probably in some cells of the Gram ribonucleoprotein, is to act as the enzyme which builds up capsular material. In the case of pneumococcus capsular material, we must consider the work of Avery and his colleagues (1944, 1946) on the transforming principle. Here we do not regard the deoxyribonucleoprotein as being so firmly attached to the cell membrane. Cell-free bacterial enzymes which will build up relatively enormous amounts of polysaccharides—as, for example, the streptococcal dextrans—can be readily obtained. We would regard this extracellular synthesizing enzyme as forming part of the cell wall and originally attached to the cell wall surface by relatively labile linkages. The enzyme, whether bound or free, when given the right conditions and the right substrate, e.g. sucrose, glucose-1-phosphate, etc., can build the capsular material on the cell wall. Electron micrographs of the pneumococcus capsular material show a basket-like network of the jelly-like mucopolysaccharide macromolecules.

We consider that the mucopolysaccharide type of macromolecule is built up by a synthesizing enzyme that is essentially a nucleoprotein, to which is attached a monomolecular layer at least of the specific polysaccharide which forms the template on

which the rest of the molecule is built. We hold also that the synthesizing enzyme remains in combination with the polysaccharide it has synthesized to form a complex mucopolysaccharide having a small nucleoprotein prosthetic group. This is a new conception of an enzyme for this particular type of synthesis, and Avery and co-workers' transformation of types in the pneumococcus group may be regarded as follows: Avery's Rough form of the Type II pneumococcus would correspond to a cell which, owing to the abnormal and inadequate growth conditions, has been able to produce the cytoplasm and the cytoplasmic membrane, but only part of the cell wall. This part of the cell wall consists mainly of protein material which is probably devoid of a large part of its ribonucleic acid and certainly lacks the special deoxyribose type of nucleic acid which normally in combination with the protein forms the capsule-synthesizing enzyme. In the Rough cell, the protein is, however, still part of the living cell wall and is still able to combine with the deoxyribose type of nucleic acid. When the latter, as in Avery and colleagues' experiments, is brought into contact with the protein and when it is still carrying its mono-molecular layer of specific polysaccharide, whether Type III or any other type, it becomes the transforming principle, combines with the protein and, when correct substrate is present, initiates capsular polysaccharide synthesis. The nature of the substrate as well as the chemical composition of the Rough cell probably has an important effect on the duration of any 'lag' period before polysaccharide syntheses takes place. It is likely to be necessary with some Rough forms for them to 'condition' the carbohydrate monomers into a form suitable for 'polymerization'. Since the nucleoprotein thus formed belongs to the class of self-duplicating molecules, the power to continue polysaccharide synthesis is transmitted to daughter cells. Moreover, it appears that the synthesis actually takes place on the cell wall. Synthesis of the nucleoprotein may be extremely vigorous in living cells and after a time the nucleoprotein may not need the cell wall as an anchor, so that part of the nucleoprotein enzyme may break away and build up polysaccharide independently of the cell, and at distances remote from the cell.

In the Gram complex ribonucleic acid from any source readily

couples with the basic protein of the cell wall and in this sense no specificity is carried by ribonucleic acid from different sources.

It is difficult at present to visualize the remarkable specificity that exists in the fifty or more types of pneumococcus capsular polysaccharides being carried by pneumococcus deoxyribonucleic acid alone, though it is likely that some structural differences do exist between nucleic acids from different sources. It is known that for enzymic synthesis of polysaccharides such as starches, glycogen, dextrans, a carbohydrate 'starter' is essential. This consists of the 'pattern' of the final polysaccharide molecule which must possess a certain minimum size and must provide ends of chains to act as a foundation on which the macromolecule can be built. I am convinced that the transforming principle does indeed carry in combination sufficient of the specific capsular polysaccharide to form at least as a monomolecular layer and that this orients itself on the deoxyribonucleic acid at the extreme surface of the cell wall.

In some cases the constituents of the cell surface may be identified serologically; in the pneumococcus, at least, these are mainly polysaccharides. In the case of most of the Gram-positive *Cl. welchii* it has been found very difficult to obtain a true immunizing antigen (or complete antigen) and this we feel sure has been due to the powerful lytic systems splitting the macromolecules into individual components devoid of full antigenic activity.

The recent work of Heidelberger and his school (1946) on successful immunization against pneumococcus types with polysaccharide preparations is relevant here. We have had the opportunity of examining two of these unfractionated polysaccharides which have successfully been used as vaccines and we find them to be, not pure polysaccharides, but to contain up to 10 per cent of extraneous material composed mainly of nucleic acid of both types.

Virulent pneumococci may be classified as is well known into a number of serological types, which differ mainly in the chemical structures of the capsular polysaccharides.

Dubos and others obtained enzymes from saprophytic soil micro-organisms which hydrolyse specifically the capsular polysaccharide of the certain pneumococci without altering (*a*) the viability of the cells, (*b*) their Gram staining capacity or (*c*) their

ability to make capsular material in correct cultural conditions. This again emphasizes the close relationship between the Gram nucleoprotein components and polysaccharide synthesis.

The striking fact that the enzymes can alter the capsules of living cells without killing these cells shows that capsules are not essential structural components of the living cells—a deduction confirmed by the fact that Rough cells have no capsules.

When the power to synthesize the capsule is lost, the organisms lose in a large measure their virulence and their ability to agglutinate in specific Smooth antiserum. Until quite recently highly purified preparations of these polysaccharides were not antigenic, though they determine the immunological specificity of the living cell. These facts suggested that the polysaccharides were simply a determinant part of a larger molecular complex, the complete antigen of the cell.

When the ribonucleic acid component of the Gram complex of the pneumococcus is hydrolysed either by animal ribonucleinase or by the ribonucleinase of the pneumococcus autolytic system, the cells become mainly Gram-negative and much smaller in size. In each case the change in staining reaction, although not affecting the morphology of the cells, results in a loss of type specific antigenicity and the type specific polysaccharide is liberated in sufficiently undegraded form as to retain the power to precipitate with homologous antisera. It follows, therefore, that the loss in antigenicity is due to a loss of a second component of the capsular antigen which one thinks must clearly be of the ribonucleic acid type—indeed it must be the ribonucleic acid of the Gram complex. Since we have actually isolated firmly bound nucleic acids from the undegraded specific polysaccharide, we consider it as proven that the capsular polysaccharides are bound to part of the nucleic acid of the Gram complex.

Thus it would appear from this evidence alone that ribonucleic acid in the pneumococcus Gram complex is located near the surface of the cell wall. The amount of this capsular polysaccharide determines in some measure the virulence of the organism which when completely devoid of it would be avirulent. When completely devoid of it the colonies would be Rough and probably mainly Gram-negative.

Abnormal Cell Division

Surface components of Gram-positive cells can be studied by observing phenomena associated with abnormal cell division.

In normal growth we get numerous manifestations of biological polymerization in the sense that we have the build-up of such big molecules as proteins, nucleic acids, polysaccharides and fats with the concomittant association of these with one another to form giant macromolecules. Origination of these such as by polysaccharide synthesis, takes place at the surface of the cell or even at distances remote from the cell.

In this biological polymerization we must have the reaction initiators, chain breakers, branching factors and so on known in chemical polymerization. In normal bacterial cell growth all these processes go on alongside one another with other growth processes and with cell division, being synchronized in a splendidly organized manner. When however any one of these becomes disrupted owing to an external influence, such as a dietary deficiency, presence of an antibiotic, a wrong pH condition, we may have a rapid disorganization of some integrated enzyme system, which is manifested as change in morphology, abnormal cell division, failure to take the Gram stain, appearance of snake-like forms, swollen forms with 'nucleation' and so on.

We have found it possible by magnesium starvation or by use of antibiotics to produce abnormally swollen forms in cocci and long snake-like forms in rod-shaped organisms such as *Cl. welchii*, and we have studied in particular the surface components. In certain magnesium starved streptococci, e.g. *Streptococcus salivarius*, the colonies rapidly lose their power to form the surface capsules and then go Rough. These Rough colonies are devoid of a special type of capsular 'levan' polysaccharide and they show mostly Gram-negative forms and sometimes swollen forms with Gram-positive material inside the cytoplasm, giving the appearance of heavy nucleation. These forms revert to the Smooth Gram-positive state on subculturing under normal conditions. Rod-shaped organisms, in presence of minute amounts of magnesium only, grow in snake-like forms, which are not segmented and are strongly Gram-positive. When snake-like forms of *Cl. welchii* are produced by growing them in presence of minute

amounts of penicillin, the fibre-like cells also are strongly Gram-positive. The surface material can be extracted in the usual way by means of bile salt and consists of both types of nucleic acid with polysaccharides, leaving behind a long strand of Gram-negative cytoskeleton faintly segmented at lengths slightly longer than the normal cells. These long Gram-negative cytoskeleton strands can be 'replated', or one might say 're-enamelled', by means of treatment with magnesium ribonucleate in the usual manner to restore their Gram-positive character.

Both magnesium starvation and penicillin clearly interfere with the normal cell dividing processes of these rod-shaped organisms though evidently not in precisely the same way. In both there is interference with some enzyme system responsible for biological polymerization. In both penicillin treated and magnesium-deprived cells, the total yield and ratio of the two nucleic acids are the same and so is their mode of union with the basic protein in the cell wall. Clearly it is the build-up of these protein chains which is affected. Indeed one could say that the length of these protein chains is enormously increased, perhaps owing to the fact that penicillin has been able to prevent a particular amino acid from being built in ordered sequence into the protein chain. In doing this, it has inhibited the action of a chain breaker and polymerization has gone on unimpeded to a relatively enormous length. It may well be, from Gale's observations, that penicillin prevents the normal build-in of glutamic acid.

With regard to the cell surface however, the stripping of the Gram complex from these abnormal snake-like cells and the coupling of the magnesium ribonucleate to reconstitute the Gram complex, provides a most striking demonstration of the location of magnesium ribonucleate in the cell wall and indeed near the surface of the cell wall.

Returning to the possibility that the basic protein of the cytoskeleton is held in long segmented fibres, we have artificially induced a similar phenomenon in Gram-negative cytoskeletons from welchii cells. There often occurs an end to end coupling up of the cytoskeletons, two or three or more at a time, and when these are replated with magnesium ribonucleate and stained by Gram's method, they appear as abnormal long rod-shaped giant Gram-positive organisms (see Pl. 1, fig. 3). It would appear that

the proteins exposed by the mild stripping of the nucleic acid and the surface polysaccharides are powerfully reactive, especially at the ends of the cytoskeleton, and they can readily unite together at these points. Such a phenomenon has been described with viruses shaken into small segments by ultrasonics and then allowed to stand in a liquid medium. These couple up end to end in a striking manner to form rods and possibly the same type of coupling mechanism occurs in the exposed protein ends of these virus nucleoproteins. This might be an example of the chemical formation of what Dubos terms the 'plasmodesmids' which then fail to snap.

The abnormal coupling to form protein chains appears to be due to chemical rather than biological processes, and again emphasizes the highly reactive property of the cell wall proteins once their nucleic acid components have been removed.

The special case of *Mycobacterium tuberculosis*

The tubercle bacillus and other acid-fast bacilli are Gram-positive in young cultures and slightly so under certain other conditions. It is possible to extract substances from the tubercle bacillus by solvents and we have used particularly cautious treatment with urea to remove gently a good deal of the waxes and fatty acids. This material also contains polysaccharides and deoxyribonucleic acid. The residual essentially fat-free cell consists largely of basic proteins, somatic carbohydrates with some ribose type nucleic acid. It is acid-fast and doubtfully Gram-positive, and is by no means a cytoskeleton. Moreover, provided that it has not become oxidized during the process of fat extraction, we can plate magnesium ribonucleate on to the fat-free cell to make a long apparently Gram-positive rod-shaped organism.

We consider, therefore, that the tubercle bacillus has a cytoplasm enclosed by a cell membrane on which is built protein material forming part of a cell wall of the type found in other Gram-positive bacteria. On this cell wall the complex nucleic acids, polysaccharides and the fatty material so characteristic of the acid-fast bacilli is then elaborated, probably in layers. The nucleic acid in this case contains a high proportion of the deoxyribose type. The fatty components do not form a capsule, but I

think that in combination with the nucleic acid and carbohydrates they are at least mainly located on the surface of the basic protein of the cell wall. Parts of this surface material are relatively loosely bound. The polysaccharides of the tubercle bacillus are highly complex and diverse and we have isolated five or six different types. Of these one readily diffuses into the medium and may be slimy, one is firmly bound to the surface lipids and yet another two, tubercle glycogen and a somatic polysaccharide, are located in the cytoplasm.

Conclusion and Summary

I regard the Gram-positive bacteria as consisting of a cytoplasm which is encased in a cytoplasmic membrane. The cytoplasm contains the nuclear elements responsible for the initiation, at least, of cell division and these consist of deoxyribonucleic acid, nucleosides, nucleotides and combined proteins. Other cytoplasmic components include lipids and carbohydrate-lipid complexes which constitute the somatic or lipid-bound polysaccharides many of which can be stained by different techniques and demonstrated as granules.

The cytoplasmic membrane itself is probably lipoprotein, formed by the union of lipids in the cytoplasm and the ends of protein chains in the cell wall and constituting a membrane boundary between these chemical components. Under some conditions, e.g. in hypertonic solutions, the membrane contracts and draws inward from the more rigid, tube-like, main cell wall.

I regard this cell wall as being built up in layers, the layer nearest the cytoplasmic membrane being composed of chains of protein material specially characterized by being built of basic amino acids and sulphydryl groups and containing the protein component of the Gram dye-retaining material. Attached to the basic proteins, and probably folded to form a layer of nucleoprotein material, is the magnesium ribonucleate of the Gram complex. The nucleoproteins thus formed, with prosthetic groups and co-enzymes, constitute the various enzyme systems responsible for cell functions and particularly for the build-up and breakdown of the cell macromolecules.

Folded into the Gram ribonucleoproteins and forming part of the cell wall—in particular the part near the cell surface—are the

deoxyribonucleoproteins which in Smooth cells under optimum conditions, can build up the capsular type specific mucopolysaccharides. In *Cl. welchii*, for example the optimum condition for capsule formation may be found only in the living animal. In detail the capsule, whether of protein or polysaccharide nature, may be regarded as a basket-like network of chain molecules located at the surface of the cell wall; it is a relatively mobile structure bound initially to the nucleic acid of the cell wall, but with the outer components, particularly in liquid medium, breaking away as individual giant molecules which then must be regarded as excreted products. The various layers of the cell wall are of such a nature as to allow the ready flow through to the cytoplasmic membrane of metabolites—generally those small molecules from which the macromolecules are built and from which energy is derived. The cytoplasmic membrane itself will allow the passage through of the metabolites particularly the ribonucleosides and nucleotides which are transformed into those of the deoxyribose type and which take part in some stages of cell division. Lipids, which may function as enzyme activators and energy reserves, are found in the cell walls of Gram-positives in varying proportions and they may also be deposited in combination with proteins and polysaccharides in the cytoplasm. In the acid-fast group, lipids are produced in large amounts, and are deposited, mainly in the bound form, near to, and indeed forming part of, the surface of the cell wall.

From the properties of cells formed under conditions of abnormal cell division, I would deduce that the various phenomena such as the formation of filamentous and giant cell forms depends on differences in the polymerization of the proteins of the cell wall. In the filamentous forms there is abnormal protein chain synthesis in a longitudinal direction, whereas in the swollen, giant forms of cocci there is a considerable and unusual increase in the thickness of the protein layers of the cell walls, i.e. there is latitudinal or cross-chain synthesis also.

The changes in properties of Smooth and Rough forms are obviously determined by the nature of surface components as decided to some extent by the cell wall nucleoproteins. The simple device of withholding all but traces of magnesium from the growing Gram-positive cell will suppress the formation of the

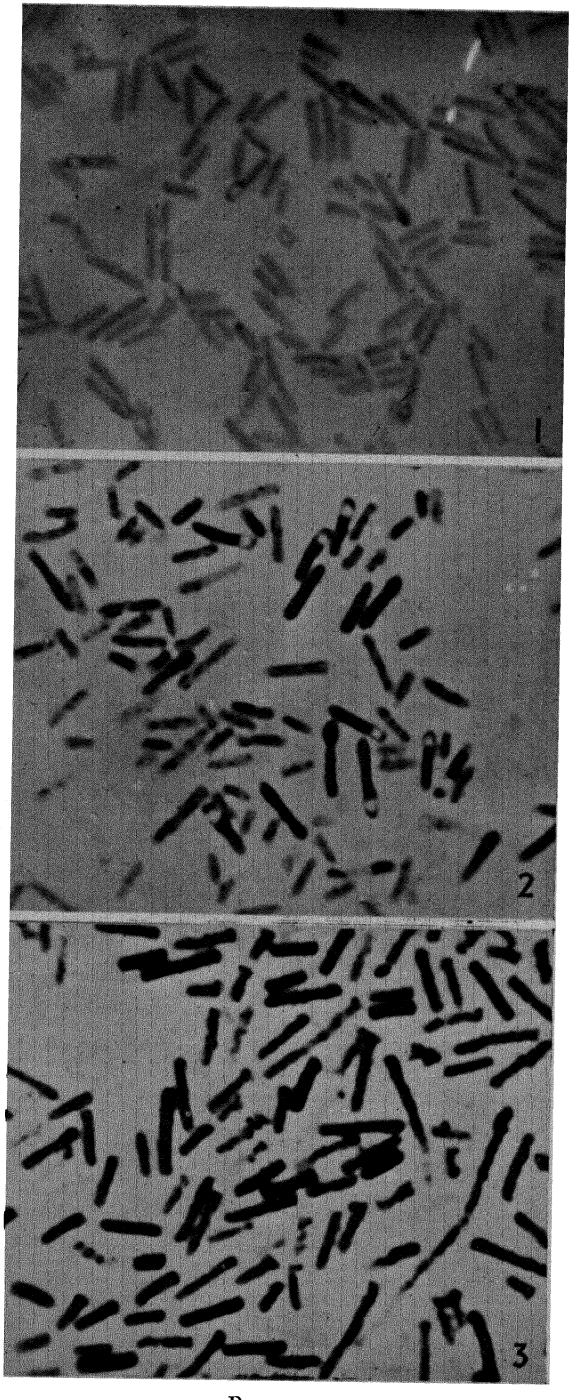

PLATE 1.

Fig. 1. Gram-negative forms of *Cl. sporogenes*. Fig. 2. *Cl. sporogenes* 'replated' with magnesium ribonucleate. Fig. 3. *Cl. welchii* cells stripped with bile salt and replated with magnesium ribonucleate. End-to-end coupling of the cells has occurred. Photographed by Dr. H. Henry. (M. Stacey.)

full quota of nucleoproteins including the Gram complex. Thus Smooth Gram-positive cells so treated will give rise to Gram-negative cells and colonies will be Rough owing to the consequent lack of the capsular polysaccharide-synthesizing enzyme on the outer cell wall.

I think that true Gram-negative bacteria differ markedly from Gram-positive bacteria in their nucleoprotein components, particularly nearer the outer surface of the cell wall; and most particularly that Gram-negative bacteria are devoid of the characteristic basic proteins which in Gram-positive bacteria combine with nucleic acids to form the Gram dye-retaining nucleoprotein. Gram-negative bacteria do contain appreciable amounts of both ribo- and deoxyribonucleic acids, but these are not combined with a large molecular basic protein in the outer cell wall. It appears that lipoproteins constitute the outer cell wall in Gram-negative bacteria though polysaccharide-synthesizing nucleoproteins are also present in the cell wall.

My observations on spore-forming organisms have been limited to a few examples only and my main comment is that a spore appears to possess a cell wall of its own and that in the mother cell it is sharply separated from the main cell wall. On stripping the nucleic acid surface component, with bile salt, from a spore-bearing cell such as *Cl. sporogenes*, the spore readily drops out leaving an actual hole in the Gram-negative cytoskeleton (Pl. 1, figs. 1 and 2). The chemistry of spores, which have a high nucleoprotein content and which before fully matured are Gram-positive, will provide a fascinating study.

It must again be emphasized that my views are based mainly on chemical work with a limited number of species.

I firmly believe that the detailed structure of bacterial cells, as with all cells, must be placed on a firm chemical basis before structure in the biological sense can be established. As a chemist, I claim that there is still far too ready a use of staining techniques which, although admittedly well tried, are completely inexplicable in the chemical sense. When used on such a variety of highly complex and often unstable macromolecules it is understandable that they produce different results in the hands of different investigators. Since interpretations of even a single observation can vary widely it is easy to see why controversies regarding

bacterial structure and cell division have arisen. Elsewhere my colleagues and I have published our views on the chemistry of the Feulgen and Dische reactions for deoxyribonucleic acid and we believe we have cleared up many controversial points in these histochemical techniques. In the problems both of composition and function, we would advocate a more intensive chemical approach to problems of the bacterial cell.

Discussion

Dr. Harriett Taylor said that no one could question the value of isolating complex molecules from bacteria and searching for the substances which endow these organisms with their antigenic and physiological properties. However, proof should be given, when a macromolecule is isolated, that it is actually a component of the organism and not an artefact. Information of this kind would be very desirable concerning the macromolecule of nucleic acid and polysaccharide, described by Dr. Stacey. As to the function of such a molecule in the bacterium, unfortunately analytical chemistry can tell us nothing.

A new hypothesis concerning the nature of the transforming principle of the pneumococcus is advanced in Dr. Stacey's paper. It is suggested that a small amount of capsular polysaccharide is attached to the desoxyribonucleic acid, and that these together constitute the transforming principle of the encapsulated pneumococcus. It is proposed that the specificity of the transforming principle is due to the attached polysaccharide and not to specific properties of the nucleic acid itself.

At the outset it is clear that undetectable amounts of polysaccharide may be present in the purified desoxyribonucleic acid fraction of the encapsulated bacterium. Certainly, a substance of high molecular weight, for the purification of which no specific techniques exist, may be supposed quite reasonably to be contaminated with small amounts of certain substances. Serological tests for capsular polysaccharide are quite negative in preparations of transforming principle from Type III pneumococci. Further, the specific polysaccharidase which hydrolyses the Type III polysaccharide is without effect upon the transforming activity of these preparations. However, by the postulates of Dr. Stacey's

theory, these observations become of little value as evidence for or against his hypotheses.

It is more worth while, therefore, to consider what his theory adds to understanding the transformation phenomenon. The essential point of the theory is that it is the polysaccharide which specifically orients the synthetic process by which it is itself formed. The assumption that a polysaccharide can orient such a synthesis is drawn from the work upon starch and glycogen synthesis, in which a polysaccharide molecule must be added to initiate the reaction (Cori and Cori 1939). This starter may be a fragment of a straight chain starch, or of one with branching chains of glucose molecules, and according to the nature of the starter, the final product is either straight or branched chained. However, experiments indicate that the role of the starter is to provide a terminal glucose unit in which an essential free group is exposed and to which the glucose-1-phosphate is linked (Cori, Cori and Green 1943). In addition to certain structural requirements, the starter must have sufficient molecular size to stabilize the combination (Hidy and Day 1944, Swanson and Cori 1948, Green and Stumpf 1942). The enzyme phosphorylase is capable of forming only the 1 : 4 linkage, and this it does regardless of whether the starter has a straight or branched structure. The specific synthetic step performed is in no way determined by the starter, but is determined by the properties of the enzyme. Indeed, the enzyme is surprisingly undemanding with regard to the nature of the starter, for except for the terminal units and the hexose residues immediately attached to these units, it seems to be relatively indifferent as to what makes up the bulk of the molecule. No new branches can be formed by the enzyme which makes the 1 : 4 linkages, for branches are made by 1 : 6 linkages. Another enzyme is responsible for this latter kind of linkage (Cori, Cori and Green 1943). When the two enzymes are present simultaneously, a branched chain polysaccharide (glycogen) is formed, regardless of the nature of the starter. There is thus no reason to believe that the starter can determine the nature of the synthetic steps leading to starch and glycogen synthesis.

The differences between the polysaccharides of various pneumococcal types are not simply configurational. They are in some cases, at least, differences in composition, viz. Type III in which

the basic unit is a disaccharide composed of glucose and glucuronic acid (Reaves and Goebel 1941), and Type I in which the basic unit is a trisaccharide of 2 galacturonic acid molecules and an amino sugar (Heidelberger, Kendall and Scherp 1936). To orient the formation of such different polysaccharides, the starter molecule would have to determine both the nature of the synthetic step and the substrates used. A theory in which a polysaccharide residue performs such tasks is contrary to existing concepts of enzyme specificity.

The theory that a polysaccharide starter is responsible for transformations in the pneumococcus is admissible if it were assumed that a pneumococcus at all times possesses a variety of enzymes for synthesizing a variety of capsular polysaccharides. Transformation could then consist of activation of one or another system by a polysaccharide starter. If this were the case, however, it is difficult to see why the desoxyribonucleic acid is also required. Further, it is difficult to see why a pneumococcus is limited to synthesizing one polysaccharide at any given time. For example, the intermediate smooth pneumococcus which synthesizes Type III polysaccharide can be transformed to Type II pneumococcus by means of the appropriate transforming principle, but when this occurs, the transformed bacterium no longer synthesizes the Type III polysaccharide.

Turning to a few minor details of the picture of the transformation process which Dr. Stacey drew, it should be noted that there is at present no reason to suppose that the R pneumococcus is idiotypically devoid of part of its surface coat of nucleic acid. Rough pneumococci are as Gram-positive as are Smooth, for example. Further, it should be added that transformation is not usually performed under obviously deficient growth conditions. In the system usually employed, for example, transformation takes place during logarithmic growth of the R pneumococci, at a moment when they are dividing every 20 minutes.

Finally, a word should be said concerning the antigenicity of purified polysaccharides. Their antigenicity is by no means a recent discovery which coincided with the use of impure commercial preparations of capsular polysaccharide. In 1927, four years after the publication of the first work of Avery and Heidelberger on the soluble specific substances of pneumococci,

Schiemann and Casper (1927) reported that capsular polysaccharide was antigenic in mice. In 1930, Francis and Tillet established that humans formed specific precipitins in response to injection of purified polysaccharides of Types I, II and III. These polysaccharides were prepared by the older methods described by Heidelberger and Avery (1923, 1925). In the preparations of these latter workers the Types II and III polysaccharides were nitrogen free. It has been generally accepted for many years that these polysaccharides are haptens in certain species, but antigens for others.

It is probable that some of the capsular polysaccharide of the pneumococcus is in combination with other cellular constituents, and that in this condition it is generally antigenic. The nature of this combination is unknown. There are no data at present which can be really considered to disclose the nature of the capsular antigen. The results of the limited autolysis experiments performed by Dubos (1937) and by Thompson and Dubos (1938) might suggest that ribonucleic acid is a part of the complete capsular antigen, were it not for the fact that there is no evidence that ribonuclease alone can replace a limited autolysis in achieving the multiple effects observed. In particular, there is no proof that incubation of intact pneumococci with ribonuclease can diminish the antigenicity of the capsular antigen, even though this treatment may render the bacteria Gram-negative.

Dr. Stacey had reported that in commercial preparations of the undegraded specific polysaccharides, an appreciable amount of nucleic acid is firmly bound to the polysaccharides. This is somewhat at variance with the published work of Alberty and Heidelberger (1948), which deserves mention. Preparations made commercially for large-scale experiments on human immunization are not as pure as those described by Heidelberger, Kendall and Scherp (1936). It was found by Alberty and Heidelberger that a commercial preparation of Type I polysaccharide contained 19 per cent by weight of nucleic acid, which was characterized by its adsorption spectrum and phosphorus content. Electrophoretic studies showed that nucleic acid and polysaccharide moved at different speeds over a wide range of pH values. Fractionation could be accomplished either by electrophoresis, or by precipitation with isopropyl alcohol, at an ionic strength somewhat

greater than that usually used in polysaccharide purifications. Capsular polysaccharide is obtained in a first fraction, and increasing amounts of nucleic acid and somatic polysaccharide in a subsequent two fractions. These results suggest that the nucleic acid and somatic polysaccharide in the commercial preparations are contaminating materials, and not firmly bound to the capsular polysaccharide.

Prof. M. STACEY said that he was of course prepared to concede that specificity could well be carried by the deoxyribonucleic acids. In their power to retain the Gram stain, the ribose types of bacterial nucleoproteins do not appear to show any specificity. Thus, for example, yeast magnesium ribonucleate could be combined with cytoskeletons from *Cl. welchii* in order to reconstitute the Gram-positive state, the same thing could be done with magnesium ribonucleate from other bacteria from plants and from any commercial source. In the combination of ribonucleic acid and protein to form a big molecule which will firmly hold the dye, there appears to be no specificity between the different nucleic acids. Prof. Stacey was prepared, however, to admit that in the future it would be found that there are quite remarkable structural differences between the deoxyribose type and the ribose type nucleic acids.

He was quite firmly of the opinion that in these polysaccharide syntheses one must have the correct structural patterns on which the enzyme could build. There was no doubt about this in the enzymic synthesis of starch-type polysaccharides where the building takes place at the ends of glucose chains. This synthesis was perhaps relatively simple involving glucose-1-phosphate substrate and forming 1 : 4 and 1 : 6 glycosidic linkages. In the case of the Type III pneumococcus polysaccharide, it might well be that cellobiuronic acid or a simple polymer thereof was sufficient to form the pattern and to provide ends of chains on which the right kind of nucleoprotein enzyme could build polysaccharide. If a mono layer of such a saccharide existed in combination with the deoxyribonucleic acid, it might not be attacked by a polysaccharide-splitting enzyme. It would certainly be difficult to demonstrate the presence of such a saccharide and also to remove it. Until much more was known about the structure of the deoxyribonucleic acids and of the way in which

they combine with proteins, the matter would have to be left open.

Dr. TAYLOR inquired whether nucleic acids do combine with polysaccharides.

Prof. M. STACEY replied that in isolating material containing polysaccharide and nucleic acid, one generally needs to work under reducing conditions. The linkage seems to be broken very easily with solvents in the presence of oxygen, and in isolating the pneumococcus polysaccharides it appeared to be a matter of luck whether in the commercial preparation some nucleic acid remained in firm combination. He had found 16 per cent in a commercial preparation kindly provided by Dr. Heidelberger. As far as he could ascertain the combination was a chemical one and was firm, since there was a high proportion which could not readily be separated by electrophoresis. It was therefore thought that there was nucleic acid combined with polysaccharide. In one specific complex polysaccharide from *Mycobacterium tuberculosis* treatment with strong sodium hydroxide was needed to split off the nucleic acid.

In regard to the point about transformation going under adverse conditions, it was meant that the transformation went the other way, i.e. Smooth to Rough. Generally, these Rough forms were got initially when the growth conditions were abnormal.

Dr. TAYLOR did not agree that this was true.

Prof. M. STACEY said that it was his experience with several organisms such as *Cl. welchii* that in the transformation of Gram-positives to Gram-negatives, which was closely connected with the Smooth forms going to Rough forms, it was under adverse growth conditions that the Gram-negative stage set in and lytic enzymes began to function.

Dr. TAYLOR stated the Rough form was a very Gram-positive form.

Prof. M. STACEY said that this may be so in the pneumococcus group but Gram-positiveness depends on ribonucleic acid, which was not necessarily connected with polysaccharide synthesis.

Dr. TAYLOR agreed with this.

Prof. M. STACEY then said that deoxyribonucleoprotein did not normally stain under conditions of the Gram reaction; that is to say, if the deoxyribose type nucleic acid was in the long fibrous

form, it could not be put back on to suitable bacterial protein to make a Gram complex. On the other hand, if it was broken down to the same molecular size as the ribose type, then it might recombine with the protein. It was thought that in the future one would need to determine the connections between the ribose type and the deoxyribose type nucleic acids in order to throw light on the build up, breakdown, transformation and so on of bacterial cells.

Dr. K. A. BISSET asked if Dr. Stacey would elucidate further his reference to violent controversies regarding bacterial structure and cell division. It was well known that morphologists wasted a lot of time which could much better be spent on chemistry, but he had not realized that they aggravated the offence by disagreeing violently among themselves. Since he had himself published a number of works on this subject, and was presumably deeply embroiled in this controversy, he wished to know with whom he had been disagreeing, and which side of the question he had been supporting.

Prof. STACEY thought Dr. Bisset would admit that there were all sorts of arguments for and against in this matter. The main arguments were whether a nucleus in a bacterium could or could not be stained. Nobody seemed to have any chemical explanation of the processes involved in staining so-called nuclei in bacteria.

Dr. BISSET replied that this controversy was rather between those who had succeeded in demonstrating the bacterial nucleus and those who, having failed to see it, usually because of unsuitable techniques, denied its existence. If the study of this question were confined to the chemical approach there might indeed be a diminution in controversy, because in the absence of a morphological conception of the nucleus there would be nothing about which to disagree.

Prof. J. R. MARRACK asked for an explanation of the mechanism by which the nucleic acid on association with the polysaccharide gives an antigen and so, by evoking antibody formation, affects the globulin metabolism of the animal which is immunized.

Prof. STACEY replied that this combination merely modified both the size and shape of the antigen and so made it the right shape to fit into the enzyme system which is making globulins.

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IV

THE OSMOTIC BARRIER IN BACTERIA

by P. MITCHELL

ONE of the most important units associated with the cell surface in bacteria is the barrier which effects the osmotic separation of cell interior and the external environment. Several names have been attached to this unit, but the associations of these with functions other than the osmotic one make it advisable to refer to it as the osmotic barrier. The osmotic barrier is a functional unit, it is defined by means of its function, and as its dimensions lie at or beyond the limits of visible microscopy and its properties are not such as to make it easily distinguished from the continuous tissue, its exact location in the cell is difficult to determine. It is usually supposed to be situated at the interface between the basophilic cell body and its weakly staining envelope, the cell wall; and because of the staining properties of this interface it has been regarded as a real structure and called the cytoplasmic membrane (Knaysi 1946 a, Bisset 1948 a and b). Electron micrographs sometimes show a structure, particularly in Gram-negative organisms, which has been interpreted as the cytoplasmic membrane, but as this generally possesses only one boundary, it would be difficult to distinguish it from the external margin of the cytoplasm (Mudd and Anderson 1944). In those organisms which will plasmolyse, however, the cleavage occurs between the cell body and wall, leaving little doubt that the osmotic barrier is situated at the so-called cytoplasmic surface (Knaysi 1946 b). It is, of course, necessary to bear in mind that the barrier has a finite thickness (5 mμ as a minimum, probably 100 as a maximum (Danielli 1942)) and that a part of this thickness may be occupied by a supporting structure which has no osmotic function and may lie outside the osmotically operative part of the barrier. It may even be that with the economy typical of living organisms, such an external structure might combine other functions with those of mechanical support (Rothstein and Meier 1948).

With the few exceptions which are usual in such generalizations, only Gram-negative organisms will plasmolyse. In Gram-positive organisms, the 'wall' and 'cytoplasmic membrane' cannot be separated by osmotic forces, and so in this case there is more doubt about the situation of the osmotic barrier.

Passive Properties of the Barrier

When the cell is in a resting condition, the passive function of the osmotic barrier is to impede the osmotic equilibration between the internal environment of the cell and the external environment. The competence with which this function is carried out can be directly assessed by determining the rate of equilibration of the diffusible substances across the barrier when the concentration differences between the internal and external environments are known.

If it is assumed that the osmotic barrier is the only impediment to the movement of a given substance across the cell surface, the rate of change of concentration of that substance inside the cell is given by the relationship

$$\frac{dC_i}{dt} = k\frac{A}{V}\left(C_e - C_i\right)$$

where C_i and C_e are the internal and external concentrations, A the area of the barrier, V the volume it encloses, and k is a constant with the dimensions of velocity and called the permeability constant.

When the substance penetrates during a time t,

$$ln\frac{C_e}{C_e - C_i} = k\frac{A}{V}t$$

and the permeability constant can be conveniently determined by finding the time for half equilibration, when

$$ln\,2 = k\frac{A}{V}t.$$

It will be noticed that for a given permeability constant, the time for half equilibration is inversely proportional to the constant A/V, the ratio of surface area to volume. Because of their small size, this ratio is particularly high for the bacteria. It is, for

instance, of the order of 1,000 times as high as for the alga *Chara ceratophylla*; so that if the permeabilities of the surfaces of bacteria are of the same order as for *Chara*, osmotic equilibration will be about 1,000 times as rapid for the bacteria. It is a matter of some interest to determine whether the high A/V ratio is compensated by a correspondingly low value of the permeability of bacterial surfaces, or whether the rapid leakage is normally balanced by a correspondingly rapid restoration, dependent upon the comparatively vigorous metabolism of bacteria.

Using the direct method, Collander (1937) has determined the permeability of the osmotic barrier of an unidentified coliform organism to malonamide, urea, glycerol and erythritol. The values, shown in Table I, were considered by Collander to fall into line with his measurements on plant cells in providing support for a modified form of the lipoid membrane theory of Overton which he refers to as the lipoid-sieve hypothesis.

TABLE I

Permeability constants k (in Å per sec.), partition coefficients α (olive oil-water) and $kM^{\frac{1}{3}}$ values for Collander's coliform organism (Collander 1937) and *Beggiatoa mirabilis* (Marklund 1936). Values of k for 10 mμ thickness of water are given for comparison of retardation.

Substance	Coliform		Beggiatoa		α (olive oil-water)	k (10 mμ water)
	k	$kM^{\frac{1}{3}}$	k	$kM^{\frac{1}{3}}$		
Urea	83	640	1580	12200	0·00015	89×10^{10}
Methylurea			1170	10100	0·00044	81×10^{10}
Malonamide	28	280			0·00008	69×10^{10}
Glycol			1390	11000	0·00049	86×10^{10}
Glycerol	55	530	1060	10200	0·00007	72×10^{10}
Erythritol	5	55	838	9300	0·00003	62×10^{10}
Sucrose			135	2500	0·00003	37×10^{10}

It has been pointed out by Longsworth (1933–4), that 'in the derivation of the equations which describe molecular diffusion processes, it is customary to assume that the velocity of migration v_i of the *i-th* constituent is proportional to the chemical potential gradient, $\frac{d\mu_i}{dx}$ of that substance

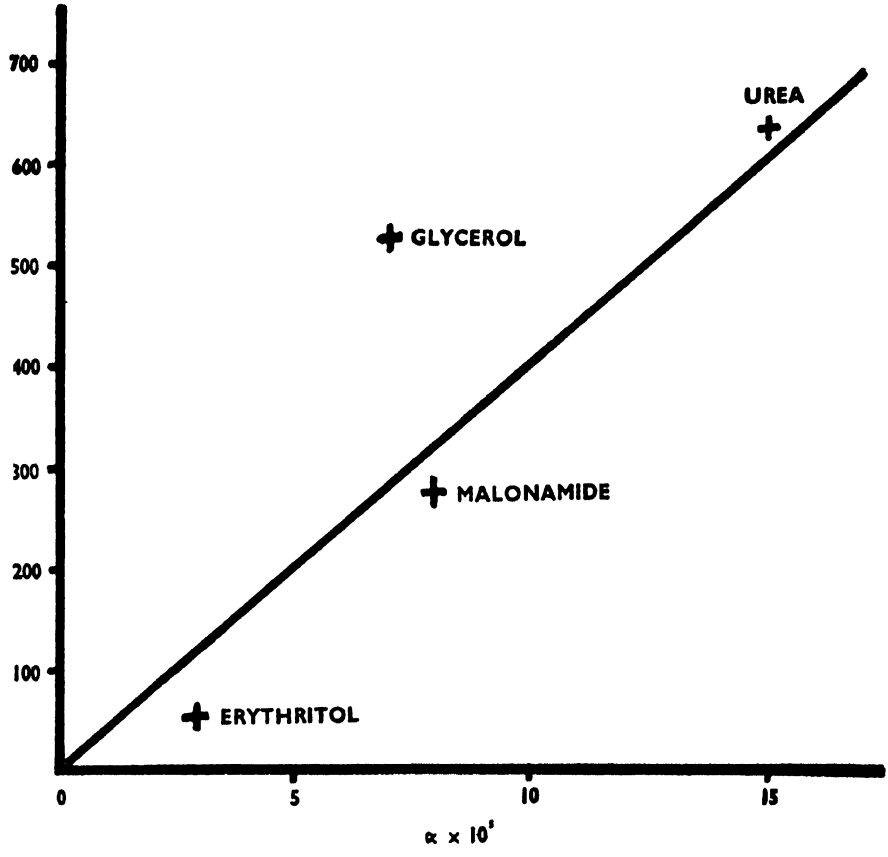
$$v_i = u_i \frac{d\mu_i}{dx}$$

u_i being the factor of proportionality or mobility. . . . According to this equation, the velocity of a constituent is proportional to the gradient of chemical potential, the latter being continuous at a phase boundary . . ., but the flux of a constituent is determined not only by its velocity but by the product of this into the concentration which is usually discontinuous at a phase boundary. It thus appears that the driving force which causes diffusion across the non-aqueous layer will be determined largely by the composition of the two aqueous phases, whereas the flux of material will depend in part upon the concentration of the diffusing substances in the non-aqueous layer. Thus, other conditions being the same, a given difference of composition between the internal and external environment will cause a greater flux of some constituent through the non-aqueous layer the more soluble that constituent is in the non-aqueous layer.' This has been stated in a more involved manner by Danielli (1943 a) and combined with the diffusion law of Thovert in the form that $kM^{\frac{1}{2}}$ is, to a first approximation, a linear function of the partition coefficient α of the penetrating molecule of molecular weight M between the lipoid and the water, when the osmotic barrier is a lipoid membrane.

The values of $kM^{\frac{1}{2}}$ and of α for olive oil-water are given in Table 1, and the plot (Text-fig. 1) of $kM^{\frac{1}{2}}$ against α shows that the behaviour of the barrier of the coliform organism studied by Collander approximates to that of a lipoid membrane.

The main alternative to the lipoid membrane hypothesis is that of the molecular sieve. This is conceived as a system of holes in an otherwise impervious surface, the retardation being determined by the ratio of the impervious area to the effective area of the holes. According to Danielli (1943 b), if it is assumed that the osmotic barrier of Collander's coliform organism is a molecular sieve about 5 mμ thick, since 'its membrane has an area of $3{\cdot}4\times10^{-8}$ cm.^{2} and a permeability of about 10^{-11} of that of a similar water layer, to molecules such as urea. If molecules were to penetrate through water-filled pores, the area occupied by the pores must be $3{\cdot}4\times10^{-8}\times10^{-11}$ cm.^{2}=34 $\mu\mu^2$, i.e. an area less than a molecular area, so small that even if the whole of this area were in one part it would be too small for any molecule to penetrate. Its membrane consequently cannot be an inert mole-

cular sieve, unless it is enormously thicker than most plasma membranes.' At first sight this argument seems conclusive, but



TEXT-FIG. 1.

The plot of $kM^{\frac{1}{2}}$ against α for Collander's coliform organism.

on inspection it appears that it proves that if urea penetrates the barrier at 10^{-11}, the rate of penetration of a similar water layer, it does not penetrate at all! It is, of course, necessary to take into account the area of a penetrating molecule in assessing the effective area of the holes, the effective area of each hole being the real area less that of the penetrating molecule. Judging from the areas occupied by straight chain ureas and monoglycerides in condensed surface films (Adam 1941), the area of the hole required for the penetration of malonamide, urea, glycerol and erythritol will be nearly the same in each case, namely about

0·26 mμ^2. But we would expect there to be a retarding effect due to the restriction of the free movement of the molecule as it goes into the hole, and this effect should be greater the longer the molecule. Bearing in mind that, dependent upon the composition of the sieve, bonding of some kind between penetrating molecules and the walls of such small holes is very likely, it is not easy to escape the conclusion that some such reasonably realistic sieve system might be held to account for the observed values of the permeability of Collander's coliform organism. There seems, in fact, to be only one difference between the lipoid membrane and the molecular sieve hypotheses when the holes in the latter are considered to be very small. In the former it is supposed that as a result of thermal agitation, the material of the barrier yields before the penetrating molecule and closes behind it, while in the latter the path of the penetrating molecule is already traced and maintained by the rigidity of the substance of the barrier. In either case, the composition of the barrier is of prime importance, and it is very doubtful whether even quite detailed knowledge of the permeability properties will provide much information about the organization of the barrier in the absence of detailed information about its composition and physical properties obtained by more direct means. Until such information is available, it is wise not to visualize the penetration process in terms of either extreme but to accept a lipoid-sieve image.

This opinion is strengthened by the fact that while Collander's coliform organism has been held to show a lipoid membrane, the accurate figures obtained for the large sulphur organism *Beggiatoa mirabilis* by Marklund (1936) (Table 1) indicate that for five out of the six substances to which the permeability of the cells were measured, the values of $kM^{\frac{1}{2}}$ are constant and independent of the lipoid-water partition coefficients, and that penetration takes place through water-filled pores. Danielli has pointed out, however, that there are several alternative explanations, and that it is not possible to decide between them until more direct evidence is collected about the nature and location of the barrier or barriers in these cells.

From the point of view of the normal functioning of the organism, the competence with which the barrier impedes the osmotic equilibration between the internal environment of the

cell and the external environment is of more consequence than the composition of the barrier. If we consider its thickness to be of the order of 10 $m\mu$, the retardation imposed by the barrier of the coliform organism is of the order of a factor of 10^{10} and that of *Beggiatoa* about 10^9. While the retardation in *Beggiatoa* is of the same order of magnitude as for most other cells that have been studied, that of the coliform organism is about one order of magnitude higher. Thus, the very high A/V ratio of the coliform organism is partially compensated by the low values of the permeability constant.

In the course of work on amino-acid metabolism of bacteria, Gale (1947) has made some direct permeability measurements of *Streptococcus faecalis* (Lancefield Group D) to lysine and glutamic acid. The resting cells of the streptococcus resemble red blood corpuscles in being only very slightly permeable to free glutamic acid. For lysine, however, the time for half penetration was found to be about 10 minutes at pH 7·2 and 37°. The rate of penetration of the isoelectric amino acid at pH 9·5 was approximately double this, and taking the cell area as 3×10^{-8} cm.^{2} and the volume 5×10^{-13} cm.^{3}, the permeability constant of the zwitter ion comes out as 5×10^{-8} cm./sec. Evidently, the barrier of the streptococcus, like that of the unidentified coliform organism, gives a retardation of the order of a factor of 10^{10}. The temperature coefficient for the penetration of lysine was found to be 1·4, slightly higher than that for free diffusion, and indicating an activation energy of only 6,000 cals. for the penetration process when this takes place inwards. On the other hand, it was found that when osmotic equilibrium was reached in lysine, the internal concentration of the amino acid was usually about ten times as great as the external concentration and that the rate of penetration of the amino acid outwards was extremely low. Presumably the activation energy of the penetration process is higher when this takes place outwards. Unfortunately, the temperature coefficient of this process has not been determined.

Although detailed information is not available, it seems that the permeability of most Gram-positive organisms to glutamic acid and lysine are similar to those of the *Str. faecalis* (Taylor 1947). It is a pity that we have no comparative values for Gram-negative cells.

Practically the only qualitative observations on the differences of the permeabilities of bacteria which are clear cut enough to be relied upon are those which show the low permeability of the osmotic barrier of the mycobacteria to dyes. It seems here that the retardation produced by the barrier must be at least one order of magnitude higher than for other bacteria (Yegian and Vanderlinde 1947) and would completely compensate for the high A/V ratio. It is, perhaps, more than a coincidence that the mycobacteria have generally a rate of growth and metabolism which is substantially less vigorous than that of other bacteria. Teleologically, we may consider that the impermeability of their surfaces makes it unnecessary for them to work as hard as the more permeable organisms to compensate the leakage between internal and external environments.

Active Properties of the Barrier

The passive properties of the barrier are such as to impede the equilibration between the internal environment of the cell and the external environment. The equilibrium is nevertheless approached quite rapidly unless processes of active transfer operate to balance the rate of loss and appearance of the substances within the cell. Such active processes have been shown to operate in the passage of glutamic acid through the surfaces of Gram-positive organisms (Gale 1947, Taylor 1947). A resting suspension of cells behaves as if it were only slightly permeable to glutamic acid, but if an energy source such as glucose is provided, after a short delay the glutamic acid passes rapidly into the cells. When the internal concentration of glutamic acid has risen to a value which is usually about ten times the external concentration, it appears to reach an 'equilibrium' and remains steady. Not only does the metabolism of glucose facilitate the passage of glutamic acid into the cells, but it also allows it to pass out quite rapidly if the external concentration is low; thus lending support to the impression that the steady value which is reached is an equilibrium value. It was found, however, that in the presence of very low concentrations of crystal violet and other dyes, the 'equilibrium' concentration of internal glutamic acid attained was considerably higher than in untreated cells (Gale and

Mitchell 1947). This suggested that the internal concentration of glutamic acid should not be represented as an equilibrium but as a steady-state value which is determined by the competition of at least three rates, namely: the rate of accumulation by an active process which performs osmotic work, the rate of loss by penetration through the osmotic barrier and the rate of loss by chemical change in the metabolic pool. This interpretation was confirmed by the finding that low concentrations of crystal violet raised the steady-state concentration by decreasing the last of the three rates, whereas high concentrations of the dye lowered the steady-state concentration by lowering the first-named rate.

In the accumulation of glutamic acid by the streptococcus, osmotic work is performed, for the 'glutamic acid' passes through the osmotic barrier against a concentration gradient. The free energy required to perform this work is transferred in some way from the glycolytic system. The manner in which the transfer is accomplished is a matter of particular interest because it concerns the connection between metabolism and the organizational activity of the cells.

Although our information is rather limited, in order to have a working hypothesis, I believe it is worth while to consider what is the simplest system by which glutamic acid could accumulate compatible with the experimental facts.

During glutamic acid accumulation, the movement of the 'glutamic acid' molecules through the osmotic barrier must be caused by some kind of force directed inwards at the barrier. The simplest force which we can invoke is that of solution in the barrier. The experimental results seem to show that while the barrier is almost impermeable to glutamic acid, glutamine passes into it and through it more readily. Thus, conversion of glutamic acid into glutamine at the external surface of the barrier would accomplish the first step in the penetration. That this may take place is indicated by the fact that though there is a delay before the onset of accumulation of glutamic acid when the cells are suspended in glutamic acid and glucose, the accumulation starts immediately in glutamine and glucose.

The second step which is now required to cause the internal accumulation of glutamic acid is the reconversion of the glutamine at the inner side of the osmotic barrier into glutamic acid

or into some other derivative which has only a very small solubility in, and tendency to pass through, the barrier. The fact that the internal glutamic acid is able to pass out of the cells (as glutamine?) only when they are glycolysing, suggests that such an interconversion at the inner side of the barrier may take place. Also McIlwain (1946) has shown that streptococci contain a deaminating system for glutamine which is active only during glucolysis.

Thus, the simplest mechanism of accumulation we may propose is conversion of glutamic acid to glutamine on the outside of the barrier, penetration of the glutamine and its reconversion to glutamic acid on the inner side of the barrier. In this way, the work done in converting the glutamic acid to glutamine and back is transformed into osmotic work, and since we are familiar with the way in which the chemical transformations may be linked with the glycolytic system, it is possible to see how the free energy of the glycolysis may be utilized for the accumulation of glutamic acid.

It will be noticed that the permeability of the osmotic barrier is of considerable importance in determining the concentration of internal glutamic acid and thereby presumably in determining the rate of growth of the cells.

The effect of reversible changes in the permeability of the barrier upon the internal environment of bacteria has not been studied, but the irreversible destruction of the barrier has been shown to result in the loss of the diffusible substances and to cause the death of the cells.

Damage to the Barrier

Hotchkiss has shown that the killing of bacteria by surface active agents is accompanied by a type of cytolytic injury which appears to be special to this type of disinfectant (Hotchkiss 1946). The bactericidal concentrations of the detergents are shown to cause a leakage of nitrogen and phosphorus compounds from the cells of staphylococci, streptococci, *Escherichia coli* and baker's yeast; and to reduce the metabolic activity of the cells to a very low level. The depression of the metabolic activity is not due to a direct effect of the detergent upon the enzyme systems, for the concentrations of detergents which are bactericidal are usually

well below those necessary to depress the activity of the enzymes in a suspension of broken cells. The effect is due to the osmotic equilibrium of intern..l and external environments with consequent drastic dilution of metabolically active components. The cells are now no longer able to repair themselves and the autolytic systems rapidly extend the damage.

The initial breakage of the barrier is not, however, accompanied by any obvious morphological changes which can be detected in the light microscope, although it has been reported by Kivela, Mallmann and Churchill (1948) that under dark ground illumination, cells that have been killed with cationic detergents have a higher scattering power ('refractive index') than normal, and this appears to be confirmed by Hotchkiss' turbidity figures.

Results similar to those of Hotchkiss have been obtained on the leakage of amino acids from streptococci caused by bactericidal concentrations of various detergents; and it has been shown by means of the electron microscope that for the cationic detergent tyrocidine, the amount which is just bactericidal causes damage to the surface of the cells. The external envelope is apparently cleanly removed, the body of the cells, however, remaining practically normal (Gale and Taylor 1947, Mitchell and Crowe 1947). Similar morphological changes are caused by long-chain fatty acids and also by phenol in bactericidal concentrations. The effect of phenol on the cell surface is not instantaneous, but requires about an hour to become complete at a concentration of 1 per cent (unpublished observations of Mitchell and McQuillen), and it is interesting to note that the leakage of amino acids in 1 per cent phenol follows the same slow course. Presumably the sharpening of the boundary of the cells which the electron micrographs show to occur in the detergents, and the consequent increase in reflecting power, is one of the factors which is responsible for the increased light scattering which has been observed.

Provided that the concentration of tyrocidine is as great as about 1/50,000, there is a linear relationship between the number of cells lysed in a given suspension and the weight of tyrocidine present, independent of its concentration. In this case, the breakage of the osmotic barrier must therefore be an all or none process. This kind of effect is not peculiar to bacteria, but

occurs also in the denaturation of proteins with detergents, a proportion of the protein exercising its full combining power while the remainder remains native (Putnam 1948).

The mechanism of the process probably depends upon the exposure of hydrophobic groups (belonging either to detergent or to the structure attacked) by the combination of the first few molecules of detergent. In this way, the probability that a molecule of detergent will be taken up by a cell or by a molecule of protein is much greater for those in which the process of breakage has begun than for those in which the initial activation has not occurred, so that some exercise a high combining power and reduce the free concentration of detergent to the level where the remainder will be free from attack.

The work on the denaturation of proteins shows that the primary binding of detergent depends upon electrostatic interaction and ion exchange, the binding of detergent being greatest when the coulombic forces are greatest and consequently showing the expected pH denaturation relationships. With anionic detergents, binding is greater when the protein is in a more acid solution and is consequently more cationic, and for the cationic detergents, binding is greater in more alkaline solutions, when the protein is more anionic. Similar relationships have also been thought to hold for the breakage of the osmotic barrier and disinfection of bacteria by detergents (Glassman 1948). Quisno and Foter (1946), however, report that cetyl pyridinium chloride is almost equally bactericidal over the range from pH 2 to pH 10. Measurements by M. J. Salton (to be published) show that for the cationic detergent Cetavlon, the bactericidal efficiency-pH relationships are different for Gram-positive and Gram-negative organisms; with the Gram-positive, the expected pH relationship is obtained, but the Gram-negative show greater sensitivity the lower the pH. The osmotic barrier of the Gram-negative organisms seems, therefore, to be more readily attacked by the cationic detergent the less negatively charged the barrier becomes. This appears to be somewhat of a paradox if we accept the idea that the coulombic forces are important in the attachment of detergent. It seems likely, however, that the osmotic barrier is a composite structure containing both anionic and cationic components.

In visualizing the titration of bacteria and of its effect upon the adsorption of dyes by ion exchange, it has been customary to consider that the ionisable complexes of the surface have one ion, such as a protein ion, which is fixed in the surface and the other, such as a magnesium ion, which is free to migrate out from the surface in exchange, say, for a basic (cationic) dye molecule. If the pH is lowered, the magnesium ion is replaced by hydrogen ions which show a smaller tendency to dissociate from the protein ion and consequently less tendency to exchange for the dye molecule; so that less cationic dye is supposed to be taken up as the surface becomes less negatively charged (Dubos 1946). If we consider, however, that both ions of the complex are anchored in the surface, and that their association forms an essential link in the osmotic barrier, the change of susceptibility to attack of that link with pH will depend upon the relative degrees to which the two component ions of the complex tend to titrate. The only merit of this explanation is that it is probably the simplest that can be advanced to account for the slope of the pH curves. But whatever the real mechanism, the least that can be said is that it must depend upon a change of susceptibility of the barrier to breakage with pH and not simply upon the change in the overall affinity for detergent.

The idea that the cell surface contains positively charged areas in spite of the predominant net negative charge at physiological pH values, assists also in explaining the susceptibility of Gram-positive organisms to lysis by anionic detergents when they bear a large net negative charge. It is just as likely, however, that these detergents operate by disorganizing lipoid constituents of the osmotic barrier, with which they associate by van der Waals' forces in spite of coulombic repulsion (Burdon 1946). But though in this event the charge on the detergent does not assist in the process of adsorption, it apparently assists in the disorganization of the barrier, because the nonionic agents such as Tween 80, although adsorbed on the cells, are nevertheless very inactive as bactericides.

It has been shown by Dubos and his collaborators that Tween 80 at a concentration of 0·05 per cent causes cultures of tubercle bacilli to grow diffusely instead of forming granules or flakes. This dispersing action of the detergent was stated to be accom-

panied by a substantial increase in the rate of growth resulting from an increase in the permeability of the cells to the nutrients of the medium. Sattler and Youmans (1948) have not, however, been able to confirm these findings. They observed even slight inhibition of the growth of a virulent strain of tubercle bacilli in Dubos' medium. On the other hand, Youmans and Youmans (1948) showed that the tuberculostatic activity of 15 out of 20 compounds was increased to a small extent in the presence of Tween 80, and this might be interpreted as an indication of increased permeability of the cells to the compounds tested. It seems quite as likely, however, that the increased penetration occurred simply as a result of the dispersion of the cells in the Tween-containing medium; for in the absence of Tween 80, the compounds had to penetrate into the relatively thick surface growth, whereas in its presence the diffuseness of the growth allowed direct access to the cells. It is difficult to see why, as a result of an increase in the permeability of the osmotic barrier of the organisms, which might be caused by Tween 80, the advantage gained by the more rapid inward diffusion of nutrients should necessarily outbalance the disadvantage of an increased loss of internal diffusible constituents.

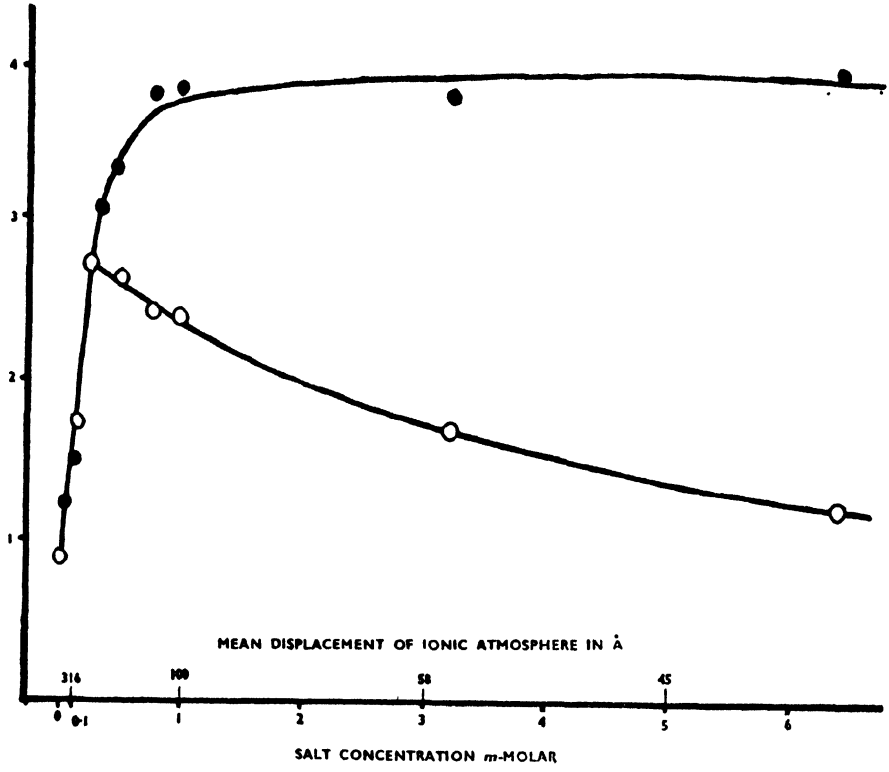
The Depth of the Cell 'Surface'

Although a number of salient facts can be established about the behaviour of the osmotic barrier in bacteria, the more direct physical data have so far provided very little information about its composition and location. The immunochemical and enzymic evidence is likely to lead to more specific information about the composition of the surfaces of bacteria, and to point the importance of the fact that the cell 'surface' has a depth.

In the physical field, however, the experimental electrophoresis studies of Moyer (1936) and of Stearns and Roepke (1941) on Rough and Smooth forms of *E. coli* and *Brucella abortus* provide data bearing on the 'depth' of the cell surface and the location of the osmotic barrier, which have not so far been interpreted.

The plot of electrophoretic mobility against salt concentration for the Rough and Smooth forms of *E. coli* are shown in Text-fig. 2. The curves for Rough and Smooth forms of Brucella are substantially the same.

It will be observed that, at high salt concentration, the Rough form has a high mobility while that of the Smooth form is



TEXT-FIG. 2.

Mobility-salt concentration curves of Rough (●) and Smooth (O) forms of *Escherichia coli* after Moyer (1936).

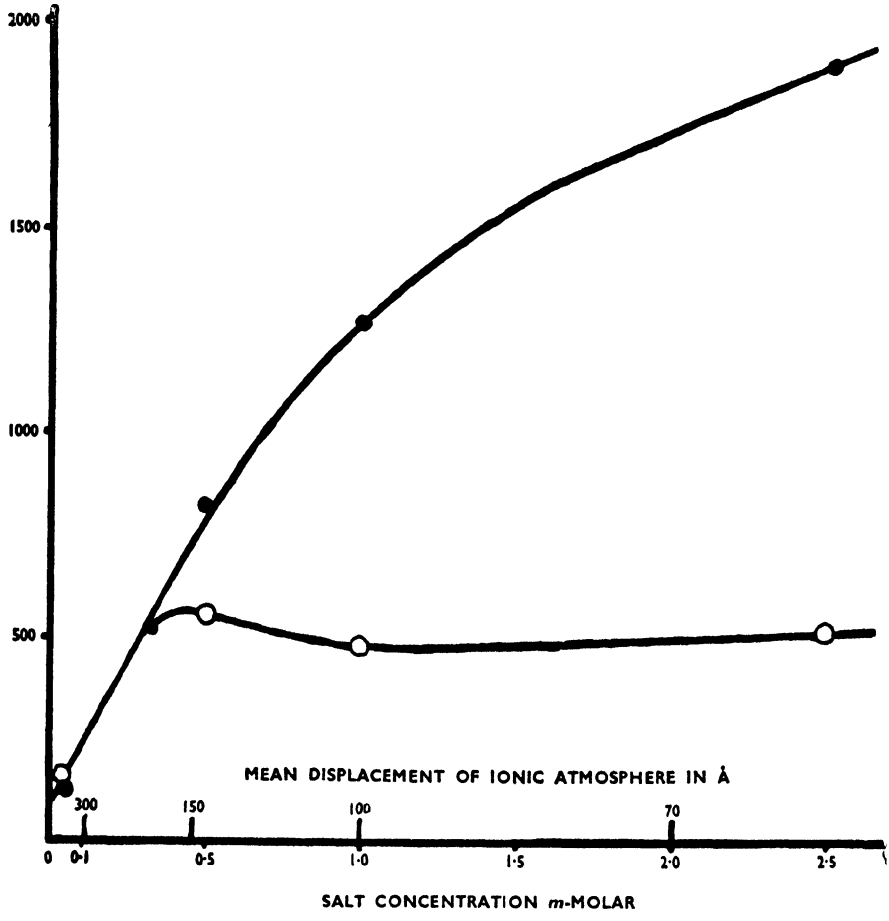
relatively low. But as the salt concentration is lowered, the mobilities of the two forms converge until, at a concentration of between 10^{-3} and 10^{-4} M uni-univalent salt, their electrophoretic behaviour becomes almost the same.

When a particle of a substance, which gives a more or less two dimensional surface, is in contact with a water phase, as a result of the physical forces between the aqueous phase and the material of the particle, and of the effects of thermal agitation, there is an exchange of ions across the interface. Some of this exchange is due to adsorption of ions from the water phase upon the particle surface and some is contributed by the ionization of the material

of the particle, but the final result is the displacement of ions across the interface causing an equal and opposite charge to appear upon the particle surface and in the water. The charges on the particle surface are fixed, while the ions carrying the excess charge in the water form a diffuse cloud about the particle because of the competition of coulombic attraction forces and the dispersing forces of the thermal agitation. The effective mean displacement of the ion cloud from the surface is dependent upon the salt concentration in the water phase, being inversely proportional to the square root of the concentration for uni-univalent salt, i.e. less the higher the salt concentration.

When this model system is placed in an electric field, the particle and ion cloud move in opposite directions, the water undergoing a shearing motion between the cloud of ions and the particle surface. If, however, the particle were covered by an envelope of some material which, while preventing the shearing motion of the water, would allow the exchange of ions between the water phase and particle surface, that part of the ion cloud situated inside the envelope would subtract its value from the net charge of the particle. With such a system at very high salt concentrations, almost the whole of the ion cloud from the particle surface would be situated inside the envelope and the mobility would therefore be practically zero, while, on decreasing the salt concentration, the ion cloud would move out through the surface of the envelope until the effective mean displacement of the ion cloud from the particle surface exceeded the thickness of the envelope, when the particle would migrate almost as if the envelope were not present. With this model in mind, and noting the calculated values for the effective displacement of the ion cloud (thickness of double layer) in Text-fig. 2, the implication is obvious. The Rough form of *E. coli* studied by Moyer has a fairly highly charged surface which, in the Smooth form, is covered by an envelope about 5 mμ thick with the properties of the envelope of our model, modified only in that it carries a small charge. (The method of evaluating the thickness of the layer will shortly be published in another place.) Added support for this interpretation of the electrophoretic observations is given by the charge density-salt concentration figures obtained by Stearns and Roepke.

Text-fig. 3 shows the Rough strain (strain 80) of Brucella and a Smooth strain (strain 517) for which the electrophoretic data



TEXT-FIG. 3.

Charge density–salt concentration curves for Rough (strain 80) (●) and Smooth (strain 517) (○) forms of *Brucella abortus* after Stearns and Roepke (1941).

are given by Stearns and Roepke, but the figures for the latter were not plotted by them. Both Stearns and Roepke and Moyer interpret the surface density of charge-salt concentration curves as adsorption isotherms. The fit of the theoretical curves on their points is not good for those which they plot, but for strain 517 of *Br. abortus*, the very large deviation in the region of 5×10^{-4} M

salt leaves little doubt that for the Smooth forms at any rate a different interpretation is required.

Evidently, in the Smooth forms of these Gram-negative organisms, the external envelope (wall?) plays little or no part in osmotic regulation, shows only a low charge density and does not interfere substantially with the electrical properties of the underlying Rough surface. Since ions do not move freely through the osmotic barrier, the external margin of the barrier must be situated at or below the external surface of the Rough forms and 5 mμ or more below that of the Smooth variants. More detailed information of this type could almost certainly be obtained from suitably designed electrophoresis experiments.

Electrophoretic studies on *Str. faecalis* (McQuillen and Mitchell 1948) suggest that the organization of the surface of the Lancefield Group D streptococcus is rather similar to that of the smooth form of *E. coli*.

To summarize, the evidence suggests that Claude Bernard's concept that all free living organisms must possess a relatively isolated internal environment is true for the bacteria.

As well as offering passive resistance to the movement of substances into and out of the cell, the osmotic barrier is the seat of processes of active transfer which maintain the differences of composition of the internal and external environments in actively metabolizing or in growing cells; and the steady concentrations of the substances within the cell generally represent steady-state conditions and not equilibria.

The barrier is probably about 10 mμ thick, and probably lies at the so-called cytoplasmic surface. The permeability values which have been obtained indicate that retardation by a factor of about 10^{10} takes place if the barrier is 10 mμ thick, and it seems likely that this large retardation would have to be caused by a non-wettable layer.

Our knowledge of the anatomy of the osmotic barrier is extremely poor because, even if we possessed quite detailed information about its permeability, we should still not safely be able to infer very much about its composition and structure. The more direct anatomical information must be sought in the fields of immunochemistry and in the study of specific enzymic

degradation of the surface and other relatively direct methods. Amongst the direct methods the technique of electrophoresis may be able to assist in describing the three-dimensional layout of the surface.

Discussion

Dr. K. A. Bisset asked whether the layer mentioned in the last part of Mr. Mitchell's paper had definitely two surfaces as well as thickness.

Mr. Mitchell said that the retarding forces on a particle migrating in an electric field are caused by the shear in the medium near to the surface, and the force which moves the particle is determined by its net charge. If we imagine an envelope which holds some of the external cloud of ions that is attracted near to the surface, it will thereby reduce the net charge on the particle and decrease its rate of migration.

A particle like the Smooth bacterium can be regarded as a Rough bacterium with a surface of Smooth substance which has thickness but not necessarily definable boundaries. When the Smooth bacterium moves, say, through water, then the water does not shear in the Rough surface, but at the external margin of the Smooth surface. The inner margin of the Smooth envelope presumably lies on the Rough surface, and the external surface would be about 5 mμ away in Smooth *E. coli*.

Dr. Bisset suggested that this thickness was not that of the semi-permeable membrane (given in the early part of the paper as also having a lower limit of thickness of 5 mμ) but of something lying outside.

There was evidence that the cell membrane has a definite thickness of its own with a front and a rear surface; because in incipient division a cell forms a transverse septum, which is a visible structure with cytoplasm on both sides. It must be an extension of the inner surface of the cell membrane. Therefore, the cell membrane itself must have a definite thickness of its own, quite apart from any estimates of thickness depending on an external boundary whose position is dubious.

Mr. Mitchell said that it would not be possible for a structure not to have a thickness.

Dr. Bisset replied that the thickness need not be defined

inwardly by a surface. Mr. Mitchell had suggested that it is not known whether the cell membrane has an inner surface or not; whereas it appeared anatomically to have an internal surface, because when bounded by cytoplasm on both sides and not only on one side, it was still a positive structure.

Mr. MITCHELL said that, judging from the permeability properties, he would expect the osmotic barrier, which may be identical with the cytoplasmic membrane, to be about 10 mμ thick, which happens to be of the same order of magnitude as that of the thickness of the Smooth layer.

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V

ON THE MECHANISM OF ADSORPTION OF BACTERIOPHAGES ON HOST CELLS

by T. F. ANDERSON

IN the cases which have been carefully studied bacteriophages are adsorbed only by strains of bacteria that are capable of supporting their multiplication. The mechanism of the adsorption process must therefore be related in an intimate manner to the molecular architecture and function of the surfaces of the two.

Early work suggested (Delbrück 1942) that the adsorption of virus particles on host cells is mediated by the steric fitting of specific and therefore complicated structures of the virus to complementary structures (receptor spots) on the surface of the host. This is the mechanism proposed (Pauling and Pressman 1945) for the equally specific antigen-antibody reaction in which the forces involved are supposed to be of the exchange or van der Waals type and fall off as the inverse sixth power of the distance between adjacent atoms of the combining structures. Such forces would be negligible for separation between adjacent atoms of a fraction of an Ångström unit.

Actually a number of observations suggest the inadequacy of the simple receptor spot theory. If the surface structure of virus and host were to be rigidly attached to these bodies and possessed of a degree of complexity compatible with the specificity of the adsorption reaction one might expect that the chances of adsorption in a random collision between the two would be very slim indeed—like throwing a key at a door and having the key stick in the keyhole. But Schlesinger (1932) showed years ago that a virus particle adheres to the host almost every time the two collide in quiet media. It seems almost axiomatic that the two processes might well be quite different—antibody has merely to combine, phage has a job to do; namely, to penetrate and convert the host cell to virus production.

Burnet and others (see Delbrück 1942) have concluded that

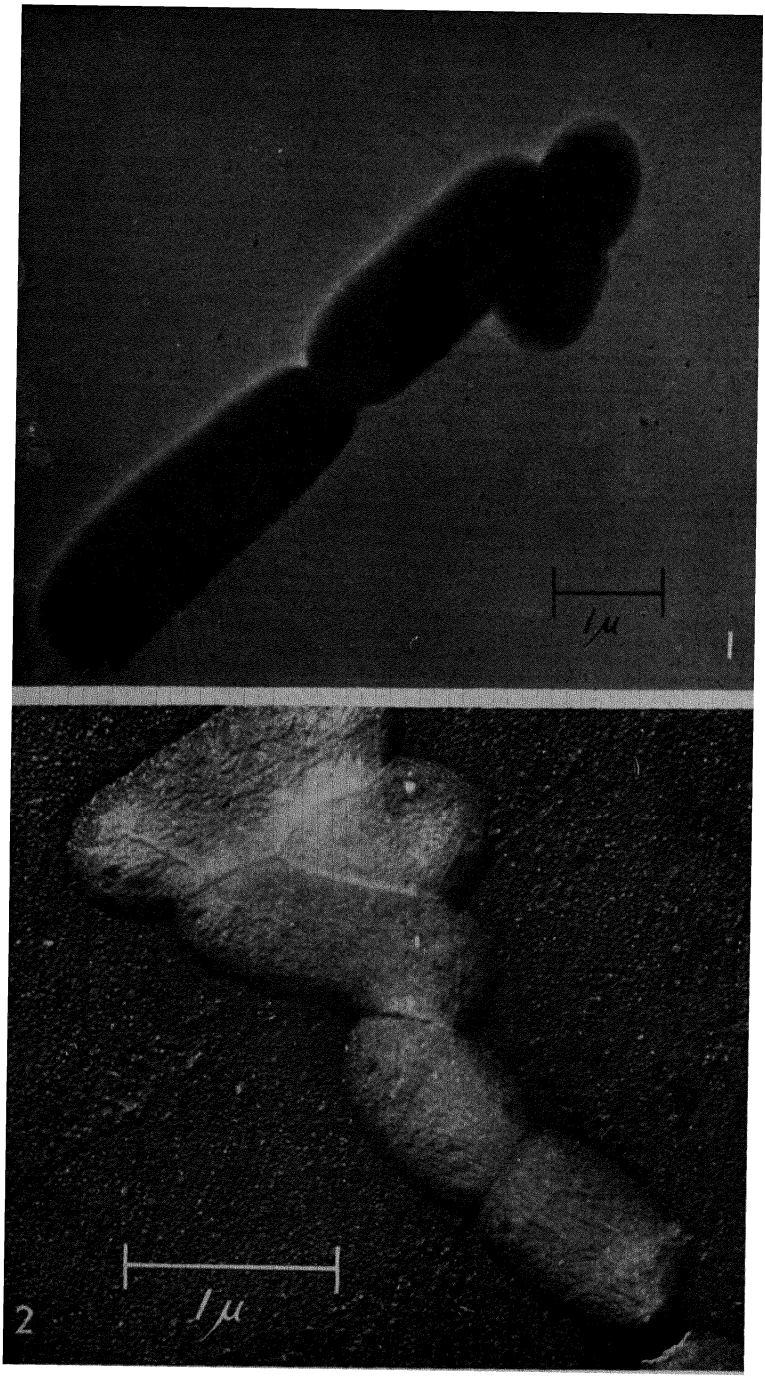

PLATE 2.

Fig. 1. *Escherichia coli* strain B grown on the supporting collodion membrane as seen in the electron miscroscope, showing tenuous threads of capsular material radiating from cells, the cell walls and the outlines of the cytoplasm. Fig. 2. *Escherichia coli* strain B shadowcast with gold. The surfaces of the cells seem to be covered with fuzzy layers of material. (Dr. T. F. Anderson.)

surface polysaccharides are important in adsorption, for cell-free extracts containing these substances inactivate those phages which were active on the cells that had produced the polysaccharides in question. Beumer (1947) has recently shown that the inactivating substances derived from different host strains but active against the same phage have similar but not identical antigenic properties.

In a number of cases susceptibility to phage action has been correlated with Smoothness or Roughness of the colony type, the colony type being determined by the presence or absence of surface polysaccharides. Furthermore, A. Pirie (1940) has observed that after treatment with lysozyme, a strain of *Bacillus megatherium* no longer adsorbs a phage which had been adsorbed on the untreated cells. Presumably in this case the surface mucopolysaccharides which are attacked by lysozyme are essential to the activity of this virus on the cells.

In the past few years a group of workers in the United States and France have chosen to concentrate their attention on a set of seven bacteriophages all active on a single non-motile strain 'B' of *Escherichia coli*. Their work up to three years ago has been beautifully reviewed by Delbrück (1946).

The work with this system has indicated new complexities in the nature of resistance and sensitivity of bacteria to phages; and, though the results do not yet give a clear-cut picture of the adsorption process, they seem to put the problem in a new perspective. The physical properties of the seven phages are summarized in Table II. It is seen that morphologically the viruses fall into four groups: three large viruses, *T* 2, *T* 4 and *T* 6, have similar tadpole-shaped structures and belong to the same serological group; the small spherical viruses *T* 3 and *T* 7 belong to another serological group; the large tadpole-shaped virus *T* 5 and the small tadpole-shaped virus *T* 1 are unrelated either to each other or to the other viruses in the *T* set.

The small viruses *T* 1, *T* 3 and *T* 7, which form large plaques, survive treatment by intense sonic vibration, and the large viruses *T* 2, *T* 4, *T* 6 and *T* 5 are relatively sensitive. Another property which relates *T* 2, *T* 4 and *T* 6 is their sensitivity to what might be termed osmotic shock. When these viruses are suspended in 4M NaCl and the ionic strength suddenly reduced by

the addition of large volumes of water, these viruses are destroyed; in the electron microscope the particles are seen to have lost the dense internal structures within the membranes of their heads. On the other hand, when the salt concentration is gradually reduced by the successive addition of small volumes of water the viruses survive. The membranes of the heads of the even-numbered viruses thus have osmotic properties. The odd-numbered viruses do not display this sensitivity to osmotic shock.

In the electron microscope the host cells, *B*, may be seen to have three types of surface structure: (1) a light capsule-like material which may be attached to the cell in long streamers (Pl. 2, fig. 1) or as a fuzzy coating (Pl. 2, fig. 2); (2) the cell wall, which retains the cell's shape after sonic disruption; and (3) the cytoplasmic membrane which bounds the cytoplasm as it shrinks from the cell wall on plasmolysis.

In the process of adsorption, then, the virus must have to deal with one or more of the above surface structures since multiplication must occur within the cell wall (Luria, Delbrück and Anderson 1943). Electron micrographs of mixtures of trytophan-activated *T* 4 virus (Anderson 1948 b) and host cells seem to show a series of stages in the adsorption process. Some particles appear to be just touching the cell wall, but are otherwise visibly unchanged (Pl. 3). Yet others have their tails missing with their heads resting against the host. Still others appear as mere blebs on the host cell wall, most of the internal structure having disappeared, presumably into the cytoplasm of the host. At some point in this process, the reaction becomes essentially irreversible: the virus is committed to forming a virus-host complex from which the infecting particle cannot be recovered; its function now is to produce more virus particles. This is the point at which we speak of the virus as being adsorbed in a physiological sense.

If a large number of host cells are coated with many particles of a virus, say *Tn*, most of the cells lyse, but a few cells (designated *B*/*n*) prove resistant and multiply to form colonies. Luria and Delbrück (1943) have shown in an ingenious but convincing way that these cells arise independently of virus action by sudden stepwise changes or mutations in the inherited properties of the cells. Such mutation must alter the surfaces of the cells, for the virus *Tn* is not adsorbed on *B*/*n*. In many cases cells which

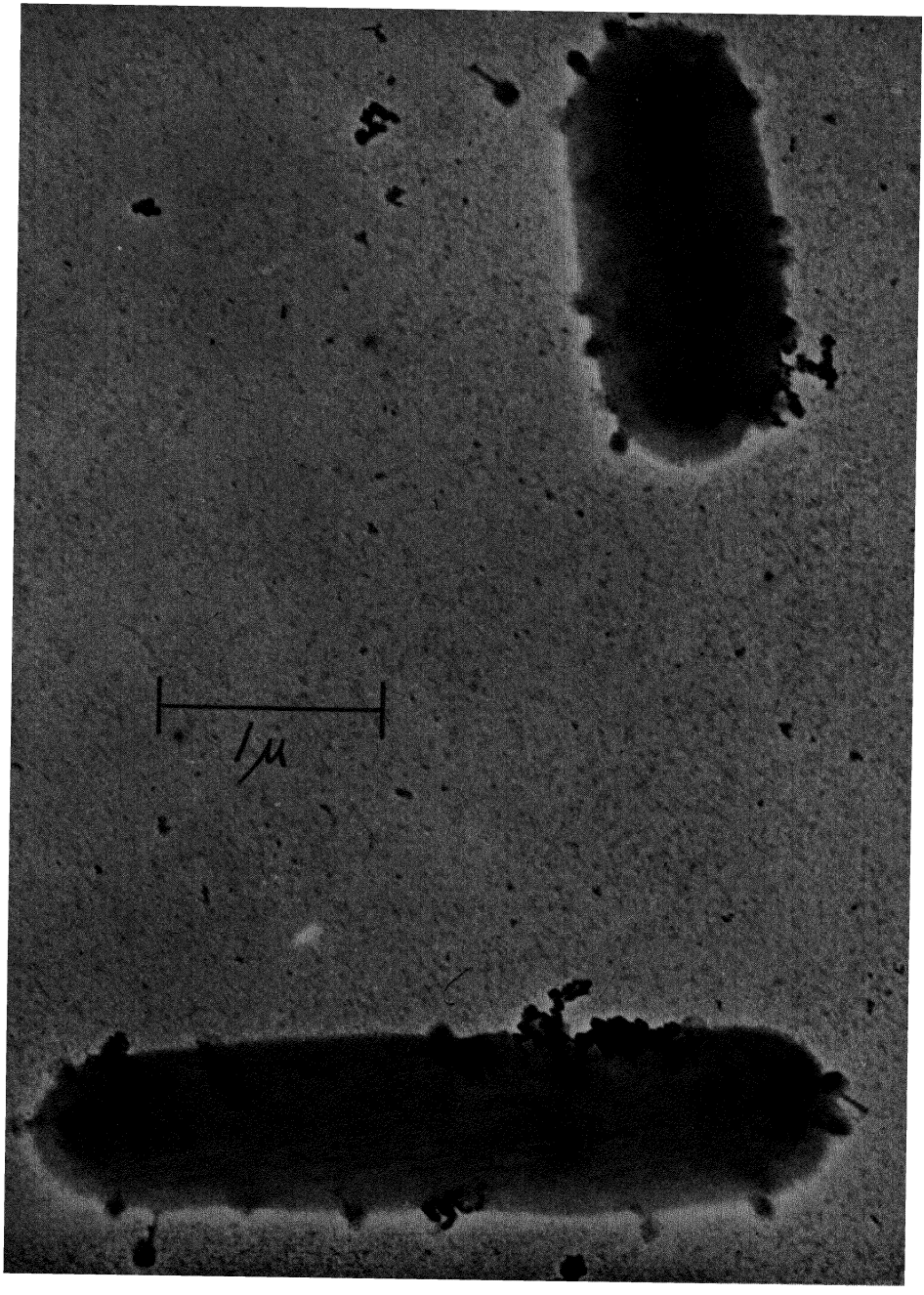

PLATE 3.

A mixture of *Escherichia coli* strain B and tryptophan-activated *T* 4. To 0·9 ml. of ammonium lactate medium containing about 10^9 cells were added 0·05 ml. of *T* 4 ($2{\cdot}5 \times 10^{11}$ per ml.) and 0·1 mg. of L-tryptophan in 0·1 ml. of ammonium lactate. Thirty seconds later the mixture was mounted for study in the electron microscope. Progressive stages in the adsorption of the tadpole-shaped virus particles can be seen. (Dr. T. F. Anderson.)

mutate to resistance to one of the viruses, say *T* 1, prove to be resistant to one of the other viruses in the *T* set as well, say *T* 5. Its character is indicated by the symbol *B*/1, 5. In this way Demerec and Fano (1945) isolated and tested 377 resistant mutants, which on further test fell into the eight different groups indicated in Table II. It may be seen that resistance to a given virus may arise in a number of different ways. We thus have *B*/1, 5 on the one hand which fails to adsorb either *T* 1 or *T* 5; and on the other hand we have *B*/1, tr, which is sensitive to *T* 5, but requires tryptophan for growth; still again, we have *B*/1, 5, 3, 4 which is resistant to and adsorbs none of the viruses *T* 1, *T* 5, *T* 3 or *T* 4. Resistance to *T* 3 and *T* 4 has always been linked; in all mutants so far tested resistance to one implies resistance to the other although *T* 3 and *T* 4 are quite unrelated serologically or morphologically. Multiple successive mutations are readily obtained and eventually a strain of *B* was secured that was resistant to all the viruses in this set of seven.

It has been suggested by E. H. Anderson (1944) that host mutations might appear to link viruses in this way by breaking metabolic pathways at points which are required in common for the multiplication of the several viruses against which the mutation confers resistance. If this were true one might expect the mutations to link the serologically, morphologically and phylogenetically related viruses, which might be expected to have similar compositions and hence similar growth requirements; such linkage is not evident. Another explanation is that these mutations may be simple chromosomal deletions reflecting the proximity of various genes for host sensitivity. Eventually it should then be possible to separate resistance to *T* 3 from that to *T* 4 provided the two viruses have different receptor mechanisms on the host.

Whatever the genetic mechanism of resistance may be it leads to the deletion or masking of the receptor mechanism for the excluded virus: the mutant does not adsorb viruses to which it is resistant (Delbrück 1944). We may express this in another way: the host cell adsorbs to its surface only those viruses whose multiplication it can (or could) support as though its surface contained *frank, specific displays of its range of suitability as a host for the viruses to which it is sensitive.*

On the other hand, the adsorption mechanism is not a sufficient condition for virus multiplication; for virus is adsorbed on heat killed or severely irradiated cells or on cells which already have been infected by an unrelated virus with the result in each case that the newly adsorbed virus fails to multiply and is inactivated. Besides the adsorption mechanism other elements within the cell are therefore required for virus multiplication. The adsorption mechanism may simply prepare the way in some specific manner for the virus to encounter and utilize its intracellular requirements. It is thus possible that mutant resistant cells contain within them the same elements for virus production possessed by the parent strain, but that, in deleting the adsorption mechanism from the surface, the mutation makes these elements unavailable to an extracellular virus particle. Ultra-violet irradiation may be thought of as damaging the necessary intracellular elements which, although still capable of combining irreversibly with an entering virus particle, are now too severely damaged to promote its proliferation (Anderson 1948 a).

The dynamic nature of the adsorption process is suggested by the action of *T* 2 on irradiated cells (Anderson 1945 a). After *E. coli* strain *B* has been irradiated with some hundreds of lethal doses of ultra-violet light, the adsorption of only a few particles of the virus *T* 2 leads to almost immediate lysis of the cell, and incidentally to the inactivation of the virus particle. Such cells are also as susceptible to lysis by lysozyme as is the classical test organism *Micrococcus lysodeikticus*. Since normal cells are apparently unaffected by this enzyme it would appear that the irradiation somehow exposed its substrate to attack. In an attempt to separate a lysin from the main body of the phage particle, the virus was exposed to intense irradiation, a treatment known to disrupt the virus. Tests of supernatants after high speed centrifugation of such treated virus suspensions showed the presence of a lytic activity greater even than that of the untreated control virus. The virus lysin did not act on *M. lysodeikticus*, although in other respects, i.e. sensitivity to iodine and other agents, it resembled this enzyme.

Further work on the virus lysin was interrupted by the discovery of other phenomena which seemed to shed further light on the mechanism of virus adsorption. In the experiments just

described we had selected a medium containing ammonium lactate as the sole source of carbon and nitrogen because it was transparent to the radiation used and supported alike the growth of the host and the formation of plaques by the virus *T* 2. When we turned to a comparative study of the viruses *T* 4 and *T* 6 which, as pointed out previously, are related to *T* 2, we found that our stocks failed to form plaques on this medium, but efficiently formed plaques on nutrient agar. In the ammonium lactate medium *T* 4 and *T* 6 did not lyse heavily irradiated *B* as *T* 2 had done. To make a long story short, it was found that these viruses were not even adsorbed on the host unless the virus particles were first 'activated' by L-tryptophan or by some other aromatic amino-acid. The virus activating substances were therefore called 'adsorption cofactors'. Once activated *T* 4 and *T* 6 were readily adsorbed on heavily irradiated cells of their host and were also able to lyse them as *T* 2 had done (Anderson 1945 b).

L-tryptophan is the most active of the cofactors tested so far, but D-tryptophan and many derivatives of L-tryptophan in which the L-amino acid grouping was tampered with in any way were found to be inactive. The specificity therefore resides in the L-amino acid grouping. Other aromatic amino acids such as phenylalanine, diiodotyrosine and tyrosine show progressively lower activities. In addition, many synthetic amino acids, among them 2- and 5-methyltryptophan, 2- and 3-pyridylalanine, *p*-brombenzylcysteine and *p*-bromphenylcysteine are active (Anderson 1946). Histidine itself is inactive but diiodohistidine shows activity. Oddly enough, in this company, *nor*leucine displays a slight activity (Delbrück 1948).

The cofactors activate the virus particles rather than make the bacteria 'receptive' to the virus (Anderson 1948 b). L-tryptophan appears to be inactive at concentrations below 0·1 mg./l., and the proportion of virus activated at increasing concentrations rises rapidly at a rate which indicates that at least six molecules of the cofactor must be required for activation. The activation by a given concentration of tryptophan is a maximum at 37° and a pH of 7·5.

Besides the original strain of *T* 4 we have isolated a number of lines which appear to require much less cofactor for activation,

so that at 37° but not at 15° they derive sufficient amounts from the medium containing growing bacteria to be activated and adsorbed to form plaques (Anderson 1948 b). Our strains are inhibited only by very large concentrations of indole. But Delbrück (1948) has isolated two additional types: *T* 4,11, which requires L-tryptophan or its analogues and is inhibited by indole; and *T* 4,12 which requires calcium in addition to L-tryptophan or an analogue and is more strongly inhibited by indole. We thus see two metabolic facets in the nature of resistance or sensitivity of a potential host: (1) the host may supply cofactors necessary for certain viruses and be sensitive to them; or (2) it may elaborate substances, as *E. coli* elaborates indole, which block the activity of other viruses and so be resistant to them.

Once our cofactor-requiring strain has been activated by L-tryptophan it can be deactivated by dilution to a point where the tryptophan in the suspensions falls below about 0·1 mg./l. By adding aliquots of such diluted suspensions of activated virus to suspensions of strain *B* at intervals after dilution, the rate of loss of adsorbability of the virus has been followed. The reaction appears to be first order with a rate constant, k, equal to 0·09 sec.$^{-1}$. At the present time it is difficult to say whether the first order nature of the reaction means that the loss of only one tryptophan molecule results in complete deactivation and loss of adsorbability, or whether different classes of virus particles lose activity at different rates to yield spurious results which appear to be first order. Also, it is difficult to tell whether the deactivation involves a simple dissociation of the virus-tryptophan complex or a very slow metabolism of tryptophan by the virus. In any event, once deactivated by dilution, the virus can be reactivated by the addition of more tryptophan.

The rates of adsorption of activated *T* 4 have been measured. In the presence of adequate concentrations of tryptophan the virus is adsorbed on host cells as follows:

$$B + T\,4^* \longrightarrow B : T\,4$$

where *T* 4* represents activated *T* 4 and *B* : *T* 4 is the virus-host complex. The free virus concentration [*T* 4*] decreases with

time at a rate which is proportional to the bacterial concentration, $[B]$.

$$-\frac{d[T\,4^*]}{dt} = k_2\,[B]\,[T\,4]$$

so that at any time after mixing virus and host we have

$$ln\,\frac{[T\,4^*]}{[T\,4^*]_0} = k_2\,[B]\,t$$

where $[T\,4^*]_0$, the initial virus concentration, is less than the bacterial concentration.

Experimentally the adsorption-rate constant k_2 is found to vary in general with the type of phage, the salt concentration and the physiological state of the host cells. For example, using optimum conditions Schlesinger (1932) obtained $k_2 = 3 \times 10^{-11}$ ml./sec. for the adsorption of a phage similar to T 4 on a non-motile strain of *E. coli*. We obtain similar values on $2{\cdot}3 \times 10^{-11}$ ml./sec. for the adsorption of tryptophan-activated T 4 on B.

Now assuming that diffusion of the phage alone determines the collision rate the collision frequency K can be calculated using von Schmoluchowsky's relation

$$K = 4\pi Dr$$

where D is the rate of diffusion of the virus and r is the radius of the host cell. For a spherical particle of diameter d, the diffusion rate is given by

$$D = \frac{RT}{3\pi N\eta d}$$

where R is the gas constant, T is the absolute temperature, N is Avogadro's number and η is the viscosity of the suspension. Combining these two relations we get

$$K = \frac{4}{3}\frac{r\,R\,T}{N\,\eta\,d}$$

for the collision frequency. In the electron microscope r, the radius of the host, appears to be about 4×10^{-5} cm. and d, the effective diameter of T 4, appears to be about 10^{-5} cm. Substitut-

ing into the above relation we obtain for the collision rate constant

$$K = 2{\cdot}4 \times 10^{-11} \text{ ml./sec.}$$

The close agreement between the calculated collision frequency and the observed value of the adsorption rate constant, $k_2 = 2{\cdot}3 \times 10^{-11}$ ml./sec., although fortuitous, clearly indicates that the activated virus *T* 4 is adsorbed on its host almost every time it approaches its surface.

Our experiments have failed to reveal any partial degree of activation of *T* 4. A given virus particle seems either to be completely activated at any one moment during which it is adsorbed at the above rate or it is completely deactivated and not adsorbed at all. It is as though the virus must carry with it a single complete set of tryptophan molecules to be adsorbed at the normal rate or, lacking the complete set, it is not adsorbed at all.

When activated virus suspensions are added to suspensions of *B* in such proportions that the tryptophan concentration falls below 0·1 mg./l., two reactions compete with each other for the virus particles:

(1) deactivation of the virus,

$$T4^* \xrightarrow{k_1} T4 + \text{tryptophan}$$

and (2) adsorption on the host,

$$T4^* + B \xrightarrow{k_2} B : T4$$

Theoretically the ratio of the products of the competing reactions should be given by the expression:

$$\frac{[B : T4]}{[T4]} = \frac{k_2 [B]}{k_1}$$

and should increase indefinitely as [*B*] increases. Experimentally the ratio is indeed proportional to [*B*] for low values of [*B*], but levels off to values near unity as [*B*] exceeds values of 10^9 ml., indicating that only half the activated virus is adsorbed under these conditions (Anderson, unpublished observations). A number of explanations for this phenomenon are possible. For example, the data fit nicely if it is assumed that a fraction of the

virus particles are deactivated at such a rapid rate that they are deactivated before encountering a host cell. Another possibility is that the adsorption process requires time for the formation of a permanent bond and that during this time some of the virus particles lose cofactor molecules and are unable to complete their bonds to the host.

Another independent observation makes it seem more probable that some time element is involved in adsorption. Namely, during violent agitation of the medium the adsorption of *T* 4 on *B* is blocked even in the presence of 0·02 g. L-tryptophan/l.; but when the stirring of such an adsorption mixture is stopped the virus is adsorbed at the normal rate (Anderson, unpublished observations). Agitation itself should increase the rate of collision between virus and host cells and if the adsorption reaction is completed in extremely short times, as it would with steric fitting of rigid complementary surfaces, agitation should have increased the adsorption rate.

If we avoid the currently inscrutable possibility that long range forces are involved we must assume that the virus comes into material contact with the host's surface. One might tentatively view the process of adsorption of *T* 4 in the following manner.

1. The initial contact may occur between thin projecting elements on one or both the surfaces. Such substances as mucoid chains would have high thermal velocities relative to those of the heavier bodies to which they are attached and could make so many collisions as the more massive bodies approach each other in suspension that in almost every approach a steric fit of complementary surfaces would occur between projecting elements. In quiet media the adsorption process would then be highly efficient, as observed, but in violently agitated media one might expect the shearing forces in the liquid to break the bonds on the fragile chain which hold the two together.

2. The virus particle must get to the cell wall. This it may do by working down the projecting element by steric refitting or by enzyme action or by both, retaining its attachment as it progresses to the surface. In this connection the hemagglutination reactions of the influenza group of viruses provide a clue, for these viruses, as well as an enzyme from *Vibrio choleræ*, digest mucins of the blood group substances. Both viruses and the enzyme are ad-

sorbed on red cells of the proper type and eventually come off the cell as though they had been moving about on its surface digesting the receptor substances until, having finished the job and still infectious, they are eluted; and the cell, having had its receptor substances destroyed, is no longer capable of adsorbing the virus to whose action it had been subjected. The influenza viruses are not irreversibly adsorbed on the red cell; the cell does not serve as a host for virus production.

3. On reaching the cell wall the virus particle seems to lose its morphological identity as seen in the electron microscope. It therefore seems likely that it is at this point that a more intimate fusion of virus and host material occurs and the process becomes essentially irreversible. The irreversibility of this fusion is illustrated by the fact that once adsorbed, the infecting virus particle can no longer be recovered from the cell either by lysis of the cell by an excess of another virus (Doermann, unpublished results) or by sonic disruption of the cell (Anderson and Doermann, unpublished results).

We are now obviously faced with more unanswered questions than we had at the start. For example, which stage in the postulated sequence of reactions determines the high degree of specificity exhibited by the adsorption process? If we assume that the initial contact with mucoid material determines specificity we are faced with the question of why the suitability of the cell or its contents for virus production should be expressed by the molecular structures of such substances. Could they be by-products of past or patterns for future specific syntheses of the cell? In this connection it might be recalled that the presence of a type specific polysaccharide on the surface of the pneumococcus indicates that the cell also contains the corresponding nucleic acid transforming principal. Or the specificity in the adsorption of phage may be finally settled in molecular terms with the contact of the virus with the cell wall where at least some of the elements required for virus production might presumably be located.

And where do the adsorption cofactors enter into the adsorption reaction? We would like to know what part of the virus makes the initial effective contact with host material, head or tail, and which of the adsorption steps requires cofactor. The inactivity of D-tryptophan suggests that the α-amino group is

TABLE II

Properties of Bacteriophages Active on *E. coli* Strain *B*

Phage (1)	Morphology (2)		Susceptibility to Sonic Vibration 9000 cps. (3)	Susceptibility to Osmotic Shock by NaCl (4)	Serological Relatives (5)	Plaque Size (1)	Principal Resistant Mutants of B (1)	
	Head	Tail					Type	Relative Frequency
T 1	Round, 50 mμ	150 × 15 mμ	Resistant	Resistant	Alone	Medium	*B*/1, tr. (6) *B*/1, 5 *B*/1, 3, 4, 5	High High Low
T 5	Disc shaped, 100 mμ	200 × 15 mμ	Sensitive	Resistant	Alone	Small	*B*/5, 1 *B*/5, 1, 3, 4	High Low
T 2	Pointed membrane 65 × 80 mμ containing dense internal structures	120 × 20 mμ attached to membrane; thicker at distal end to which filaments are frequently attached	Sensitive	Sensitive	*T* 4, *T* 6	Small	*B*/2	Very low
T 6			Sensitive	Sensitive	*T* 2, *T* 6	Small	*B*/6 *B*/6, 1, 5	High Low
T 4			Sensitive	Sensitive	*T* 2, *T* 4	Small	*B*/4, 3 *B*/4, 1, 3, 5	High Low
T 3	Round, 45 mμ	None	Resistant	Resistant	*T* 7	Large	*B*/3, 4 *B*/3, 1, 4, 5	High Low
T 7			Resistant	Resistant	*T* 3	Large	*B*/7, 3, 4 *B*/7, 1, 3, 4, 5	High Low

(1) Demerec and Fano (1945); (2) Anderson (1946); (3) Anderson, Boggs and Winters (1948); (4) Anderson, unpublished results; (5) Delbrück (1946); (6) Anderson, E. H. (1946).

of critical importance as though it may be involved in a synthetic step, possibly of peptides. If this were true, the fact that other amino acids can substitute for tryptophan in activating the virus would suggest that they might be able to take each others places in protein synthesis in general. This possibility can be examined critically by the use of tracer techniques and seems worth investigating.

In brief, the mechanism of adsorption of phages on bacterial cells appears to be much more complex than was once thought. Instead of considering the process as a simple steric fitting of complementary rigid surface structures we shall have to look for dynamic processes involving enzymic steps of degradation and synthesis.

Discussion

Dr. A. Felix was interested in the relation of the mucoid materials of the cell surface to the suitability of the surface or the contents of the cell for virus production and was impressed by Dr. Anderson's question whether these materials might not be by-products of past or patterns for future specific syntheses of the cell. He was also impressed by Dr. Anderson's conclusion, that the mechanism of adsorption of phages on bacterial cells appears to be much more complex than was once thought, and that instead of considering the process as a simple steric fitting of complementary rigid surface structures we shall have to look for dynamic processes involving enzymic steps of degradation and synthesis.

This conclusion approached very nearly to the view first expressed by Bordet, that bacteriophages are indigenous products of the bacterial cell and not separate viruses parasitic on the bacteria. He had himself held this view for some years in the face of severe criticism. If the work of the American group had been carried out on more than one single strain or one single organism, it is very likely that their conclusions would have been quite different.

In Table II the correlation between the size of the phage particle and the size of the plaque produced is emphasized. But in the large Salmonella phages this correlation does not exist, i.e. the same phage when propagated under identical conditions

on different strains may produce plaques varying in size by one-hundredfold or two-hundredfold.

The Salmonella phages differ in many ways from the *Bact. coli* phages, and Dr. Anderson's opening sentence simply does not apply to all phages. There was no doubt that some Salmonella are adsorbed by strains which are not capable of supporting their multiplication. Dr. Craigie also would probably strongly oppose the conclusion that specific adsorption of the bacteriophage is possible only when the living cell is capable of producing or allowing multiplication of bacilli.

The Salmonella phages are no longer classified in terms of the Salmonella species they attack, but as phages acting on certain well defined antigenic components, e.g. the O-O, O-A and even O-R phages. Now *Bact. coli* contains antigenic components of the same kind; and if a correlation were attempted between the antigenic composition of the *Bact. coli* cell and the phage, the results would be similar to—if not analogous with—those ob-obtained in the Salmonella group. In this group the observed facts make it difficult to resist the conclusion that it is impossible to accept the bacteriophage as an independent entity, and that the genetics of the bacteriophage are identical with the genetics of the bacterial cell.

Dr. ANDERSON replied that the choice of a single host cell with many phages was made in order to collect as many facts as possible about a few related things rather than collect many facts, however important they might be, about a great number of objects whose relation to each other is obscure.

He was glad that Dr. Felix took exception to the first sentence of his paper; he didn't like it either, because the unfortunate word 'carefully' implied that workers who had reported adsorption of phage on resistant bacteria had not been careful; but the word did apply to the *T* phages and the mutant strains of *E. coli B.* An understanding of the apparent exceptions is instructive. For example, *T* 2 or *T* 4 is readily adsorbed on *E. coli B* in the presence of 5-methyltryptophan, but if allowed to remain for long in the presence of this inhibitor of tryptophan metabolism the resulting virus-host complex is irreversibly prevented from producing phage and the infecting particle is lost (Cohen and Anderson 1946). Similarly, complexes between *T* 1 or *T* 5 and *B* require

calcium ion for their development (T. F. Puck, unpublished, and M. Adams, unpublished) even though these viruses are readily adsorbed in the absence of calcium ion. In each of these cases the complexes would have been able to produce virus if the substances (e.g. tryptophan or calcium ion) essential to some metabolic steps in their development had been made available in adequate concentrations.

There was a rough inverse correlation between the size of a phage and the size of the plaques which it forms, though there was considerable variation between different strains of a phage. Of two possible explanations, the first seems to involve the phenomenon of lysis-inhibition as discussed by Hershey and Rotman (1949). Here certain lysis-inhibiting strains (designated *T* 2*r*$^+$) form small clear areas on the bacterial smear, but interfere with the growth of plaques formed by non-inhibiting strains (*T* 2*r*) at a considerable distance from the area of clearing. Properly speaking the *r*$^+$ plaques should thus be considered to be much larger than the cleared areas which they form. Plaque sizes might also be limited by the production of substances such as indole by the growing bacterial population on the smear. Such substances inhibit the adsorption of certain cofactor-requiring strains of *T* 4 (cf. Delbrück 1948). In such a case a sensitive plaque could grow until the concentration of such inhibiting substances reached values which blocked adsorption while plaques of a non-sensitive strain of the same phage could continue to increase in size. If the inhibiting substance were volatile like indole it should be possible to test this possibility by seeing if one could vary the size of sensitive plaques at will by flushing out the air in the petri dish or by introducing vapours of the substance at any desired time after plating.

With regard to the origin of the bacteriophages, since the phages cannot multiply without specific host cells, they are certainly intimately dependent on host cells. But such a dependence could hardly be taken as evidence that the phages *originated* from normal host cells. Indeed, the only direct datum, the antigenic distinction of phages from their host cells, would suggest that they did *not* arise from their present hosts.

It is difficult to see in what sense the genetics of the bacterial cell could be proven to be identical to that of the phages. Aside

from the inherent difficulties in proving an identity between component parts of biological systems (cf. N. W. Pirie 1940) this concept is questionable on more general grounds. Certainly the phages are not only phenotypically different from the bacterial cell but differ greatly from each other. If two forms like phage and host cell were to be genetically identical they would have the same mutation patterns, the same potentialities and mechanisms for expression of phenotypic characters and the same mechanisms for exchanging genes to produce hybrid types. The *T* phages have mutation patterns which seem to be quite unrelated to those of the host cells. Moreover, an identity in potentialities for the expression of phenotypic characters would mean that the phages should not only have arisen from the cells but be capable of reverting to the cellular form and back to the form of the original phage or even back to any one of the other types of phage active on the cell. This would mean that if one of the phages were genetically identical to the host cell all the phages active on the same cell would be genetically identical to each other!

If Dr. Felix would use a less stringent word than 'identical'—a word like 'analogous', for example—they might be able to agree, but only to the extent that the genetics of the bacteriophages may be analogous to those of other biological systems. Indeed, it was largely with the hope of discovering something regarding the fundamental mechanisms of genetics as a whole that the detailed study of an apparently primitive system like the phages had been made. An account of their pioneer work on the genetics of bacteriophage has just been published by Hershey and Rotman (1949). When two or more related phages of the '*T*-even' type (i.e. *T* 2, *T* 4 or *T* 6) infect the same host cell, the mixedly infected cell produces hybrid types of virus. Hershey and Rotman have determined the frequencies with which hybrids of various types result from such crosses and have found evidence for the existence of three linkage groups connecting the thirteen genetic loci with which they worked. One of these loci concerns host range, the others plaque type. Both this work and the independent work of Luria (1947) suggest that sub-units of the viruses multiply independently and eventually combine to form

finished virus particles. Whether analogous mechanisms exist in higher organisms remains to be seen.

Dr. A. L. HOUWINK said that at Delft they had seen fine threads, similar to those shown by Dr. Anderson, radiating from *E. coli* and *Pseudomonas pyocyanea*. These threads had not been described before and they could only be detected with the electron microscope. They occur only when the bacteria are cultivated on a surface, e.g. the surface of agar or of a collodion membrane or simply the surface of a fluid medium. The function of the threads was not clear, but apparently it was not to attach the bacteria to the surface.

Plate 4, fig. 1. showed *Ps. pyocyanea*, cultivated on agar and 'stripped off'. These bacteria bear young flagella at their lower end, whereas fine threads are seen at the upper end. Flagella and threads may also occur at the same end. Pl. 4, fig. 2, showed a motile strain of *E. coli* which has been cultivated in a fluid medium, to wit 0·1 per cent peptone water. These cells attached themselves to a collodion membrane which floated on top of the medium. They have flagella and a large number of fine threads all around. Dr. Anderson had suggested that these threads are capsular material. This would mean that they represent dried slime. Dr. Houwink thought that this was improbable, as they are seen to intercross in many places.

Dr. ANDERSON asked why the fact that the threads crossed each other made it unlikely that they were dried slime.

Dr. HOUWINK replied that if they were slime they would fuse and appear to branch from the junction rather than cross. The structures must have been present before the drying of the preparation.

Dr. ANDERSON agreed that the structures probably did not originate only in the drying process, but wondered whether a suspension of thread-shaped structures of these dimensions could have the slippery and viscous properties which on a macroscopic scale would be associated with a slime.

Dr. D. HERBERT asked for fuller details of the conversion of *Bact. coli* from a lysozyme-resistant to a lysozyme-sensitive state by ultra-violet irradiation. He had found—admittedly with a different strain of *Bact. coli*—that after various periods of irradia-

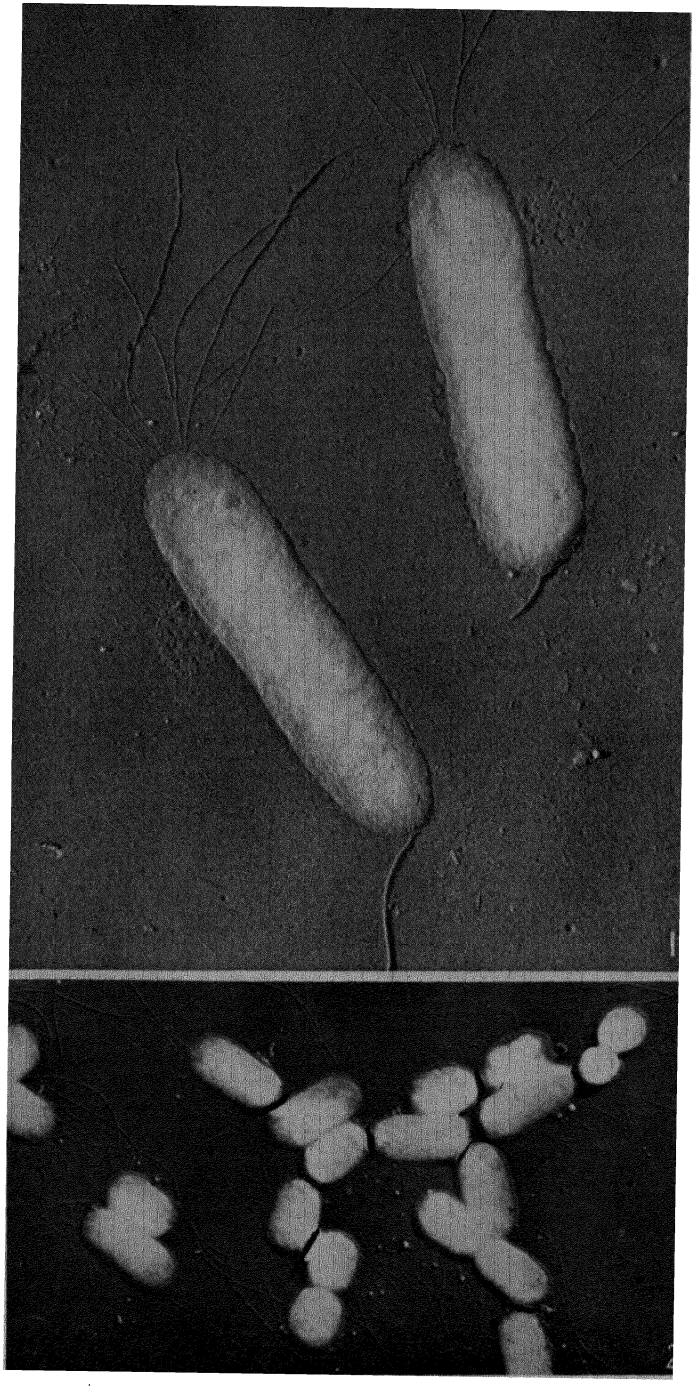

PLATE 4.

Fig. 1. Shadowcast electron micrograph of *Pseudomonas pyocyanea*, showing a flagellum at the upper end and fine threads at the lower. Fig. 2. Shadowcast electron micrograph of *Escherichia coli*, grown on the supporting collodion membrane, showing flagella and a large number of fine threads. E. M. Delft. (A. L. Houwink.)

tion it remained resistant, even when tested immediately after fairly heavy dosage.

Dr. ANDERSON replied that he had used a strain called P.C. by Bronfenbrenner but that he had made no survey of the different strains; though it would be interesting and easy to do so. He used 200 or more lethal doses of radiation and tested immediately afterwards because susceptibility to lysozyme is lost by cells on standing. He thought it possible that irradiation stripped something off the cell and so uncovered a substrate for lysozyme.

Prof. M. STACEY suggested that the lysis was the end-result of a sequence of events initiated by a key treatment. In Gram-positive organisms, he and his colleagues contended that an enzyme released the polysaccharide from the nucleic acid, and then the normal lytic process did the rest.

Dr. ANTOINETTE PIRIE asked whether the evidence was more in favour of the view that the lytic substance made from the phage by ultra-violet irradiation is an enzyme produced by the phage or that it is a substance, a part of the phage, which then is adsorbed and activates the autolytic enzymes of the host bacteria. The lytic agent does not lyse heat-killed bacteria, but will only lyse bacteria that are still in an autolysable state.

Dr. ANDERSON said it was possible that the lysin from *T* 2 is not an enzyme at all, that it merely trips some autolytic reaction of the irradiated cell; or it might be an enzyme which acts directly on structural components of the cell. In the latter case we may well be faced with a problem in semantics, for a virus may well contain some components designed to carry out a special reaction once and others to carry out other reactions many times. Even an enzyme might, in certain circumstances, act only once, but in this case there would be no evidence that it was an enzyme. The problem is how many molecules of substrate we should ask a protein molecule to convert to some product before calling it an enzyme. The no less interesting protein components of cells which are designed to do a special task only once have so far been largely unsuitable for study because, except when (as is the case with trypsinogen) they themselves are converted into enzymes, the biochemist lacks the techniques for detecting them on the macroscopic scale. We can detect single particles of a

virus or transforming principle because they are self-reproducing, yet when we come to investigate their separated parts we might expect to find that some of them have a single task to do and take no direct part in their own synthesis or require the co-operation of other elements for their reproduction.

Further study of lytic substance from phage would soon start, to see if lysis requires cofactor in some cases. They had been unsuccessful in separating lysin from *T* 4 but succeeded in the case of *T* 2, the former virus requiring cofactor and the latter not requiring a cofactor for adsorption. Since the intact *T* 4 effected the lytic reaction when provided with tryptophan it is possible that a lysin analogous to that in *T* 2 exists in the particle.

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VI

THE STATUS OF SOME ARGUMENTS ABOUT THE BACTERIAL SURFACE

by A. A. MILES

My remarks on the bacterial surfaces will be restricted to a few comments on points raised by the opening speakers; and to a more detailed examination of one particular field, where I shall take a stand, not as a participant criticizing the technique of play, but as spectator commenting upon the rules of the game.

In an attack on the bacterial surface by diverse techniques, there is some risk of confusion in our ideas of the word 'surface'. Reducing ourselves to a disembodied intelligence of no dimensions proceeding towards the centre of the bacterium from a place well outside it, we shall at some point encounter something that differs from the surrounding medium in which we started. The first material encountered would probably be a cloud of loosely held ions, mixed perhaps with soluble bacterial products, ranging from simple salts to enzymes, diffusing away from the bacterium. A little later we may meet a more or less diffuse, hydrated, 'slime' layer, in some cases carbohydrate, in others polypeptide; and then a complex structure of proteins, lipids and carbohydrate, with potential or real holes for the passage of certain molecules. Where did the surface begin? The classical microbiologist would say where the light-absorbing power and the refractive index of the material, whether in the native or the stained conditions, differed substantially from that of the surrounding medium; and in some cases would consequently exclude capsular material and perhaps flagella, and also material like those which determine, for example, the electrophoretic surface. The electron microscopist might define it as the plane at which a stream of vapourized gold is held up; but, being defined by work with dried and perhaps distorted cells, this plane is almost certainly artefactive. Nevertheless, microscopical technique deter-

mines with some precision where the common-sense surface starts; and though within the microdimensions we are considering, this common-sense surface may be a long way from the surface that is important for the bacterium, nevertheless, it forms a useful reference point to which we can relate other equally valid surfaces.

From consideration of the passage of small molecules into the cell, we postulate a surface of a certain limited permeability, but there remains the problem of the outward passage of large molecules like the ectoenzymes and toxins. This problem may be unreal in the sense that the free enzymes and toxins found in a culture have come from disintegrated cells, but its unreality has yet to be demonstrated. Moreover, since in unicellular organisms like amœbæ we must postulate a surface that without leak is selectively permeable to small molecules, and yet visibly permits the ready passage of large inert particles, it is not unreasonable to suppose a bacterial surface permeable to particles ranging in size from simple ions to macromolecules, a surface whose permeability will vary with the tool we use to explore it.

Serological Arguments

On first thoughts, the serological surface is likely to be somewhere near the common-sense surface, because we argue that a large molecule like a mammalian antibody is unlikely to penetrate very far into the bacterium, at least without producing some determinable effect; and by assuming that agglutination is a surface phenomenon, we have made quite pretty pictures of the antigenic surface of various bacteria. However, if we choose to invest the bacterial surface with the properties claimed for the multiple superimposed films of antigen and inert material invented by Rothen (1946), bacteria could be agglutinated by antibody specific for substances buried deep under the common-sense surface. Moreover, since this long-distance action of antibodies (if it exists) may be enhanced non-specifically by overlying or underlying layers of other substances, a great deal of what we nowadays interpret as steric interference with the combination of antigen and antibody at a topographically simple surface, may be due to the properties of a system of considerable depth.

That serological analysis could be falsified by interference in one plane is clear, for example, from Bawden and Pirie's (1944) antigenic preparation of bushy stunt virus from tomato sap, which failed to precipitate with its antibody until a chromoprotein was removed from the complex; and if similar antigenic but 'non-reacting' complexes were present in bacteria we should be ignorant of their presence until some lucky variation of treatment uncovered them to make an agglutinable cell.

But even when, with the help of an Ehrlichian exuberance of hypotheses, we have located the antigens in their various topographical layers, we are left with the problem of determining their number in a given antigenic surface. The mosaic hypothesis of Durham (1901), fruitful though it has been in providing an easy qualitative notation for characterizing bacteria by antigens, is clearly not universally applicable. For example, serological analysis in terms of antibody nitrogen in bacterium-antibody complexes by the chemical methods developed by Heidelberger, suggests that differences between two antigens regarded as qualitative by other tests are quantitative only, because, in given circumstances, both antigens will absorb all of one type of antibody from an antiserum.

The Flagellar Controversy

It is one of the functions of this symposium to reconcile the surfaces of the chemist and physicist, the morphologist and the serologist. Reconciliations are also required within, as well as between, disciplines. We are to discuss the nature and function of bacterial flagella. During the past decade the orthodox view has been vigorously questioned by Prof. Pijper, who has thereby stimulated many other investigators to a re-examination both of their material and their evidence. At present the nature of bacterial flagella is largely a matter for morphologists to debate, about the function of a particular bacterial surface whose existence is agreed. I propose to examine the arguments in this debate, not in a premature attempt to decide the issue, but to indicate the weight we ought to attach to the different kinds of evidence adduced on both sides.

The problem is simply stated. 'Are flagella the cause or the

consequence of bacterial motility?' and this we may translate into a convenient shorthand, 'Are flagella active or passive?'

At the outset we must be clear about the universe of discourse. In earlier days viruses were described as 'filter-passing and ultramicroscopic'; and by a natural process these initially descriptive terms came to be regarded as definitive, with the consequence that when an infective agent proved to be resolvable or at any rate visible under the microscope, or failed to pass certain filters, it was by some people automatically demoted from the rank of a virus, and declared to be something else. We must avoid the analogous danger, when flagella are proved to be active or passive in a given microbe, of denying that the microbe is a valid example of the kind of bacteria we have in mind; and for the present purpose limit the discussion to representative and typical eubacteria like *Salmonella typhi*, *Bacterium coli* and *Proteus vulgaris*.

The fundamental observation is the association of motility with the presence of flagella. That in some strains of a motile species flagella cannot be demonstrated, or that some flagellated strains do not move, is irrelevant in view of the extreme tenuity and fragility of flagella and the susceptibility of bacterial motility to environmental changes in artificial culture. The knowledge about this association, which has grown out of the use of flagellation as a character in classifying bacteria, is relevant to the discussion only in that it provides the statistical justification for saying, for example, that if the flagella prove to be passive in a strain of *S. typhi*, then they are likely to be so in the species, and, because of the obvious affinities within the genus, in all the motile Salmonella.

In this association of flagella and motility there is no logical indication which one is the cause of the other, or whether the two are causally related at all. The assignment to flagella of the role of prime mover appears to have been based on analogy with the more demonstrably organized and active flagella and cilia in larger organisms, and on the wholly unwarranted belief that Nature is too neat and economical in her works to have provided bacteria with such organs unless they had a function.

Nevertheless, the association demands an explanation if not in logic at least in poetic justice; and the hypothesis of flagella as passive distortions of outer layers of the cell must be considered

as seriously as that of the active flagellum. The nature of the substance distorted is immaterial, so long as we can prove that flagella and the outer layer consist of one and the same substance. Their identity would support the passive flagellum: lack of identity, however, would not prove the active flagellum, for there is no reason why cytoplasm should not extrude passively in fine threads through apertures in the outer cell-wall—as it does, for example, in the Foraminifera; but in this case one of the arguments for a passive flagellum—namely, the absence of any connection with the cytoplasm—would disappear.

In bacteria of the *Bacterium*, *Salmonella* and *Proteus* groups the slime layer seems in most cases to be predominantly polysaccharide; and since 1904, when Beyer and Reagh showed that flagella were heat-labile, evidence has accumulated about the lability of flagellar material during mild treatment by heat, acid, ethanol and other agents; facts which, together with the conspicuous antigenicity of flagella, point more to protein than polysaccharide. In *Pr. vulgaris*, at least, that indication has been fulfilled; the flagella consist of a fibrous, myosin-like protein (Weibull 1948, Astbury and Weibull 1949).

The demonstration of a distinct flagellar substance has other consequences. In the absence of a protein layer on the outer surface, the passive protein flagellum must be created by forces that bring deeper protein to the surface, and then pull it out into threads. We must also endow a motile strain with peculiar powers of synthesizing the distinctive flagellar substance. But we have in this no good disproof of the passive flagellum: it is tidier to assume that the motile bacterium has specially organized flagella that are both active and chemically distinct from the rest of the cell, but the bacterium is not necessarily as tidily organized as the bacteriologist's mind.

Observations of Fixed Preparations

Let us turn now to more direct observations. The relatively brutal treatment used in preparing material for the light and the electron microscope rightly make us suspect the resulting preparations; but we must not suspect too much. We know that flagella are easily detached and we must not expect to see a field of intact

and perfect cells; and with due regard for the way in which artefacts and detached flagella are distributed over the field, it is reasonable to assume that everything of flagellar dimensions attached to the cell, allowing for the exigencies of drying, are the flagella themselves. The positive evidence that in some preparations the flagella traverse the cell wall to make a direct connection with the central material of the cell, is therefore of more value than absence of such appearances. But there is as yet no clear proof that the threads have not arisen superficially on the upper or lower surface of the cell as it lies on the collodion membrane, and, being superimposed across the dried remnant of the cell wall, only appear to traverse it. Indeed, the only possible proof to the contrary would be statistical; namely, to demonstrate the same thing in such a large majority of preparations that the probability that in all cases chance superposition is the basis of the observed appearance becomes negligibly small.

The fairly constant polar or circumpolar origin of the flagellum in monotrichate cells has little weight as a fact in favour of an active flagellum, because, according to d'Arcy Thompson (1942), surface tension would be lowest at the exposed point, corner or end of a cell, if and when the surface was in a fluid or semi-fluid condition; and the pole would therefore be the place where passive threads form. Whether the numerous uniformly thin tenuous threads in a lophotricate or peritrichate cell can be as easily envisaged as the consequence of the shear and turbulence developing in the wake of a moving bacterium will depend on the observer's expectation, based on his experience of the behaviour of matter in the macroscopic world, of how physical forces will act within microscopic dimensions upon substances about whose physical properties we know very little. In other words, the distinction between functional organs and passive distortion of a cell surface, even in the most clean-cut electron micrograph, is largely æsthetic; and though an æsthetic sense developed from a long and loving familiarity with the morphology of living objects may be exceedingly useful as a guide in biological investigation, and may prove in the end to have been well-founded, it cannot be accorded much weight in a controversy of this kind. If we do not know the forces that might move the active flagellum we are in no position to deny that the elegant

flagellar threads could have been spun as a result of bacterial motion.

For similar reasons we must also reject arguments based on the spiral shape of the cell, because without an appeal to teleology we cannot distinguish a spiral resulting from adaptation to easy motion through liquids, however engendered, from a spiral adapted for the easy translation of somatic constrictions into forward motion; or from a spiral resulting from wholly undeterminable circumstances. The proof of a fundamentally spiral structure is therefore irrelevant to our problem, unless it is claimed to be *necessary* for somatic self-propulsion; in that case disproof of the spiral structure in a motile microbe (as in the motile sacrinæ and in the motile, penicillin-induced globular forms of *Vibrio choleræ* described by White (1950)) would be evidence against the passive flagellum.

Observations on Living Cells

Leaving arguments from analogy, and the discussion of differing æsthetic judgements, we come to observations on living bacteria. The proponents of the passive flagellum have the more difficult task. The obvious contractions and waves of motion seen in the bacterial cell may be the result as much as the cause of flagellar motion; and it is quite possible that active somatic and active flagellar motion may occur in the same cell, as appears to be the case in certain protozoa. What is required, then, is an unequivocal proof that flagella follow and do not initiate bacterial movement; or that motility is not affected by removal or immobilization of flagella. Proof of the first point depends on arranging certain very rapid events in a demonstrably correct time sequence; events like the precedence of a bacterial somersault or reversal of the direction of motion (both admittedly rapid) over the re-orientation of flagella. The somersault, which may be due to a sudden check of motion in one direction, is not crucial, because we may postulate relatively prolonged passive periods even for active flagella, when they would stream behind a cell carried forward by its own momentum.

As regards active or passive 'tails', the distinction required is like that of deciding from purely external evidence whether a

submarine seen from above is turning because the rudder is actively deflected from the axial line, or was bending as the boat turned.

Flagella can be shaken off bacteria without killing them, and without destroying their motility. We cannot thereby deny that flagella are not an integral part of the cell, any more than that the tail is not an integral part of certain lizards, because they leave it behind in the hand of a would-be captor; and without proving that *all* the flagella were removed, motility after losing flagella is not a crucial point. With regard to immobilization, a specific immobilizing agent is required if we are to resolve conflicting claims based on the use of colloids that are said to clog both flagellar and somatic movements. An antibody to the flagellar substance immobilizes motile cells without killing them; and if flagella and cell surface are antigenically similar, or even related, then flagellar antibody might inhibit somatic contractions. The proof, likely to be exceedingly difficult, that the flagellar substance does not in any way contribute to the serological properties of the rest of the body surface, would favour the hypothesis of a direct action of *H* antibody on active flagella. The well-known phenomenon of the agglutination of motile bacteria by a pure *O*-antibody, producing clumps of cells that are still motile, proves that the absorption of antibody to the surface does not necessarily affect the contractibility of the cell itself; and if we suppose that *H* antibody has no special effect of a surface antigen, immobilization by matting together of flagella with *H* antibody is significant.

The crucial proof of the active flagellum is the demonstration of its independent motility. Given a more or less fixed cell in the field of observation, flagella activity would be revealed by the agitation of visible suspended particles: but a convincing quantitative distinction between agitation due to molecular bombardment, to convection, to currents produced by undetectable movements of the cell itself on the one hand, and to the churning produced by flagella on the other hand, will be difficult.

A less equivocal proof is possible. By Ørskov's (1947) elegant method of observing the motion of *Pr. vulgaris* in a thin watery fluid loaded with indian ink particles, it should be possible to photograph the invasion of a patch of ink by a motile filament of *Pr. vulgaris*, and in the absence of any gross 'shouldering aside' of

the ink by lateral movements of the bacterium itself, to demonstrate clearance of the ink from a wide area round the filament, with a margin separated from it by a distance not only equal to the effective length of the flagella, but also well beyond the range of disturbance of the medium by transmitted tremors or vibrations in the body of the bacillus.

I have dealt with only some of the numerous arguments that have been advanced by both sides in the flagella controversy, but the principle of my criticism is, I think, valid for all of them. In rejecting many of them as inconclusive or irrelevant I do not necessarily deny their relevance to other aspects of work on flagella; but I contend that, without assuming for bacterial functions and substances properties for which we have little evidence, we cannot apply them with any profit. To argue, for example, for the passive flagellum because it is difficult to conceive how energy could be transmitted from active somatic centres to a flagellum or how kinetic energy could be generated in a flagellum itself; or, on the other hand, to argue that bacteria are unlikely to propel themselves through water as a fish does, because they are not organized metamerically; is to put the cart before the horse. It will be time enough to consider how a flagellum works when we have proved beyond doubt that it does so; alternatively it will be time enough to find out how the bacterium manages to move like a fish when flagella have been proved not to move it.

Arguments from analogy have both value and force; but in the present circumstances they serve mainly to illuminate the Weltanschauung of the biologist using them, rather than the problem he is investigating. Nevertheless, it is both right and inevitable that each of us should display his own Weltanschauung in controversy; and even though we fail to solve all the immediate scientific problems, we shall have done a great deal if as a result of this symposium we understand each other's views.

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VII

THE NATURE OF BACTERIAL SURFACES

by E. T. C. SPOONER

THE doctrine of the serological activity of surface antigens, which would locate some antigens in the bacterial surface and others deeper in the cell is presented by Topley and Wilson (1946) and by Dubos (1945) with great caution, and with a general warning against fallacies due to such things as quantitative changes in the antigens of bacteria undergoing variation, steric hindrance by neighbouring groups on the cell surface and non-specific physical influences in agglutination tests. However, the doctrine is now an integral part of the mental picture of the cell surface carried by most bacteriologists and is therefore worthy of detailed study.

A bacterial antigen is taken to be located on the surface of the bacterial cell if the following three conditions are satisfied.

1. Immunization with the whole bacterium readily evokes a specific antibody which will react with the antigen.

2. An antiserum so produced will agglutinate suspensions of the living or undegraded bacterium.

3. Absorption of the antiserum with a thick suspension of the whole bacterium will remove the antibody specific for the antigen.

Flagellar and capsular antigens satisfy these requirements; their position on the surface of the bacterium is not doubted because they can easily be shown to be parts of surface structures which can be separated from the cell by simple mechanical means.

Flagella themselves, whatever view one takes as to their nature, are thread-like appendages to the cell surface, 20–30 mμ thick, rather than a part of the functional surface itself. Probably their own surfaces are important to their own function; it seems clear that the deposition of a monomolecular layer of antibody on the flagellar surface is enough to stop flagellar activity. Capsules may also be regarded as outgrowths from the functional surface rather

than part of the surface itself, although those molecules which give rise to the capsular material are presumably themselves in the cell surface, so that the distinction is rather artificial.

The capsular antigen of the pneumococcus can very easily be removed by allowing a glucose broth culture to grow for twenty hours or so, by which time most of the capsular material dissolves off and the cocci, which at eight or ten hours were agglutinable only by their own type-specific anti-polysaccharide antibody, become freely agglutinable by sera from which that antibody has been absorbed, or by sera made against other types which contain R antibody common to all types. A similar conversion of a type-specific pneumococcal suspension into a non-type-specific suspension can be effected by simply washing a suspension of 'young' type-specific pneumococci in saline, thereby washing off their capsules and making them agglutinable by the R antibody. Once the capsule has been removed, either mechanically or by the production of a Rough strain, the R antigen, which is probably itself a carbohydrate-containing molecule, stands revealed as satisfying the three requirements of a surface antigen. While it possesses a capsule the pneumococcus cannot be agglutinated by antibody for the R antigen; and the evidence of washing and degradation experiments convinces us that the normal position of the R antigen is deep to the capsule. The capsule of *Hæmophilus influenzæ* is equally easily washed off (Pittman 1931).

Capsular, or mucoid, antigens have been shown to be possessed by a wide range of different bacterial species. They are apparently not necessary to the functional integrity of the cell, and indeed may not appear in freshly isolated strains, but in some cases develop only in certain variants arising during artificial cultivation, as for instance at low temperatures on media containing sugar.

Certain other antigens, such as the *Vi* antigen, which with their antibodies are responsible for the agglutination of living cells, or cells which have been subjected to minimal injury, are accepted as being on the cell surface because they satisfy our requirements, and because cells that possess them are inagglutinable by antibodies to 'deeper' antigens. As Dubos points out, it is unlikely that big antibody molecules can penetrate far into the

cell substance; and if they did it seems unlikely that they would cause agglutination.

Some antigens are thought to be in the surface of the cell even though they do not satisfy all three of the conditions. For instance, *Brucella abortus* and *Br. melitensis* are not agglutinable by monospecific sera against the minor antigen of the *A* and *M* pair; but the ability of the minor antigen to absorb antibody specifically presumably means that it is near enough to the cell surface to encounter and bind its specific antibody, and therefore it is most probably at the surface. Miles (1939) ascribes the failure of the minor antibody to agglutinate to its quantitative inferiority, whereby, when all superficial minor antigen groups are satisfied with their specific antibody, the degree of sensitization of the cell remains insufficient to upset the suspension stability conferred by the rest of the unsensitized surface.

The evidence that an antigen lies below the cell surface is a great deal more difficult to interpret. There are many bacterial antigens, such as the R antigens of the Salmonellæ, which take no part in agglutination of the living, unaltered and undegraded bacteria and the presence of which in the cell is revealed by precipitation tests on extracts of various kinds, or by agglutination tests on 'degraded' strains which have lost one or more of the antigens possessed by the parent strain. For the detection of these 'deeper' antigens, one may use sera prepared against either degraded forms or the undegraded organism. Sera against degraded variants are often the more satisfactory; although the 'deep' antigens will usually give rise to specific antibody formation when undegraded bacteria containing them are injected into animals, even though the undegraded bacteria in question cannot be shown to be agglutinable by the 'deep antigen' antibodies, and may not absorb such antibodies satisfactorily from an antiserum.

Can one argue that an antigen which prevents agglutination by antibody specific for a second antigen is necessarily more superficially located than the second? *Vi* forms of *S. typhi* are, in unheated suspensions of young cultures, agglutinable by *Vi* antibody, not by *O* antibody. Partly degraded strains may be agglutinable by both antibodies; simple heating or even ageing of culture will render the *Vi* strain *O* agglutinable. It seems

unlikely that the destruction of the *Vi* antigen by heat causes a complete disintegration of the molecule, which is of the same class, chemically, as the other gluco-lipid somatic antigens and is, in the purified state, fairly stable (Boivin and Mesrobeanu 1938); more probably heating leads to something in the nature of an intramolecular change, involving loss of agglutinative specificity, and possibly altering at the same time that molecular configuration which blocks the neighbouring *O* antigen.

The relative quantities of *Vi* and *O* antigens, and the relative predominance of each in the agglutinative behaviour of the bacillus, seem to depend on cultural circumstances of temperature, age of culture and the presence of glucose in the medium (Gladstone 1937).

We are left uncertain whether the *Vi* antigen is really more superficial than the *O* antigen, or whether the *Vi* antigen so affects the bacterial surface that, by what is called steric hindrance, union of the *O* antigen, also at the surface, with its own antibody is prevented.

The conception of steric hindrance is a rather difficult one for the non-physicist; but clearly different kinds of steric hindrance are possible, and steric hindrance does not mean a complete covering-over. On the human red blood corpuscle, the Rh antigen is presumably a surface antigen, for it can combine with antibody, including the 'incomplete' anti-D antibody. Treatment of the red cell surface by Receptor Destroying Enzyme of the kind found in cholera filtrate or influenza virus alters the surface in such a way as to remove the steric hindrance that prevented the incomplete antibody from agglutinating (Chu 1948, and references given by him). In this case, treatment of the red cell with R.D.E. of the influenza virus has been shown to alter the suspension stability of the cell in such a way as to decrease its cataphoretic velocity (Hanig 1948). What appeared to be a steric hindrance may really be a non-specific decrease in suspension stability.

Apart from capsular and 'mucoid' antigens, the class of antigens which seem to be most closely associated with the cell surface are the carbohydrate-lipoid-protein complexes sometimes known as 'Boivin antigens', including the *O* and *Vi* antigens of Gram-negative bacilli. Their existence has been demonstrated in many

kinds of bacteria. Like the capsular antigens, their molecules seem to be well endowed with hydrophilic polar groups, so that the surfaces of bacteria which possess them are in general well hydrated, suspensions having a high stability and cultures showing the usual properties of 'Smoothness'. Like the capsular antigens also, they are comparatively easily removed from the cell without a complete lytic breakdown of its architecture, as in the Boivin technique of treatment with trichloroacetic acid, and variants lacking them arise in cultures.

The methods usually employed to separate antigens of this class from bacteria are considered by Miles and Pirie (1939) usually to involve some degree of degradation of a much larger molecule. Their own work with the antigen of *Br. melitensis* revealed an antigen with very pronounced physical properties indicating a marked asymmetry of structure, and a capacity to form orderly oriented aggregates both on its own and with specific antibody.

Consideration of the quantities of Boivin antigen obtainable by simple trichloroacetic acid extraction before and after tryptic digestion of a Salmonella would suggest that, if these antigens are confined to the cell surface, there cannot be room in the surface for a large number of other molecules; that protein molecules are not a dominant constituent of the surface of Smooth variants is indicated by the fact that they give a negative Millon test. Probably, however, the Boivin antigens are not confined to the cell surface.

The R antigen of the Salmonellæ also seems to be a carbohydrate-lipoid-protein molecule, differing from the *O* antigen in lacking species specificity, and in being less abundant. It is not clear whether a Rough form possesses more of this antigen than does a Smooth form. In the Rough form, the R antigen satisfies the requirements of a surface antigen; whether it is also located in the surface of the Smooth form, but obscured in its effect there by the more abundant *O* antigen, is uncertain. The decreased suspension stability of the Rough form seems to be due less to any property of the R antigen itself than to the strongly hydrophobic quality of the *Q* protein which, in the absence of abundant *O* antigen, can render a suspension unstable in saline, and consequently difficult to study (White 1932).

White's study of the *Q* protein indicates that this substance is

present in all three of the Smooth, Rough, and ρ forms of a *Salmonella* sp. Antisera to the *S* and *R* forms did not always contain anti-*Q* antibody, and anti-*Q* sera did not agglutinate *S* forms; and yet absorption of anti-*Q* sera with either *S* or *R* suspension would remove the *Q* antibody.

One is left by this confusing evidence very uncertain whether to locate the *Q* antigen in the surface along with the carbohydrate-lipoid-protein antigens, or whether to assign it to the depths of the cell. The effect of the *Q* antigen on the suspension stability of *R* forms suggests strongly that it is located in their surfaces. Removal of *Q* antigen from the cells is comparatively easily effected by treatment with acid ethanol. Whether such treatment could be expected to release an intracellular constituent, one does not know. That protein antigens, as opposed to the more complex antigens of Boivin type, can occur on the cell surface seems clear in the case of the *M* and *T* antigens of the hæmolytic streptococci. Here again the position of the carbohydrate *C* antigens is uncertain.

The comparatively gross techniques of serology probably give only a partial picture of the antigenic complexity of the cell surface. In the Salmonellæ, the serological multiplicity of *O* antigens must correspond to a multiplicity of different polar groups, or of patterns of group arrangements, on the *O* antigen molecules. To what extent it corresponds to a multiplicity of chemically distinct antigens is unknown.

In Gram-positive bacteria, it seems probable that the protein ribonucleate on which the Gram reaction depends is itself superficially placed. The apparent thickness of an organism is increased by Gram staining, and occasionally, especially among the aerobic spore-bearing bacilli, an individual bacillus is to be seen in which the surface stains in patches, some Gram-positive and some Gram-negative. When this happens, the Gram stain has the appearance of being heaped on those parts of the surface which take it (see also Dubos 1948).

The multiple specificities of bacteriophages increase the number of specific molecular or polar group patterns which it is necessary to postulate on the surface. As Burnet and others (Gough and Burnet 1934, Levine and Frisch 1934) have shown, some bacteriophages have their specificity determined by known *S* and *R*

antigens; and there is little doubt that a bacteriophage specific for an *R* antigen may be unable to attach itself to a Smooth bacillus, even though that bacillus contains the appropriate Rough antigen. On the other hand, among *Vi* phages for *S. typhi* the range of phage specificity is much greater than the demonstrable range of *Vi* antigen specificity, which suggests that the point of attachment of the phage may not necessarily be always the same grouping that determines antigen specificity.

The work of Anderson (1948) and Delbrück (1948) on the *T* 2 and *T* 4 bacteriophages of *Bacterium coli* tells us that certain phages can attach themselves to their host bacteria only in the presence of certain amino-acid and other 'adsorption cofactors', but does not throw light on the actual surface groupings to which the attachment is made.

The part played by desoxyribose nucleic acid in the synthesis and specificity of the undoubtedly superficial capsular antigen of the pneumococcus suggests that the desoxyribose nucleic acid itself may not be located far from the cell surface, unless it represents a remote step in a lengthy chain of molecular evolutions which culminates at the surface (McCarty 1946).

These various considerations lead one to suppose that the bacterial surface must contain many different kinds of molecule, some antigenic, others probably not. Whatever the molecular arrangement of the surface, it must be such as to allow small molecules to penetrate the cell. The microscopic appearance of a capsulated bacterium, such as a pneumococcus, suggests that, since capsular development is maximal during the phase of logarithmic growth when metabolic exchanges are presumably themselves maximal, surface capsular antigen must be arranged in a network or gel pattern, probably containing much water, such that the vital exchanges of the pneumococcus can be conducted through it. That a complex antigen can form an orderly 'patterned' gel when mixed with its specific antibody was clearly shown by Miles and Pirie (1939) in their work on the undegraded somatic antigen of *Br. abortus* and *melitensis*. Even when the capsular gel is enormously thickened by the addition to it of specific antibody, it probably remains permeable, for pneumococci are able to grow in antiserum—though evidence on the effect of antibody on the growing capsule is somewhat conflicting

(Barber 1919, Blake 1917). Sevag and Miller (1947) have shown that Gram-negative bacilli continue to respire normally in the presence of specific antibody, even when complement is also present, unless lysis occurs. These workers found that lysis produced an acceleration of respiratory exchange for a short time, but that this short phase of hyperactive respiration was succeeded by a cessation of respiratory processes. Morris (1948), on the other hand, describes a lag in growth of *S. typhi* growing in antibody and complement. There is uncertainty, therefore, about the effect of plastering the bacterial surface with antibody: probably the conditions of the experiment are important in determining the result. We cannot use this evidence at present to tell us anything significant about the structure of the surface.

That a hydrophil gel of very loose texture may be formed around a bacterial cell is made clear by the calculations of Johnson and Dennison (1944) on the amount of specific antibody involved in producing capsular swelling of pneumococci. Accepting Hershey's estimate that one pneumococcus may be expected to combine with about 4×10^6 antibody molecules, which, packed, would occupy about 0·8 μ^3, they found an actual increase of volume on 'quellung' of the order of 2·8 to 9·8 μ^3, indicating a very loosely woven texture for the swollen capsule, and one through which diffusion would probably be easy for small molecules. Jacox (1947) recently claims to have shown that many proteins other than specific antibody globulin can produce a capsular swelling of the pneumococcus. If this is so, it would seem to be a property of the capsular substance to form with protein molecules a loose hydrated gel of the kind under consideration.

If one accepts a picture of the cell surface such that large antigenic molecules of various kinds lie in it in some sort of mosaic, they and other molecules sharing the actual surface between them, it seems probable that the mosaic is held together in some molecular manner to form a gel with considerable structural rigidity. The presence in the typical surface antigens of highly soluble haptene groups suggests that the gel in question probably contains much water bound into its substance.

The properties which must be attributed to this gel are, that it must be sufficiently permeable to allow the ordinary metabolic

exchanges of the cell, that it must account for the electron microscope and plasmolysis experiment pictures of a cell wall over an underlying protoplasm which can shrink away from it, and that it must be liable to dissolution as a result of any of the treatments which lead to bacteriolysis.

I am not competent to discuss the significance of electron microscope pictures which show an apparently rigid cell wall, which can be torn in such a way as to show a jagged torn edge. Since, however, solid films possessing considerable rigidity and elasticity can be produced in protein and various monolayers at interfaces, there would seem to be no inherent difficulty in supposing a molecular arrangement, one or many molecules thick, conferring such properties on the cell surface. The rigidity and elasticity of a membrane of this kind need not, it seems, prevent that membrane from taking part in various molecular interchanges and transformations. It is to be remembered that the processes of suspension in water, drying and perhaps washing, to which the cell must be subjected before electron micrographs can be taken, possibly affect the appearances of rigidity, as may the ionic insults offered by plasmolysis experiments. Plasmolysis might perhaps reveal a cleavage between a surface layer of protoplasm and the bulk of the cell, which does not exist in nature, and which need not indicate a second surface in the unplasmolysed cell. Indeed, it seems possible that plasmolysis experiments do little beyond revealing a difference of textural quality between the surface layers of the cell and its deeper parts, which might be expected at any interface between two phases so different and so complex as bacterial protoplasm and the surrounding medium.

Lysis presents a rich field, and a difficult one, for speculation. It seems clear that there are many different kinds of lysis, some involving the complete disintegration and solution of the cell, others leading to more localized damage, and some, not usually referred to as lysis, but certainly comparable with it, resulting in the liberation of cellular constituents without much visible structural change in the bacterial morphology.

Agents producing this last class of effect include protein precipitants such as trichloroacetic acid and mercuric chloride. According to Mesrobeanu (1936) treatment of Gram-negative

bacilli with appropriate concentrations of trichloroacetic acid leads to the rapid escape of most of the smaller molecules of the cell, including a large proportion of the larger molecules of the Boivin antigens which we have decided probably lie predominantly at the cell surface. The framework of the cell remains intact, and, with Gram-positive bacteria, the Gram-staining property is retained.

I do not know precisely what changes the trichloroacetic acid produces in protein molecules; its solvent action on the bacterial cell suggests that it disconnects from the framework of the cell other molecules which are normally held in their proper places in the protoplasm. It seems most unlikely that protoplasm is a homogenous gel. Localization of chemical function within the cell is indicated by the well-known presence of the so-called nuclear structures, and by the ability of some bacteria to form within themselves visible granules of insoluble products of metabolism such as sulphur, iron or tellurium. One or more of the probable internal phases of the protoplasm probably allow more or less diffusion; but many of the intracellular components of the bacterium may be held in place by the configuration of interfaces between different phases or by phases with a structure which does not permit free diffusion. The action of a protein precipitant seems to be to upset the holding mechanism, so that small molecules are free to escape.

White (1932, 1933) used 96 per cent acid ethanol to liberate the protein *Q* antigen of *Salmonella* spp., and 75 per cent acid ethanol to liberate the rather more dubious *T* protein antigen. Formamide was used by Fuller (1938) to disintegrate streptococci; it has a similar action on many bacteria. Again, its action seems to be related to the cell proteins; but formamide produces a more thorough disintegration of the cell than the other protein precipitants I have mentioned.

Pulvertaft and Lumb (1948) have recently shown that many protein-active disinfectants, such as formalin, phenol, mercuric chloride, merthiolate and sodium hypochlorite, if used at concentrations only a little above their bacteriostatic strengths, will produce lysis. The mechanism which they suggest is one whereby various vital enzymes are selectively destroyed, leaving untouched the autolytic enzymes of the cell.

A similar mechanism has been suggested for various other forms of lysis, as for instance lysis of pneumococci by saponin, or by desoxycholate. Klein and Stone (1931) showed that lysis by saponin depended upon sensitization of the pneumococci with cholesterol, or with a sterol-containing fraction from such animal sources as ascitic fluid or blood. Whether or not disintegration of the bacterial body is due, as Avery and Cullen (1923) and others have suggested, to the action of autolytic enzymes in the pneumococcus, which are extractable from it, the joint action of saponin and cholesterol is highly suggestive of a surface penetration effect similar to those discussed by Schulman and Rideal (1937). The surface in question would not, of course, be necessarily the cell surface corresponding to the outline of the cell; it might be a hypothetical interface between phases either at the cell surface, or deeper in its substance.

Many surface-active anions and cations have been shown to have bactericidal powers (see Rahn 1945); but it is not clear that these always act at the cell surface rather than in the cell protoplasm. In either situation, they may be expected to produce a disorganization, though not at present of a nature to reveal anything very clear about the nature of the surfaces at which they presumably act.

Lysis by complement is a more complex phenomenon, possibly more directly related to the cell surface proper because the antigens on which complement lysis, when it happens, depends are in general the somatic, probably surface, antigens themselves. In our general ignorance of the complement mechanism, we cannot say whether the antigen-antibody reaction which is a part of it acts by presenting a complex surface, predominantly globulin, into which one or more of the smaller non-globulin components of complement can penetrate, with strong disruptive effect, or whether the antigen-antibody reaction which must presumably be located at the cell surface enables some smaller component to penetrate deeper into the cell. Most of the work on complement lysis of bacteria has dealt with death of bacteria rather than with proved lysis; and it is doubtful whether it is legitimate to transfer the arguments based on complement lysis of red blood corpuscles direct to the problem of bacterial lysis, as is commonly done. This is a field which demands more investigation before much

can be drawn from it to help our conception of the nature of the cell surface.

Lysis of bacteria by lysozyme is more probably a true surface effect, at any rate in the first instance. Lysozyme is a protein enzyme, of molecular dimensions such as to make its ready penetration into the cell unlikely. Its action was shown by Epstein and Chain (1940) to be one of hydrolysis of large carbohydrate complexes, such as may reside in the cell surface. Mere disruption of the cell surface is probably, however, not the sole effect of lysozyme action, for Penrose and Quastel (1930) found that suspensions of *Micrococcus lysodeikticus* which had been subjected to the action of lysozyme lost their power to activate hexose sugars and glutamic acid.

The lytic effect of NaOH may also be hydrolytic in essence, though I know of no evidence as to the true nature of this reaction.

Lysis of bacteria by bacteriophage presents other problems. The current conception of this reaction is that a phage particle, having attached itself to the cell surface, penetrates the cell and multiplies in the protoplasm. Disruption of the surface membrane follows when phage concentration within the cell has reached a certain 'burst size', and the contained protoplasm and phage particles flow out (Wyckoff 1948). The story rests largely on electron micrographs of bacterial cells which, in addition to anything the phage can do to them, have received grievous battering in their preparation for the electron beam. Perhaps the sequence of events may not be quite what the electron microscope has suggested; but in any case it tells us very little so far of the nature of the cell surface.

It is doubtful whether one should regard the surface of the bacterial cell as an integument, even though its molecular configuration is specialized and lends it some rigidity and elasticity; even if the electron pictures of phage lysis mean what they appear to mean, it seems clear that other reactions may cause rapid disintegration of the cell surface, along with disintegration of the rest of the protoplasm. Whatever its molecular configuration, it seems clear that the cell surface must be a readily penetrable boundary between those intracellular phases in which diffusion can occur, and the outside. Its nature must be closely related to

that of the protoplasm which underlies it, and with which it is in functional continuity. Probably the secrets of its functional texture are hidden in and protected by an orderly arrangement of water molecules held in the hydrated shell of the carbohydrate-lipoid-protein complexes which seem to be chiefly associated with the surface, and which are produced in greatest abundance during active growth, splitting off their smaller haptene molecules which pass away into solution, as the complete antigen is regenerated in the surface.

The ordinary bacteriologist probably carries in his mind some such picture of the bacterial surface as I have tried to draw. The outline of this picture is not clear, for there is no clearly defined separation of any one surface from the rest of the cell, and there is a possibility that some of the reactions usually referred to the surface actually take place inside the cell.

Discussion

Mr. J. Smiles referred to the contractility of the bacterial cell wall. He had occasionally observed qualitative differences of considerable magnitude in the rheological properties of the membranes and of the cytoplasm of different types of micro-organisms. The group of organisms to which that of bovine pleuropneumonia belongs appears to possess membranes which have an extremely low modulus of extensibility. When preparations of young cultures of the organism of bovine pleuropneumonia are examined by darkground illumination, a number of interesting phenomena can be observed. The organisms are usually in the form of long thick filaments which settle on the slide if the medium in which they are mounted is not disturbed by currents. When in contact with the slide they slowly change their form. A tendency to divide by segmentation is evident; the filaments break down into chains of short filaments or into round and granular bodies, which may revert to the original long filamentous forms. The short filaments may spread out over the slide and develop into large flat round bodies. The fact that the organisms spread out on the slide indicates that the membranes have a low modulus of extensibility and enclose substances of a fluid nature. If the medium is disturbed when the long forms

have settled on the slide, currents develop which cause the organisms to stretch. The cell contents divide into two or more segments held together by fine filaments which can be stretched considerably before rupture occurs. These filaments are permanently deformed before the breaking strength is reached, but if the pressure on the segments is released before deformation takes place the segments will be drawn together by the elastic fibres. Further evidence for the fluid nature of the cell content need not be considered here.

In bacteria the modulus of extensibility of the cell membrane is considerably higher, but varies from one species to another. Ultra-violet microscopical studies of the effect of air- and freeze-drying on bacteria disclose a variation in the shrinkage of different organisms which depends on whether or not the organisms are in contact with a flat surface at the time of drying, and, if in contact with such a surface, on the area of the contact surface.

When *Bacillus megatherium* is air-dried in contact with a flat surface there is a slight but definite shrinkage of the cell wall and the cytoplasm, but when a suspension of organisms in water is frozen and then dried, shrinkage and distortion of the organisms are excessive. Where the rheological properties of the membrane and cytoplasm are such as to allow a moderate area of an organism to be in contact with the surface this area is increased on drying by the surface tension of the last traces of the fluid between it and the surface. In effect the drying of this fluid tends to flatten the organism still further, and as drying continues, shrinkage in directions parallel to the surface is reduced to a minimum. In general, the smaller the area of contact when an organism in a fluid medium first settles on a flat surface, the greater will be the shrinkage.

Dr. I. M. DAWSON referred to the shrinkage which occurs when bacterial cells are dried before examination in the electron microscope and agreed that it is rather difficult to identify cell-wall material in dried preparations of intact cells.

King and Alexander (1948) measured the lethal effect of shaking bacteria with glass beads. Recently he had found at Hampstead that this method can be used to prepare cell-wall suspensions from several bacterial species. In a typical experiment with

Staphylococcus aureus a suspension of the bacteria in distilled water was shaken with an equal volume of 200 mesh glass beads for one hour in the Mickel microshaker. The resultant suspension, on centrifugation at 8,000 r.p.m. for 15 minutes, gave a supernatant fluid which contained most of the cell cytoplasmic material and a deposit containing the cell-wall material and a few intact organisms. Plate 5 shows a palladium shadowcast micrograph obtained from this deposit. There are three intact cocci characterized by the relatively long shadows they cast and by their electron opacity. The free cell walls in the micrograph are electron transparent and throw very short shadows, indicating that the cytoplasmic material flowed out in the shaking process and remained in the supernatant fluid on centrifugation; several show clearly where rupture of the cell wall took place.

The staphylococcal cell wall, therefore, is mechanically more rigid than the cytoplasm; its thickness in this micrograph is 15–20 mμ. No detailed microstructure had yet been observed in the free cell wall at the present limit of resolution of his electron microscope.

Dr. D. McCLEAN said that he had repeatedly grown pneumococci in homologous antiserum, and asked Prof. Spooner to elaborate the doubts he had expressed about the effect of antibody on the capsulated organisms.

Prof. SPOONER said the question was whether, when growing in antiserum, the pneumococci virtually became rough; that is to say, would a pneumococcus which carried a specific capsular antigen on its surface grow in anti-capsular serum without changing into something else.

Dr. H. TAYLOR said that when a Smooth strain is grown in the presence of homologous antibody, for several sub-cultures, there is no observable change. Then, at perhaps the sixth or seventh sub-culture the cells agglutinate in a different fashion and generally prove to be Rough on sub-culture.

PLATE 5.

Palladium shadowcast electron micrograph of *Staph. aureus* cell walls. (Dr. I. M. Dawson.)

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VIII

CAPSULE FORMATION IN THE PNEUMOCOCCUS

by HARRIETT E. TAYLOR

CAPSULE formation is very widespread among bacterial species. In the group Pneumococcus, with which the present report will be concerned, most of the commonly recognized members which possess differentiating or type-specific antigens become surrounded by a gelatinous envelope of polysaccharide when cultural conditions are favourable for growth. The presence of this capsule at the surface of these bacteria is one of the essential conditions for their virulence, and its chemical composition determines the specific serological reactions of each encapsulated strain. In the course of evolution the capsular antigen has undergone tremendous variation, there being at present over seventy different types described, although other antigens of the pneumococcus seem to have undergone very little differentiation. The nature of the capsular antigen, and the circumstances which determine its formation, plays a dominant role in determining the character of the interface between pneumococci and their environments.

Several kinds of evidence indicate that the capsular antigens of the pneumococci are complex. For example, all of the type-specific antibody of a given antipneumococcal serum can be specifically precipitated by the purified capsular polysaccharide obtained from a homologous strain. Yet the polysaccharide alone is not antigenic in certain animal species, notably the rabbit, although injection of the bacterium as a whole induces the formation of precipitins specific for the capsular polysaccharide (Avery and Niell 1925). Thus, while there is ample reason to believe that the antigens differentiating encapsulated races are endowed with their specificity by the same polysaccharides which are found in their capsules, there is also reason to believe that the polysaccharides alone are not the complete antigens. The com-

plete antigen does not appear to be located in the capsule, which may be removed from killed bacteria by a specific polysaccharidase leaving the bacteria still competent to induce the formation of antibody directed specifically against homologous polysaccharide (Dubos 1939). To explain these and other facts, it has been proposed that the capsular antigen is a dual structure composed of the antigen proper lying in the surface layers of the bacterium, and of a capsule, external to these layers and readily accessible to the action of a polysaccharidase (Dubos 1945).

The capsule probably consists of little more than polysaccharide, contaminated with extracellular products of metabolism. Because the polysaccharides are water soluble, the capsule is continually going into solution, and does not, therefore, have a constant size or definite boundary. The thickness of the capsule is probably governed by the interplay of two factors: the rate of synthesis or secretion of polysaccharide, and its rate of dissolution. In the presence of serum, the capsule is found to be very large. As Dubos (1945) has pointed out, this is probably due to the increased viscosity of the medium which retards the diffusion of the polysaccharide away from the bacterium. Although composed of carbohydrate, the capsular material does not seem to be utilizable by the pneumococcus itself, and is not apparently a storage polysaccharide. Dubos has suggested that the capsular polysaccharides are waste products, and that capsules are not so much structures as they are regions about the bacterium which are modified by the slow diffusion of a waste product of high molecular weight. This view is sustained by what has since been discovered concerning the metabolic origin of the dextrans and levans of other bacteria in which polysaccharides having high molecular weights are formed from the residue of the carbohydrate which the bacterium does not utilize.

While this much can be said about the capsule, the nature of the capsular antigen is unknown. In no case is there convincing evidence that cell-free extracts of pneumococci can induce the formation of antibody which specifically precipitates capsular polysaccharide, except when these extracts were injected into species of animals in which the purified capsular polysaccharide itself is antigenic. In a study of the capsular antigen, Dubos showed that when pneumococci pass from the Gram-positive to

the Gram-negative state by a process of limited autolysis, they no longer can stimulate the formation in rabbits of precipitins specific for capsular polysaccharide. The material released by this autolysis represented from 4 to 10 per cent of the dry weight of the bacteria, was composed largely of ribonucleoprotein and ribonucleic acid, and was apparently incapable of stimulating the formation of specific precipitins by rabbits (Thompson and Dubos 1938, Dubos 1938). Thus, the problem of the nature of the capsular antigen has become associated with the more general problem of the nature of the Gram reaction (Dubos 1937).

Though the capsular antigen is believed to be the most superficial antigen of the pneumococcus, it may not be actually more superficial than some of the protein antigens of the bacterium. The presence of a capsular antigen usually accompanies the formation of a capsule at the bacterial surface, and the serological reactions of the capsule completely dominate the agglutination reactions of the bacteria. Once the capsule is removed by repeated washing of the killed bacteria, or by enzymic treatment, antipneumococcal antibodies specific for protein constituents, common to the Pneumococcus group, can act as bacterial agglutinins (Avery and Dubos 1931). This suggests that these protein antigens also are localized in the surface layers of the pneumococcus. Furthermore, while the antigenicity of the capsular structure is associated with the intactness of the complex which gives the Gram-positive reaction, variant Rough (R) forms which have permanently lost the capacity to form capsules are no less Gram-positive. Hence, if the capsular antigen is a part of the complex which retains the Gram stain, it is very likely a small part.

How the capsule and the capsular antigen are related to each other functionally is not known. It is conceivable that the capsular antigen is the enzyme which synthesizes the capsular polysaccharide, but there is no evidence that this is actually the case. However, a large body of data has been obtained concerning the factors intrinsic in the pneumococcus which are responsible for the presence of the capsular antigens and the corresponding capsules. These intrinsic factors will be discussed below.

Permanent, inherited differences exist between races of pneumococci not only with respect to the chemical structure of the

capsular polysaccharides formed, but also with respect to whether they form capsules at all. Unencapsulated or Rough (*R*) forms have been isolated from nature, and also obtained from encapsulated (*S*) races of many types, in culture, under laboratory conditions. Most of the Rough strains obtained in the laboratory are stable variants of *S* strains, and as long as they are maintained in ordinary culture media, no reversion appears to occur. But the behaviour of R races when they are cultured under conditions which favour the growth of *S* pneumococci permits one to divide these races into two groups. There are strains in which reversion to the encapsulated condition can occur, and those in which reversion does not occur even after prolonged cultivation under such conditions. It is remarkable that when an *R* strain spontaneously reverts to the encapsulated state, the kind of polysaccharide synthesized by the reverted bacterium is always that kind which was synthesized by the encapsulated strain from which the *R* pneumococci were derived. *R* strains obtained from different types of *S* races resemble each other closely as antigens, and apparently do not stimulate the formation of type-specific antibody when injected into animals. Thus, these bacteria have lost both the capacity to form a capsule, and a capsular antigen. However, strains have been isolated which have properties intermediate between the *R* and *S* conditions (Blake and Trask 1933, MacLeod and Kraus 1947, Taylor 1948). These strains possess the capsular antigen, but some of them synthesize or secrete so little polysaccharide that they do not appear to be encapsulated. These races, too, are genetically stable under usual cultural conditions, but like certain *R* strains, can give rise to normally encapsulated forms under environmental circumstances which strongly favour the growth of *S* pneumococci.

The experiments of Griffith (1928), followed by those of Avery and his associates (Dawson 1930, Dawson and Sia 1931, Alloway 1932, Avery, MacLeod and McCarty 1944, McCarty and Avery 1946 a, McCarty and Avery 1946 b, McCarty, Taylor and Avery 1946) have greatly advanced the understanding of the intrinsic differences between the strains just described. It is now well established that certain *R* strains, derived from encapsulated strains, can be transformed specifically into the encapsulated state

by means of material extracted from encapsulated pneumococci. The kind of polysaccharide produced by the transformed bacterium is determined by the source of the pneumococcal extract used to perform the transformation, and appears to be quite independent of the origin of the R pneumococci. These transformations can be achieved only under special cultural conditions, some of the details and possible significance of which will be discussed presently. It is a unique aspect of this transformation that the transforming agent not only induces the R bacteria to form a capsular antigen and a capsule, but also induces its own perpetuation through successive bacterial generations.

Chemical and biochemical studies have shown that the transforming substance is present in a highly purified, viscous desoxyribonucleate fraction of the encapsulated bacterium. This has been demonstrated using Type II, III and VI pneumococci as a source of the nucleate (McCarty and Avery 1946 b). Transforming activity of these fractions is destroyed rapidly and specifically by small amounts of crystalline desoxyribonuclease, which depolymerizes the nucleate. Further, studies upon extracts in the course of purification show that as protein, polysaccharide and ribonucleic acid are removed, transforming activity remains proportional to the concentration of desoxyribonucleic acid in the extracts (Hotchkiss 1948). All evidence thus far obtained is compatible with the view that the transforming principle is a desoxyribonucleate of high molecular weight. No other substance seems to be involved in producing the transforming activity of the pneumococcal extracts. If the transforming principles are composed solely of desoxyribonucleic acid it is clear that desoxyribonucleates must be substances having diverse biological and chemical properties. At present we have no notion of what kinds of variations in chemical structure could endow these substances with diverse biological specificities. Quantitative analysis of the products of hydrolysis of pneumococcal desoxyribonucleate indicate that it resembles desoxyribonucleates obtained from other sources (Hotchkiss 1948). It should be noted, however, that none of the desoxyribonucleates studied with sufficient care have proven to have the composition expected if the basic unit of the polymer were simply a repeated tetranucleotide.

While very small amounts of desoxyribonucleic acids isolated from *S* races of pneumococci can transform susceptible *R* pneumococci into encapsulated bacteria, a chemically similar fraction isolated from an *R* strain is ineffective in inducing such transformations (McCarty and Avery 1946 b). It may be argued from this that the transforming activity found in the desoxyribonucleates of *S* pneumococci is due, after all, to a contaminating substance present in extremely minute amounts, and that its action is dependent in some way upon the simultaneous presence of the highly polymerized nucleate. However, it may be equally well argued that the desoxyribonucleate of the *S* pneumococcus is composed of molecules having diverse configurations, compositions and biological specificities, only one or a few of which molecules are involved in the transforming reaction. Biological evidence has recently been obtained in support of the second view, which already seemed the more satisfactory interpretation of the biochemical and chemical studies on the transformation principle. A second kind of transformation can now be performed using the desoxyribonucleates of either *R* or *S* pneumococci as the source of transforming principle. In this second kind of transformation, the bacterium undergoing the transformation is a variant of an *R* strain, and differs from the parent *R* clone in that the pairs of diplococci do not become detached from each other as growth proceeds. This leads to a characteristic alteration of mode of growth in liquid and on solid media. Transformation of bacteria of this strain, designated extreme rough (*ER*), converts them into typical *R* pneumococci (Taylor 1949 a). *ER* pneumococci cannot be directly transformed into encapsulated bacteria, but must first be transformed into *R* forms. The circumstances surrounding this transformation in two steps suggest that at least two transforming agents of different specificity are present in the desoxyribonucleic acid fraction of the *S* pneumococcus: one responsible for the change of *ER* into *R*, the other for the change of *R* into *S*. However, only one of these two agents appears to be present in the corresponding fraction of the *R* pneumococcus, i.e. the agent which transforms *ER* into *R* (Taylor 1948). Thus, the desoxyribonucleate fraction of the *R* pneumococcus is not devoid of all transforming activity, but appears to be devoid of a capsular transforming principle.

The apparent loss of a capsular transforming principle from the *R* strain analysed in these studies can be interpreted as an actual physical loss of this agent. It should not be overlooked, however, that transforming principles are autoreproducing, and resemble in this respect genes and viruses. These latter agents can mutate, and in doing so, lose certain of their manifestations without interrupting their propagation. Similarly, while the *R* pneumococcus has lost the capacity to form a detectable capsular transforming principle, this bacterium may none the less be propagating a homologous agent having modified activities. It is difficult to see how certain strains of *R* pneumococci could undergo reversion to the encapsulated state if a total loss of the capsular transforming principle were always the cause of disappearance of capsule and capsular antigen.

This speculation is more justifiable in view of experiments which have been made upon forms of pneumococci intermediate in characteristics between *S* and *R*. These intermediates are stable clones of pneumococci which possess capsular antigens, but in which capsule formation is diminished under the same conditions of culture which permit normal capsule formation by typical *S* strains. Three such intermediates have been isolated from a Type III encapsulated strain, two of which have been studied in some detail (Taylor 1948). These three clones form a series in which the manifestation of a capsule is progressively suppressed. The strain in which a capsule is least in evidence resembles an intermediate isolated from a Type II race, and studied by MacLeod and Kraus (1947). Intermediates of this extreme kind carry capsular antigens, as demonstrated by the antibody reactions they evoke in rabbits, but they are not virulent, nor do they give the capsular swelling reaction in homologous antiserum. The colonies of these races cannot be distinguished with certainty from the colonies of *R* races, for they are not mucoid as are the colonies of normally encapsulated races. In agglutination reactions with homologous type-specific antiserum, pneumococci of the intermediate races are agglutinated with less type-specific antibody than are the pneumococci of the corresponding normally encapsulated strains. This can be accounted for if it is assumed that the secretion of soluble polysaccharide is diminished in the variant strains. Such an hypothesis is also in

agreement with the observations that less capsular polysaccharide can be isolated from cultures of these variants. Some polysaccharide is, however, formed at least by the Type III intermediate of this extreme kind, for a type-specific precipitin present in cell-free extracts of this strain can be destroyed by the specific polysaccharidase which attacks the Type III polysaccharide (Taylor 1948). While several kinds of evidence indicate that the polysaccharides formed by the intermediates are modified only quantitatively, as yet no adequate chemical study has been made to verify this point.

The desoxyribonucleate fractions of these strains transform *R* pneumococci, under appropriate cultural conditions. The characteristics of the transformed bacteria are invariably those of the pneumococci used as a source of transforming principle. Thus, if this agent is obtained from a Type III pneumococcus in which capsule formation is suppressed, the transformed bacterium is a Type III organism of the same modified nature. Provided the *R* pneumococcus is transformed first into an intermediate form in which little polysaccharide is synthesized or secreted, a second transformation can be performed with the transforming principle of the normally encapsulated pneumococcus, rendering the doubly transformed bacterium normally encapsulated. Hence, an *R* pneumococcus may be converted into a fully encapsulated one by two transformation steps as well as by one. The following two-step transformations have been performed: *R* into Type II intermediate into Type II normal (MacLeod and Kraus 1947); *R* into Type III intermediate into Type III normal (Taylor 1948); *R* into Type III intermediate into Type II normal (Taylor 1948). Thus, the intermediate can be transformed into an encapsulated form of heterologous type.

In interpreting the significance of two-step transformations of this kind, MacLeod and Kraus have emphasized the possibility that in passing from the *R* to the *S* condition the *R* bacterium may have to acquire more than one element from the *S* transforming extract, the intermediate condition resulting from the possession of only part of the necessary elements. However, if this were the case, one should expect forms intermediate between *R* and *S* to appear frequently in transformations of *R* pneumococci with transforming principles obtained from fully encapsulated

races. The appearance of such intermediates in these transformations is, on the contrary, a very rare event. This alone indicates hat the transforming principle of an intermediate race is not at normal constituent of the transforming extract obtained from the corresponding fully encapsulated strain. Moreover, strict specificity of the transforming principle obtained from the fully encapsulated race is not due to an artificial selection by the transforming mechanism of only one kind of transformed pneumococcus. It has been shown that when R pneumococci are transformed with mixtures of the transforming principles of normal and intermediate races, both fully encapsulated and intermediate forms appear as transformation products (Taylor 1948). It seems more likely, therefore, that the strains of pneumococcus which are intermediate in character between R and S, are strains in which the capsular transforming principle has undergone mutation, affecting the capacity of the agent to induce polysaccharide synthesis, but not its powers of autoreproduction.

It has been demonstrated in one of the two-step transformations described in the paragraph above (R into Type III intermediate into Type III normal) that after completion of the second step, the resulting bacterium proves to possess only the second of the transforming principles with which it interacted (Taylor 1948). This suggests that a given pneumococcus can possess only one capsular transforming principle at any given moment. Furthermore, it indicates that the second step of the two-step transformation just described consists of a replacement of the transforming principle in the bacterium by an analogous principle introduced from the environment.

Although it is unlikely that we now recognize all of the intrinsic factors responsible for capsule formation, it is none the less clear that the capsular transforming principle, apparently a desoxyribonucleic acid of high molecular weight, must be present in the bacterium if capsule formation is to occur. This agent plays a major role in determining the properties of the surface of the pneumococcus, since it determines the kind of capsular polysaccharide formed and the perpetuation of the capsular structure through an indefinite number of cell generations. The capsular transforming principle is apparently autoreproducing, and can

mutate to give rise to new lines of pneumococci in which capsule formation is reduced.

In discussing the nature of the encapsulated pneumococcus, three elements have been discerned: the capsule proper, the capsular antigen and the capsular transforming principle. Each seems to be a distinct part of the encapsulated bacterium, but how these elements are related to each other, structurally or functionally, is not known. It has been suggested that the transforming principle initiates a series of reactions, leading first to the formation of a specific enzyme, which in turn forms the polysaccharide (Avery, MacLeod and McCarty 1944). This is a working hypothesis in which the problems of functional relationship are clearly implicit. The question of structural relationship has thus far only been indirectly touched upon. The remaining paragraphs will be devoted to speculations upon this question, and to a brief discussion of some experimental material which may have some bearing upon it.

Since the capsular transforming principle resembles a gene in several functional respects, it is conceivable that the capsular transforming principle is located in a nuclear body, separated from the superficial antigen, whose properties it determines. The first condition of such an hypothesis is that the pneumococcus contain a nuclear apparatus. A granule of Feulgen-positive material which appears to be duplicated before fission can be demonstrated in the pneumococcus (Taylor, unpublished) as well as in many other kinds of bacteria. Thus, it is possible that the bulk of the desoxyribonucleic acid of the pneumococcus is located in a nuclear structure. We have no notion, however, as to whether or not the capsular transforming principle is likewise located in that body.

On the other hand, it is conceivable that the capsular transforming principle is located in the surface layers of the bacterium, as is the capsular antigen. This would imply that these layers of the bacterium are composed of elements, at least some of which are autoreproducing. It has already been pointed out above that there is reason to believe that the capsular antigen may be a part of the complex which retains the Gram stain. In this connection it is a striking fact that a Gram-positive reaction is given by few biological structures, and all of these are believed to be auto-

reproducing structures, i.e. the chromosomes of certain species, mitochondria, centrosomes and centromeres. However, there is one difficulty with the hypothesis that the transforming principle is, like the capsular antigen, associated with the material which makes the pneumococcus Gram-positive. The material released by a limited autolysis of pneumococci, which destroys both the capsular antigen and the Gram-positive reaction of the cocci, does not appear to contain any desoxyribonucleic acid. It is possible, of course, that small amounts of this substance are none the less present, but difficult to detect in the presence of large amounts of ribonucleic acid.

It has been shown that a Gram-negative bacterium, *Escherichia coli*, can undergo transformation of serological type, under circumstances which are very analogous to those found for the pneumococcus (Boivin, Delaunay, Vendreley and Lehoult 1945, 1946). The similarities between these two cases of specifically induced transformation indicates that the same mechanisms determine the formation of polysaccharide capsules in both Gram-positive and Gram-negative species of bacteria. This cannot be considered as evidence against the hypothesis discussed in the preceding paragraph. Although the Gram-positive reaction may be given only by autoreproducing structures, not all such structures give this reaction.

Certain aspects of the transformation phenomenon are more satisfactorily explained by supposing that the capsular transforming principle is superficially located in the bacterium, forming alone or in combination with other constituents an autoreproducing unit. If indeed the capsular transforming principle is a desoxyribonucleic acid molecule having the same size as those making up the bulk of the fraction used in inducing the reaction, it is a large molecule having a molecular weight of about 500,000. It is somewhat easier to visualize the interaction of this molecule with a superficially located receptor, than to see how it can penetrate the bacterium and eventually become integrated in a nuclear structure.

A detailed study of the mechanism of transformation should tell us much about the functional and structural relationships of the antigen, capsule and transforming principle. Studies on this mechanism have been under way for several years, but we are

far from understanding even the first step of the transformation reaction. At present it appears that the several accessory factors required to effect transformations play a major role in permitting the effective contact between transforming principle and pneumococcus. It is known that the R pneumococcus grown in nutrient medium is unable to react with the capsular transforming principle, but when certain pathological serous fluids are added to this culture medium transformation can take place (Avery, MacLeod and McCarty 1944). Recently it has been found that transformation proceeds as well when small amounts of anti-Rough pneumococcal antiserum are added to the nutrient medium, along with slightly greater amounts of purified or crystalline serum albumin (Hotchkiss, Taylor and Avery, unpublished). The albumin may be of bovine or human origin. During the early phases of growth of an R culture, these accessory substances act upon the bacteria in the absence of transforming principle, altering the pneumococci in some manner which permits them to fix the transforming principle in as short a time as five minutes (for technique, see McCarty, Taylor and Avery 1946). Before this early phase, occupying four hours' time, is completed, transformation cannot occur. This reactive or sensitized condition of the R pneumococci is transitory, disappearing as soon as the growth rate of the culture slows appreciably. The sensitization can be rapidly destroyed if the sensitized bacteria are washed with fresh medium, or even disaggregated from their agglutinated condition and left for a few minutes at 37° before adding the transforming principle. These and other facts indicate that special environmental and metabolic factors intervene to permit the interaction of the pneumococcus with the transforming principle. These conditions are non-specific in the sense that they in no way determine the kind of transformation which ensues. Further, they are in general required to obtain any one of the transformations of pneumococcus now recognized. Thus, if the bacterium undergoing transformation is not readily agglutinated by R antibody, as is the case in transformations of Type III intermediate pneumococci, a suitable agglutinating mechanism must be substituted (Taylor 1948).

At present it is simplest to visualize the first step of the transformation reaction as a change in the surface of the pneu-

mococcus, induced by a complex of factors, of which the transforming principle itself is not a part. This change may be one which permits the transforming principle to adhere to the bacterial surface. That such a change should be required is hardly surprising, if the transforming principle is a desoxyribonucleic acid, since under physiological conditions bacteria are negatively charged. Furthermore, in the growing state, bacteria possess their maximum content of ribonucleic acid, at least a part of which is probably at the bacterial surface. Neither the charge nor the content of ribonucleic acid should facilitate an interaction with a desoxyribonucleic acid.

To understand the role of the accessory factors alone in the induction of specific transformations will require a greater knowledge of the nature of the bacterial surface than we now possess. Yet from what we do know at present it appears that the bacterial surface is a more labile structure than we had supposed. The little we know of the mechanism of transformation indicates that in the course of growth in a certain environment a profound reversible physiological change takes place in the surface of the pneumococcus, which permits interaction with a large biologically active molecule. The fact that specific transformation occurs at all demonstrates that the bacterial surface is more labile in a genetic sense than any cellular structure which has been the object of comparable study.

Discussion

Prof. A. W. Downie asked whether agglutinins are necessary in order to obtain transformations. He pointed out that in Griffith's experiments no agglutinins were employed.

Dr. Taylor replied that agglutinins of the rough pneumococci must be added to the medium in which *in vitro* transformations are performed. Experiments suggest that the essential role of the agglutinins is to produce packets of growing bacteria during the logarithmic growth phase, during which transformation takes place under the influence of the transforming principle. In the absence of these agglutinins the R pneumococci grow diffusely. The necessity for growth in the agglutinated state is indicated by the observation that even in the presence of agglutinins trans-

formation can be inhibited if the cultures are agitated during growth, thereby preventing the formation of these packets. Furthermore, a simple mechanical agglutinating mechanism can replace agglutinins in an *in vitro* system (McCarty, Taylor and Avery 1946). It is not yet known why the Rough bacteria must be grown in an agglutinated state in order to transform them. There are some indications that reducing conditions are required by the transformation process. Such conditions would be found wherever there is a dense population of growing pneumococci. In the *in vivo* technique of transformation, discovered by Griffith, localized proliferation of the injected *R* bacteria takes place in a subcutaneous region, and consequently a dense mass of proliferating bacteria is present even in the absence of agglutinins. This may be the reason why agglutinins are unessential for *in vivo* transformations.

Dr. M. R. POLLOCK pointed out that in two-step transformations a fully encapsulated pneumococcus does not possess a transforming principle which can transform *R* pneumococci into the intermediate Smooth condition. On the other hand it does possess a principle which can transform either an *R* or an intermediate Smooth pneumococcus into a fully encapsulated one. An intermediate *S* bacterium transformed by this principle is apparently indistinguishable from the normally encapsulated pneumococcus, even to the extent of no longer possessing a transforming principle which transforms *R* pneumococci into intermediate *S*. This suggests that this transformation is a replacement of something in the intermediate form by something obtained from the fully encapsulated bacterium. This being so, transformation would not necessarily consist of the filling in of a gap, as one was tempted to visualize previously, suggesting that a fully encapsulated bacterium of one type could be transformed into a fully encapsulated bacterium of heterologous type.

Dr. TAYLOR commented that thus far it had not been possible to transform in any way a pneumococcus which formed a demonstrable capsule. She pointed out that this might be due to some kind of interference between the transforming principles involved, or to a failure of the sensitization mechanism to operate due to the secretion of a capsule. Additional evidence has been

obtained, however, that suggests again that certain transformations have as an overall effect the replacement of a principle in the bacterium by one extracted from another pneumococcus and introduced in the transformation cultures (Taylor 1949 b). Notably, between the strains R and *ER*, transformations are reciprocally possible. Thus, strain *ER* contains a transforming principle capable of inducing the transformation R→*ER*, while strain R contains a principle which can induce the reverse change, *ER*→R. These are mutually exclusive principles in the sense that neither R nor *ER* bacteria contain both of them at any one time. Strain *ER* can give rise to R forms, and R to *ER* forms, by spontaneous mutation, as well as by induced transformation. Thus, it is simplest to suppose that the transforming principles of the R and *ER* pneumococci are genetically homologous, that they are two stable forms of a particular kind of determinant of cell character, and that passage is possible from one form to the other by spontaneous mutation. If this is so, induced transformation of *ER*→R or R→*ER* could be interpreted as the replacement of one principle by the other.

Prof. M. STACEY said it was a question of 'patterns' not only in the finished molecule following the complete transformation, but also in the intermediate stages where you must have the right sugar substrate for polysaccharide formation. This Rough/Smooth phenomenon is mainly a matter of polysaccharide synthesis. In dextran and levan synthesis, a sucrose substrate is necessary, and for starch and glycogen a glucose-1-phosphate substrate. In some of these early stages where deoxynucleic acid is not essential a second transforming principle or factor might be explained by the fact that the Extreme Rough organism is building up the right sugar intermediates before polymerization.

He could not see how, in the seventy types of pneumococcus, there could be seventy different nucleic acids—assuming it is the nucleic acid pattern which is determining the building. Another difficulty was that there would be two deoxy-type nucleic acids in a coccus, the R→*S* transforming principle found in the Smooth form, and a *ER*→R transforming principle, different in the sense that it will not give the full complement for the polysaccharide synthesis, in the Rough form.

He asked Dr. Taylor whether she had considered not only the

carbohydrate intermediates but also the metal elements such as magnesium, because magnesium is just the right sort of element to fit in with a nucleic acid in the co-ordinating sense. He also asked whether differences had been found in the extraction of the deoxynucleic acid from the Smooth and from the Rough forms.

Dr. TAYLOR replied that the transformation phenomenon could be regarded as mainly a matter of polysaccharide synthesis or mainly one of nucleic acid synthesis and only secondarily a matter of polysaccharide synthesis.

The change in the pneumococci which takes place in the absence of transforming principle, during the first four hours of growth of the cultures, is not at all the same kind of change as that which is referred to as transformation. Contrary to transformation, it is transitory, disappearing after active growth has ceased. It is a change which permits the bacteria to interact with a transforming principle. This change, called sensitization, may very well be concerned in part with building up the proper metabolic intermediates which permit transformations. Permanent induced transformations have been made only under the influence of transforming principles, all of which thus far discovered seem to be desoxyribonucleic acids.

The transforming principle which changes Extreme Rough pneumococci into Rough, and the principle which changes Rough pneumococci into Smooth, are both found in the desoxyribonucleic acid fraction of smooth bacteria. Of these two principles, only the former is present in Rough pneumococci. Both principles are highly susceptible to destruction by desoxyribonuclease. The experiments made thus far indicate that the agents responsible for these two transformations are independent units. They suggest, further, that to account for the differences in biological activity of the desoxyribonucleic acid fractions of Rough and Smooth pneumococci, chemical differences must exist between these two fractions.

There are no differences in the techniques used to prepare the desoxyribonucleic acid fractions of Rough and Smooth pneumococci, except that in preparations from Rough strains it is not necessary to take steps for the removal of capsular polysaccharide. The polysaccharide of Type III Smooth pneumococci is removed by treatment with a specific polysaccharidase. The capsular

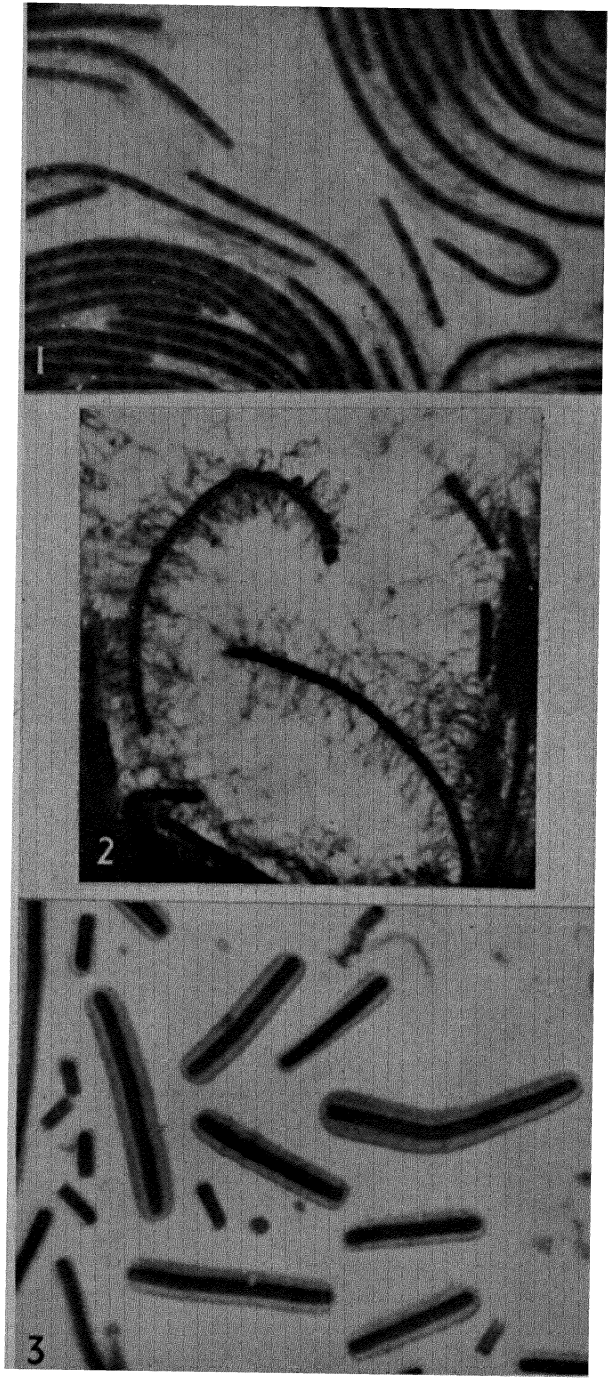

PLATE 6.

Fig. 1. Swarming *Proteus vulgaris*, fixed and stained for cytoplasm. The dark parts of the bacilli represent the cytoplasmic portions. Fig. 2. Swarming *Proteus vulgaris*, treated as the preparation used for fig. 1, with the only exception that the staining process was much extended. Fig. 3. *Bacillus anthracis*, strain H. M., grown on 20–30 per cent horse serum agar, fixed and stained as the preparation represented

polysaccharides of a number of other types can be removed by the same precipitation which separates nucleic acid from somatic polysaccharide.

As to whether carbohydrate intermediates and minerals are essential for the transformations, very little is known. Transformations are carried out in a complex medium, which presumably contains adequate magnesium. Addition of more magnesium does not affect the outcome of the experiments. Certain experiments have suggested that an excess of glucose can inhibit transformations of Rough pneumococci to Smooth.

Dr. EMMY KLIENEBERGER-NOBEL demonstrated on photomicrograms that cytoplasm (Pl. 6, fig. 1), flagella (Pl. 6, fig. 2) and capsules (Pl. 6, fig. 3) can be fixed and stained by the same method; namely, fixation by Bouin's solution through the agar followed by staining with Giemsa solution for different periods of time. Consequently it is possible that they are made up in part of similar chemical constituents. The capsule may be composed of a fine basic framework, perhaps protein in nature, in which the polysaccharides or polypeptides are deposited. This assumption would account for the definite shape and outline which capsules possess, a feature distinguishing them from amorphous slime.

Dr. D. MCCLEAN said that his main purpose in intervening in the discussion was to remind the meeting of an interesting state of affairs concerning the surfaces of streptococci which had not been referred to in any of the opening papers or comments. The position differs strikingly from that described for pneumococci, and he thought that it merited more experimental investigation.

In 1897 Bordet associated the virulence of streptococci with capsules. In 1931 Hare reported that virulent streptococci in young cultures which were capsulated resisted phagocytosis; the older cultures did not. In 1933 Duran-Reynals reported that invasive strains produced a diffusing factor which was later identified as hyaluronidase. The examination of the relation of capsulation to hyaluronidase production revealed an interesting situation.

Some strains of streptococci produce capsules and others hyaluronidase, but never both together (McClean 1941 a). Capsule or hyaluronidase production is a constant feature for strains of any one serological type. This was confirmed and extended by Dr. Crowley in 1944, when she examined over 300 strains.

One only gets capsules in young cultures up to about six hours in fluid medium liberally enriched with serum, and that raises an interesting point which demands more investigation. The secretion of capsular material by the organisms continues as long as there is multiplication, but the material passes into the medium and is no longer visible. Capsules will persist on a suitably enriched solid medium for twenty-four hours or more. The disappearance of the capsules is not related to any change in the oxidation-reduction state or pH of the medium (McClean 1941 b). Streptococcal capsules, unlike those of pneumococci, have no relation to type-specificity or group-specificity. Identical capsular material is obtained from the streptococci of group A, group C and from some other less important groups. Streptococcal hyaluronidase, on the other hand, appears to be group-specific and not type-specific. Antiserum against group A hyaluronidase will not protect against the enzyme produced by group C streptococci.

Hyaluronidase production is adaptive. In 1945 Rogers investigated the conditions under which hyaluronidase was produced by several species of organisms, and he said, among other things, that for streptococci the enzyme synthesis per unit weight of bacterial nitrogen is directly proportional to the amount of hyaluronate added to the medium. The capsular material proved on examination to be hyaluronic acid, and we have the interesting situation that the capsules are immediately destroyed by the addition of hyaluronidase from any source, and therefore these two properties of enzyme or capsule production by some strains of streptococci are mutually exclusive in any one strain. An exception may be Lack's (1948) report that a strain of streptococcus, group A, type 6, which normally developed capsulated organisms, occasionally showed hyaluronidase producing variants.

The immediate disappearance of capsules following the addition of hyaluronidase from any source can be seen under the

microscope. This phenomenon reminded Dr. McClean of an American visitor who, when shown this phenomenon, said, 'I guess your enzyme strips the pants off the streptococci'. When an attempt was made to treat mice infected with capsulated streptococci with intravenous hyaluronidase, it was found that even after very large doses of enzyme the streptococci were only decapsulated for a very short time in the animals' bodies (McClean 1941 b). The inhibition of enzymic activity is apparently due to competitive adsorption of enzyme by a substance present in the pseudo-globulin of the serum (McClean 1942).

An example of the marked difference between the enzymic inhibition of capsulation in the test tube and in the peritoneum of the mouse can be given. In the latter, after an intravenous injection of 10 units of enzyme, the capsules are already reappearing in fifteen minutes and 100 units only maintain decapsulation for one hour, whereas in the test tube the organisms remain decapsulated by one unit of hyaluronidase throughout the period of observation.

In mice infected with capsulated streptococci, the peritoneal exudate is scanty and viscous, due to the secretion of large quantities of hyaluronic acid by the streptococci, and this viscosity can be immediately destroyed by the addition of a trace of hyaluronidase. Virulent streptococci secrete large quantities of hyaluronic acid which is a powerful protective agent, because hyaluronic acid is a normal constituent of the vitreous humour, synovial fluid and other tissues, and is therefore not antigenic and not attacked by the host's defence mechanism. In 1943 Humphrey failed to obtain antibody formation by hyaluronic acid even when this was chemically linked to a protein.

In conclusion Dr. McClean thought that work should be done to find out the metabolic and other factors which determine whether streptococci produce either hyaluronic acid or hyaluronidase. In view of the situation revealed in which organisms of the same species produce either the enzyme or its substrate and what is already known about reversible enzyme systems in other fields, it seems likely that a methodical biochemical study might reveal a very interesting interplay of factors and might increase our knowledge of the part played by the capsule and by the enzyme in the life of the streptococci and in infection.

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IX

BACTERIAL SURFACE, FLAGELLA AND MOTILITY

by A. PIJPER

Fundamental Observation

My fundamental observation is that *Salmonella typhi*, a typical peritrichously flagellated bacterium, by means of sunlight dark-ground microscopy can be shown to drag a long tail behind its body when it is swimming at high speed, and that this tail becomes a definite spiral at lower speeds.

Structure of Bacteria

Through the work of several authors, chiefly Knaysi (1944), and Robinow (1945), some problems of the structure of bacteria have apparently been settled. It is generally accepted now that they contain a watery cytoplasm, of colloid nature, which through condensation at its surface, forms the cytoplasmic membrane. It is closely surrounded by a tough, thin and elastic cell wall which keeps the bacterium in shape. Around this cell wall there appears to be a layer of varying thickness, and of a slimy nature. In 1877 Koch said that if it were not for such a layer, bacteria dried on a glass slide would shrink irrevocably, and that therefore all bacteria must have it, and this is now fairly generally accepted, although for different reasons.

A differentiation between capsules and mucoid envelopes (Klieneberger-Nobel 1948) would be merely quantitative. It seems simplest to follow Dubos (1945) and call it capsule irrespective of thickness. Its consistency is mucous, and its constitution is badly known in most cases, except for pneumococci. It may be a secretion or an excretion, or it may result from gradual gelatinization, liquefaction or other modification of the cell wall. Dubos (1945) suggested that on account of its viscosity it does not

diffuse readily into the surrounding medium, and slight diffusion would explain its generally blurred outline. One must assume that 'life' stops at the cell wall, or as Dubos puts it, 'the capsule is not a constituent part of the body'. According to my theory, the so-called flagella which form the tail are derived from nothing but this lifeless capsule. Kingma Boltjes' contention (1948) that such flagella could not be thrown off without disruption of the whole cell, is therefore quite incomprehensible. Motile bacteria often throw off flagella, without damage to the cell, but this could not be if they were an integral part of the life of the cell and in direct communication with the live protoplasm. Even Zettnow, firm believer in flagella as motor organs, published photomicrographs indicating that flagella underwent a gradual transition into a slimy mass round the body of the bacterium (1918).

About the structure of capsules little is known. Burton and Kohl (1946) published an electron micrograph of a pneumococcus with a capsule in which spiral threads are visible. Indications of spiral structure are found in electron photographs of pneumococci by Mudd, Heinmets and Anderson (1943), after treatment with heavy metals. Heidelberger, Kendall and Scherp (1936) regarded the capsular carbohydrate of pneumococci to be in the form of thread-shaped carbohydrate polymers. The observations of Aschner and Hestrin (1946) probably have a bearing on the subject. They saw that the membranes produced from the slime envelopes of *Acetobacter xylinum* consisted of extremely long fibrils of cellulose, of uniform width, and these fibrils were not hair-like outgrowths of the cell wall but originated extracellularly.

Numerous electron micrographs exist in which motile bacteria lie surrounded by enormous numbers of very thin wavy threads of curiously uniform width. These are commonly regarded as flagella. Their uniform width has been used as an argument in favour of their being motor organs. Bearing in mind the probably micellar structure of the capsule, which in its turn is based on molecular chains of definite composition, the uniform width seems more in favour of the idea that these flagella are just capsular material. Johnson, Zworykin and Warren (1943) described two distinct flagella types, averaging 40 and 16 mμ respectively, in *Achromobacter harveyi*.

In this paper the word 'flagella' will be used without prejudice, and merely for convenience.

Technique

Early efforts to study agglutination phenomena microscopically brought home to me that there was no technique in existence that made bacterial flagella visible in activity (1930, 1931–2). Darkground microscopy seemed indicated, but proved satisfactory only when the sun was used as light source (1931–2, 1938, 1940, 1941 a, 1941 b, 1942, 1946, 1947 b, 1947 c, 1948 a, 1948 b, 1949 b). No artificial lightsource, including high-pressure mercury lamps, can compare with the sun. This sunlight darkground technique must be stressed here because to my knowledge it has not been used by anybody else for this purpose. My equipment does not only allow bacterial flagella to be watched in full activity, but also to be photographed and filmed at normal speed on 16 mm. film. Sunlight is brought to the microscope by a small Zeiss heliostat, standing on a glass plate which can be moved up and down to suit the season of the year. No auxiliary mirror is used and the mirror of the microscope is removed so that no light is lost through reflections. The microscope stand came from Beck, the understructure was made locally and was so designed that it allows sufficient movement to send the focused beam of sunlight through the central axis of the microscope. The Movikon film camera can replace the eye in a moment; it slides on the same bar as the microscope and this arrangement was found to cause no vibration of the microscope.

The Zeiss cardioid condenser proved the best one of those I tried, but other makes come very close to it. To see live flagella in many bacteria no high magnification is necessary, a dry objective 20× coupled with an eyepiece 20× often is enough. For cinemicrography magnification must be kept low to get enough light to the film. The Zeiss oil immersion objective 35× with a photographic eyepiece 4× and a short distance between microscope and film answered very well. For structures larger than flagella the Zeiss oil immersion 60× with iris and occasionally the Beck oil immersion 90× with funnel stop were found suitable. Still pictures were made with a Kontax camera in the

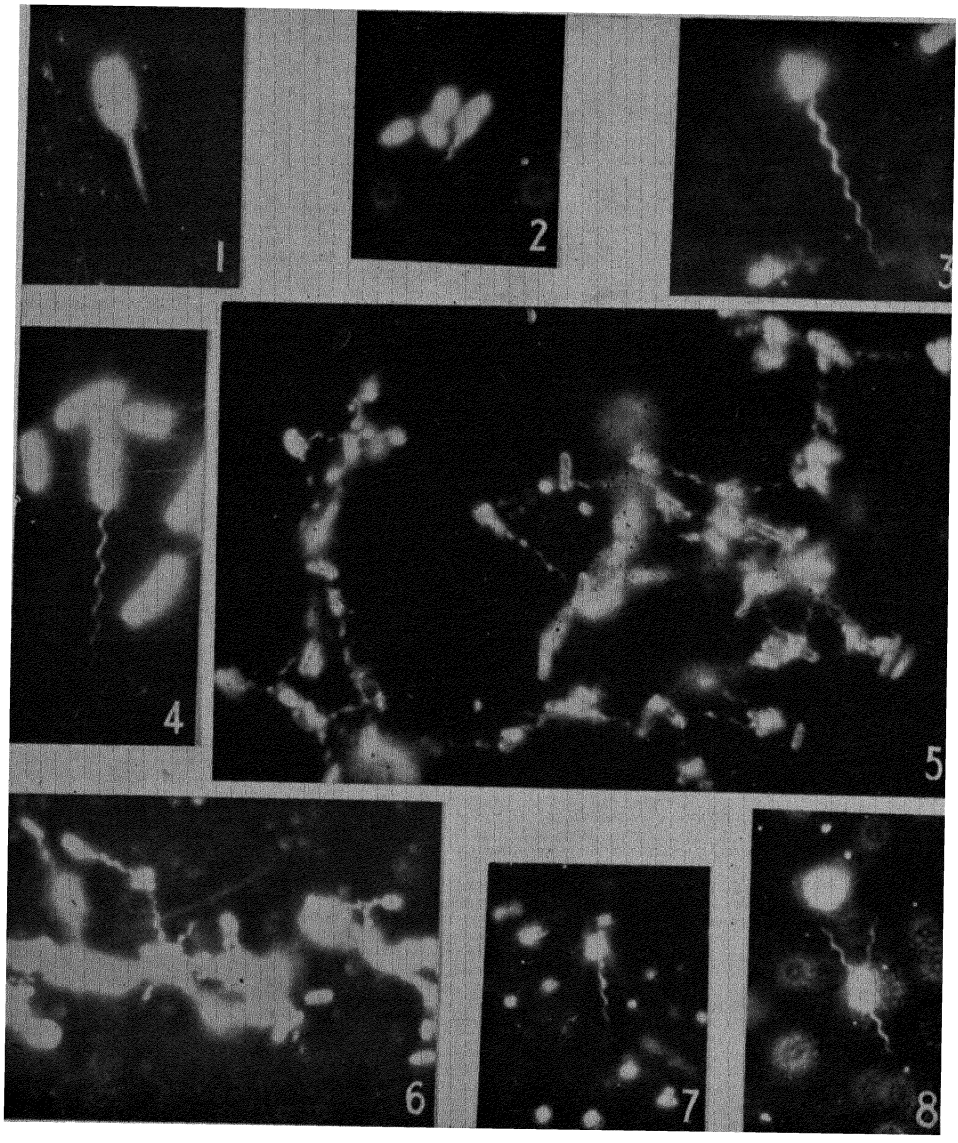


PLATE 7.

Fig. 1. *S. typhi*, in broth, swimming fast, with tail. × 2,860. Fig. 2. *S. typhi*, in broth, slowing down, spiral-like tail. × 955. Fig. 3. *S. typhi*, in gum solution, long thickened tail. × 2,860. Fig. 4. *S. typhi*, in gum solution, coating of tail beginning to break up into granules. × 2,860. Fig. 5. *S. typhi*, in gum solution, coating of tails broken up into granules. × 955. Fig. 6. *S. typhi*, agglutinated by H-serum. Note bacteria linked up by thickened flagella, covering of which is breaking down into granules. × 955. Fig. 7. *B. cereus*, in methylcellulose solution, with thickened tail. × 955. Fig. 8. *B. cereus*, in methylcellulose solution, tail split up into three thickened flagella. × 955. (A. Pijper.)

place of the Movikon film camera, with the beamsplitter-attachment belonging to the Kontax, and usually with a magnification of 300×.

Suitable photographic film for taking moving bacteria does not exist. One has to use the most sensitive material, and this is Kodak Super XX, but it records a fraction only of what the human eye can see. What is needed is higher sensitiveness coupled with greater latitude so that not every slightly brighter spot becomes a glare. The graininess of all photographic material is still appalling.

Against darkground microscopy the objection has been raised (Kingma Boltjes 1948 a), that increased brilliancy of lightsource would spoil contrast, on which the method depends, by making the background grey. This, however, is a question of proper technique, and part of this is replacing the glass slide by mica, and also very careful selection of coverslips. Most coverslips are very spotty in darkground.

It is still necessary to emphasize that motile bacteria should be studied in really watery solutions such as broth. Reichert (1909) first expressed the erroneous idea that motile bacteria can be made to show their otherwise invisible flagella by immersing them in thick colloid solutions of gelatin or gum. The idea was that this would slow them down and also would force them to twist their flagella together and these twisted bundles would then be thick enough to become visible. It was also thought that there would be a change in refractive index which would facilitate resolution. This idea was accepted by Neumann (1925), and by Neumüller (1927) and several others, and it was recently revived by Kingma Boltjes (1948 a). I have demonstrated that this idea is wrong, and that the main effect of such colloid solutions is a thickening of all microscopic structures by a precipitate of the colloid material on them (1931–2). This coating of the bacteria and their tails or flagella hampers movement, very largely prevents tails or flagella from the normal untwisting that often takes place in really watery solutions and produces nothing but caricatures and artefacts. That the thickening is a coating and not an increase in thickness by flagella having become twisted together can easily be proved by just watching and waiting. The fact that flagellar tails in suitable colloid solutions appear so

much longer than in their natural state in water, points the same way (Pl. 7, fig. 3). The precipitation makes so much more material available to be drawn out into a tail. Anyhow, after a while the coating breaks up into a number of granules, and the very thin underlying flagellum becomes visible again, in sunlight darkground microscopy (Pl. 7, figs. 4 and 5). The coated thick flagella are easily visible with ordinary darkground methods. The whole thickening process is very similar to what happens when motile bacteria are mixed with the corresponding *H*-serum. Here too I have shown (1938, 1941 a) that the flagella and bodies become covered with a granular coating of globulins, which may coalesce to a complete sheath (Pl. 7, fig. 6), and later on break down again into granules. This was confirmed by Mudd and Anderson (1941) with an electron microscope.

With certain reservations, however, which follow from the above considerations, the method of suspending motile bacteria in thick colloid solutions has its uses when one has no sunlight darkground equipment. It makes things visible with simple darkground methods which otherwise could not be seen at all (1947 a). But one should be fully aware of the fact that one is looking at artefacts. The artefacts should always be interpreted in the light of observations on perfectly normal bacteria, and these can only be made with sunlight darkfield methods.

There is a further usefulness in the suspension of motile bacteria in colloid solutions in that colloids vary in their tendency to precipitate on different bacterial flagella. Methylcellulose, kindly supplied to me at the suggestion of Prof. R. Breed, by the Dow Chemical Company of Midland, Michigan, as 'methocel', proved very useful in studying *S. typhi*. In strong solutions it produced the clumsy artefacts described above, but weaker solutions could be made up which caused minimal precipitation, so that visibility was increased without too much damage to motility and appearance (1947 a). Solutions of gelatin, gum arabic and mucin cannot be adjusted so nicely. On the other hand, methylcellulose does not precipitate on the flagella of *Rhodospirillum rubrum*, nor on those of *Vibrio metschnikovii*. Conn and Elrod (1947) tried methylcellulose with *Bacillus cereus* and saw no flagella. In my hands *B. cereus* in suitable methylcellulose solutions showed up flagella exactly like *S. typhi* (Pl. 7, figs. 7

and 8). They appeared in their normal thin state in broth or water when viewed with the sunlight darkground technique but remained quite invisible under those conditions with simple darkground methods. It was only the thickening by methylcellulose that brought them out with such methods. There may be a suitable colloid for every motile bacterium, depending probably on chemical affinities between the material of the capsule and the colloid, but conditions like pH will also have to be taken into account. The affinity need not be purely chemical, there may be physical factors; e.g. Svedberg and Pedersen (1940) have shown that methylcellulose molecules are very elongated particles with negligible solvation, and this may be a factor.

At any rate, gum and gelatin as used by Kingma Boltjes (1948 a) completely spoil the appearance of the flagella of *S. typhi* and *Proteus vulgaris* and prevent the observer from seeing flagella as they really are. Observations made under such conditions give no information on normal conditions and cannot be taken at face value.

It is awkward that normal flagella can only be properly seen and photographed by means of sunlight darkground methods, and that there is no suitable artificial lightsource available. But it may be that the advantage of sunlight is not only its supreme brilliancy. Through atmospheric filtration sunlight apparently retains just enough ultra-violet rays to be photographically effective without being biologically harmful. It is of historical interest that Koch (1877), who first photographed bacterial flagella in a fixed state, used a heliostat, and that he hinted at the usefulness of having such light reaching the preparation at an angle, thus foreshadowing sunlight darkground microscopy.

Sunlight Darkground Microscopy and the Electron Microscope

The gains accruing to microbiology from the use of the electron microscope are very considerable, but they can be overestimated. Where motility and motor organs are concerned, it seems reasonable at least to make an effort to interpret electron photographs on the basis of what can be made visible with the sunlight darkground method, instead of the other way round.

The sunlight darkground method allows continuous observation of living freely moving bacteria in three dimensions under the most natural conditions, i.e. in water. The electron microscope makes pictures of stationary flattened-out dehydrated bacteria in an absolute vacuum. What happens to the microbe in the interval between its removal from the culture medium and its post-mortem on the collodion membrane is largely unknown. It has, for example, become customary to assume that when in an electron micrograph cell wall and cytoplasmic membrane have become separated this must needs be due to shrinking of the cytoplast. Van Iterson's electron photographs of *Spirillum serpens* and *V. metschnikovii* (1947 a) are supposed by the author to show this, and as some flagella of *Sp. serpens* seem to traverse the space between cell wall and cytoplasm, she concludes that they are connected with the protoplasm of the cell. I intend showing further on that the flagella of the large spirillum-like *Sp. serpens* are such different structures from the flagella of the ordinary motile bacteria that they cannot be discussed together. But even so, continuous observation of *Sp. undulans*, which for the present purpose can be regarded as identical with *Sp. serpens*, with the sunlight darkground technique, has shown me that it is not the cytoplast that shrinks but the cell wall that becomes distended or blown out when the bacteria die (1949 a; Pl. 8, fig. 1). The distension of the polar end of the cell causes a shift in the normal polar place of attachment of the flagella, and the flagella may then appear to pierce the cell wall. The same kind of shift, in varying degrees, is seen in Pl. 8, fig. 2. It is of interest that van Iterson's own electron micrograph shows a definite bulge of the cell wall just at the pole, which seems to have been overlooked. As to *V. metschnikovii*, van Iterson (1947 a) from a similar electron micrograph 'gets the impression' that the flagellum originates from a basal granule. In a further publication (1947 b), this time without photographs, she states that in *V. metschnikovii* the one flagellum originates from a definite blepharoblast. I intend showing further on that with the sunlight darkground technique the flagellar condition of *V. metschnikovii* appears very different from what is traditionally assumed and this might at least in the future be taken into consideration when conclusions are drawn from electron micrographs. Makers of

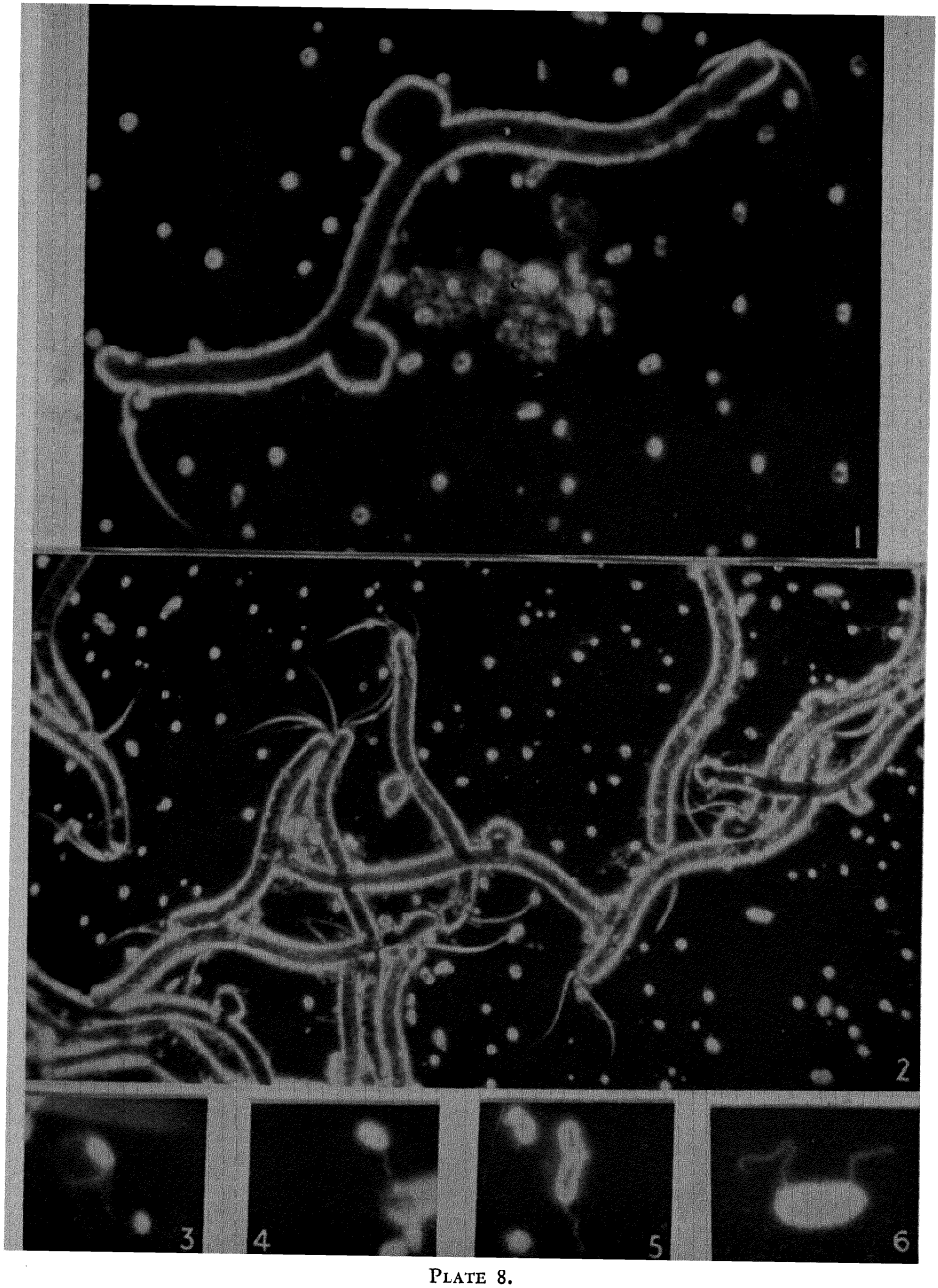


PLATE 8.

Fig. 1. *Sp. volutans*, in water, wall distended by inner pressure, attachment of flagella shifted from pole to side. × 4,000. Fig. 2. *Sp. volutans*, as Fig. 1, but varying degrees of distension and shifting. × 1,800. Fig. 3. *S. typhi*, in broth, tail splitting into two flagella. × 1,000. Fig. 4. *S. typhi*, in broth, swimming slowly, tail on side. × 1,000. Fig. 5. *V. metschnikovii*, in broth, swimming slowly, spiral-like tail. × 1,500. Fig. 6. *S. typhi*, in broth, cell contents being squirted out through two holes in wall. × 3,000. (A. Pijper.)

electron micrographs often are tempted to read more into their pictures than the conditions under which the pictures are taken warrant, and to expect other people to accept such interpretations. Conn and Elrod (1947) in a direct effort to refute my theory, used the electron micrograph of *V. metschnikovii* made by van Iterson and mentioned above, together with another one of the same subject from the same source, as evidence in their argument which followed the lines of van Iterson's without, however, publishing the micrographs, and knowing that in the ordinary course of events I could not possibly have seen them when their criticism appeared. Their own electron micrographs of flagellated bacteria are intended to show that there are monotrichate and peritrichate bacteria and that discrepancies must be explained on the basis of what they call degenerate peritrichous flagellation. As, however, both the appearance of their pictures and the way in which they were made do not offer any guarantee that the wavy threads lying near bacteria really belong to them (some seem to be attached but may be just lying on top of the bacterial body, others are supposed to be attached to the body but are separated from the body by a gap) one can understand that the authors themselves found it difficult to reach a definite conclusion in many cases.

More care should be exercised in interpreting electron microscope pictures, and, especially where motility is concerned, an effort should be made to bring them into accord with the results of observations on living and moving bacteria.

Motility of Bacteria

Whether one accepts flagella as motor organs for bacteria or not, the mechanics of their propulsion are difficult to understand. No such thorough study of the mechanics of bacterial motility has yet been undertaken as those by Lowndes (1943) and Brown (1945) for protozoa.

A bacterium provided with a tail is a very well streamlined body, and the energy needed to propel it should be small. From zoology we know that aquatic animals propel themselves through 'Schlängelung' (Hesse 1935), i.e. through waves passing over the animal, be it in the horizontal or in the vertical plane. Breder

called it 'anguilliform' or eel-like (1926). As Gray (1948) pointed out, the surface of an actively moving fish differs from a rigid model, the resistance probably becoming less as the water flows smoothly past the undulating surfaces. In fish the fins serve for steering, propelling is the work of the body musculature, producing a series of sine waves which are passed caudally by successive contractions and relaxations of the metameral body muscles (Breder 1926). In between a good deal of 'passive gliding' is done, as every goldfish bowl shows. All this can also be seen in motile bacteria, and has been described and filmed by me (1946, 1947 b). The only difference is that bacteria, being non-symmetrical organisms, can afford to perform 'Schlängelung' in both a vertical and horizontal plane at the same time, which means spiral movement, or what I have called gyrating undulating movement (1946).

With bacteria one should differentiate between 'movement' and 'directional movement'. Bacteria can change their shapes very considerably through contractions of their protoplast, which are transmitted to the cell wall, and this causes movement, but it is movement more or less on the spot. Such movements were described in general by Macé (1912), by Remlinger and Dumas (1915) and by Ruffer and Willmore (1909) for dysentery bacilli, by Starkey (1939) for his *Sporovibrio desulfuricans* when it was kept at 55°, and several others. It is this sort of movement which makes a decision as to bacterial motility often so difficult. Somewhat more 'effective' for propulsion are the bodily contractions of the Myxobacteria of which a good description was given by Stanier (1942). In properly motile bacteria these bodily contractions evidently have become definitely spiral, and the movement becomes directional. There are all kinds of transitions between disorderly contractions and organized spiral waves of contraction. Shanahan and Tanner (1948) saw motile *Escherichia coli* when affected by penicillin exhibit peculiar motility in that progression was by a sluggish swimming motion of the entire cell. Stuart, Wheeler and McGann (1946) made some enterobacteriaceae, usually regarded as non-motile, motile through 'conditioning', which meant passages through soft agar, and this was confirmed by Weil and Slafkovsky (1948). I have often seen *S. typhi* and similar bacteria exhibit peculiar swinging movements

of the middle part of their bodies whilst the poles remained steady and the whole bacterium remained on the spot, until a moment later normal spiral contractions took the place of these irregular contractions and directional movement was resumed, and the bacterium swam away as a perfect spiral (1947 b).

As Seifriz (1946) has so comprehensively pointed out, there are many spiral tendencies in animate nature, revealed in the shape, growth and locomotion of organisms. Francis (1940) demonstrated spiral structures in protoplasm. Spiral movement of bacteria therefore fits quite well into the general nature of things.

Motile bacteria often show rapid to and fro movement. All that is needed to explain this is to assume that the spiral wave suddenly changes its direction. According to Breder (1926) the spiral wave does this in fishes when they suddenly stop; some fishes have even been seen to swim backwards in these circumstances. Also, if a really fast swimming bacterium suddenly reverses the direction of its spiral wave, its momentum may carry it on in the original direction, and then it must needs throw a somersault, and this is exactly what one often witnesses in fast moving bacteria.

Tails, Flagella and Motility

Most motile bacteria examined by me so far by means of the sunlight darkground technique have shown a tail, as illustrated in Pl. 7, figs. 1 and 2, for *S. typhi*. The tail can be made visible rather easily in *S. typhi* and *Pr. vulgaris*. It is more difficult in *Bacillus subtilis*, *B. cereus* and *B. megatherium*. *Caryophanon latum* often drags two tails along, and sometimes one. In *Rhodosp. rubrum* I have so far rarely seen a tail. On the other hand, I have occasionally seen tails on undetermined motile bacteria that had got into cultures as contaminants. Most of my work has been done on *S. typhi*, and here I also found the clue why it is not always possible to make tails visible, even with sunlight darkground technique. *S. typhi* as a rule does not show tails when grown in peptone water and also not in certain kinds of broth. The pH, as pointed out to me by Dr. A. E. Oxford, must be watched, a final pH of 6·3 was found to be essential for best results. It

therefore seems that every bacterium has its own optimum medium and optimum pH for showing tails.

The tail of *S. typhi* has often been seen by me (and I am referring here to sunlight darkground microscopy of broth cultures without addition of colloids) to untwist first into two (Pl. 8, fig. 3) and then into a large number of thin wavy threads which group themselves round the bacterial body. The appearance then is exactly like a successful picture of a fixed and stained peritrichously flagellated bacterium. The wavy threads after a while become detached and disappear (1946). There are many electron photographs of motile bacteria showing similar wavy threads lying about, but they are rarely so regular as the sunlight darkground picture and as van Iterson (1947 a) has pointed out, the electron microscope often shows a very much larger number of wavy threads than the sunlight darkground technique and they also are much thinner. It is quite possible that the wavy threads seen by the sunlight darkground technique are capable of untwisting still further. The point that matters is that if these threads are to be regarded as motor organs, then the tail must be regarded as such. There is no reason to accept the suggestion of Conn and Elrod (1947) that although capsular material, which I saw becoming untwisted into the wavy threads, trails behind some bacteria when they are swimming, these bacteria still have motor flagella as well. Individual *S. typhi* can pass one another at such close range that if motor flagella were sticking out on the sides their motility would be disturbed, and this is not the case. All that *S. typhi* possesses by way of flagella is twisted together in the tail, and this is the importance of the tail. Only by studying the tail can the problem of flagella be solved. According to my theory in most of the bacteria usually regarded as motile, such a tail occurs, and it consists of capsular material. Its structure is explained by the gyrating undulating movement of the bacterial bodies, assisted probably by the thread-like nature of the capsular material. It has no motor function but is completely passive. Before this argument can be continued, it must be pointed out that not everything that looks like a tail, is a tail in the sense ascribed to it here so far.

The case of Spirillum volutans. The large spirilla, of which *Sp. volutans* is the type specimen, are frequently used in the study

of supposed flagella. Yet, as I have shown (1949 a), their tails have nothing to do with the tails or flagella of the usual motile bacteria. The main points of difference are best shown in a table:

	Sp. volutans	*S. typhi*
(1)	Outline sharp.	Outline diffuse.
(2)	Definite permanent shape, with characteristic curve, and sharp pointed end, looking like a horn.	Varying length and thickness, may be completely absent, point never sharp.
(3)	Attached to body by narrow neck.	Attached to body in indistinct way.
(4)	Place of attachment definitely at pole.	Attachment varies in place.
(5)	Darkground and staining methods show continuity with cell wall.	No continuity with cell wall, but with capsule.
(6)	Stains with cell-wall stains.	Stains with difficulty with flagellar stains only.
(7)	Easily visible with simple darkground outfit.	Can be made visible with sunlight darkground technique only.
(8)	Can easily be made visible with phase contrast microscope.	Cannot be made visible with phase contrast microscope.

The differences are great enough to place them in quite different categories. No conclusions drawn from the appearance or behaviour of the flagella of *Sp. volutans* need apply to those of *S. typhi* and similar bacteria. In a previous section reasons have been given why van Iterson's interpretation of the position of the flagella in her electron micrograph of *Sp. serpens* need not be correct. It may be added here that if her interpretation were correct, the retracting cytoplast must have drawn the flagella in through holes in the cell wall, without any visible effect on the cell wall, a somewhat surprising performance. Whatever, through continued investigation, the function of the flagella of the large spirilla may turn out to be, whether they are motor organs or just drawn out parts of cell wall, it will have no direct bearing on the function of the flagella of the ordinary motile bacteria.

Attachment of Tail

The tail of bacteria like *S. typhi*, consisting of twisted flagella, must in some way be attached to the body. If the flagella are motor organs, they must communicate with the protoplasm, and for this they would have to pierce the cell wall. This is what many people believe. If the flagella are just mucous appendages, no such penetration will exist. From a general biological viewpoint the existence of dozens or more of holes in the cell wall of, for example, *Pr. vulgaris* merely to let through a number of flagella, does not seem very acceptable. As Dubos (1945) has put it, flagella are not essential to the life of the cell. Also, motile bacteria exhibit spiral contortions of their bodies, which would suffice for propulsion, and flagella as motor organs appear rather superfluous. Here follow some pertinent arguments in favour of looking upon tails and flagella as purely passive appendages.

A broth culture of *S. typhi* showing well-developed tails, easily visible in sunlight darkground, when shaken or rather vibrated vigorously for fifteen minutes, will still show very motile bacteria, but practically all the tails will have disappeared. This experiment has been performed several times, and it shows that tails can be shaken off by purely mechanical means without any effect on motility.

Unsuitable kinds of broth, and most kinds of peptone water, may give excellent growth of *S. typhi*, with excellent motility, and practically total absence of visible tails. Here probably lies the explanation of the vagaries of flagellar staining.

A fast swimming typhoid bacillus, suddenly performing a semi-somersault through too rapidly reversing its spiral wave, as described above, will momentarily leave its tail exactly where it was, whilst the body performs these acrobatics, and the tail will follow on again when the body goes on in its original direction. This phenomenon takes place very rapidly, and one must assume that the bacterial body turns round very easily in its mucous coat or capsule, without any interference with the threads of mucus dragging behind in the shape of a tail. The importance of this phenomenon is so great that it is difficult to understand why it is so persistently overlooked. Kingma Boltjes (1948 a) has missed the point of it and seems to think that the bacterium moves for a

while with its long axis perpendicular to the direction of movement, and claims that this would only be possible through flagellar action. In reality and as described and filmed by me, the whole semi-somersault is over in a flash and the bacterium is in that transverse position for a fraction of a second only.

During rapid to and fro movement the tail also remains completely passive, and the bacterium swims backwards and forwards with complete unconcern as to the position of its tail, which flows from the one pole to the other.

When a bacterium like *S. typhi* slows down its movement the tail becomes a more definite spiral, and the gyrating undulating movement of the body becomes slower. At about this stage it often becomes obvious that the tail is not attached to any particular spot, but appears to follow the movements of the body in somewhat uncertain fashion, often apparently attached to the side of the bacterium, and leaving the caudal pole free (Pl. 8, fig. 4).

If one tries to fit the above phenomena into the conception that the flagella are active motor organs, all originating in the protoplasm and piercing the cell wall, the position becomes very complicated. It seems already very difficult to imagine how during normal movement actively motile flagella could pierce the cell wall from within, bend at right angles, run along the body of the bacterium, become twisted together into a tail and then unfold their propelling activity. What would happen to such structures during semi-somersaults and sudden reversals, or rather what they would have to do, and in complete unison, is quite beyond my imagination as to what is biologically and mechanically possible. If one, however, assumes that bacteria are normally surrounded by a mucous coat or capsule which at times can become rather voluminous and which then hangs very loosely round the body, one arrives at a simple explanation of all the phenomena mentioned in this paper. Such a coat could at least partially be shaken off by mechanical means without affecting motility; its trailing end, called the tail, could stand still whilst the bacterial body performed its semi-somersault, or swing round to the other pole during sudden reversal; it could sway from side to side during slow movement; it could on occasion be poorly developed and cause disappointment in flagellar staining.

If flagella are threads made of mucus, which gradually diffuses

into the surrounding medium, this surrounding medium must have an effect upon the tail, and a change in its pH value might affect its consistence and therewith its stiffness and visibility. A glucose broth *S. typhi* culture which had reached a pH value of 4·98 showed no motility and no tails. A drop of 1 per cent sodium carbonate solution added underneath the coverslip brought about good motility, and tails, but also several bacteria swimming with their 'tail' in front. Evidently the sudden change in pH had hardened the mucous and when a bacterium possessing a tail reversed its direction of swimming, the capsule did not as usual flow to the other end, but stayed where it was.

Observations on *V. metschnikovii* with the sunlight darkground technique so far have shown me that in broth cultures at least as many individuals swim with their tail in front, so that they resemble a ship with a bowsprit, as with their tail at the back, like the usual appearance of *S. typhi*. There also were individuals with a tail and a bowsprit at the same time. Frequent reversals of direction changed tails into bowsprits and bowsprits back into tails. Sometimes a bowsprit got bent, probably through the resistance of the medium. In many cases either a bowsprit or a tail during periods of slower speed changed its rigid rod-like appearance into a definite spiral (Pl. 8, fig. 5), which occasionally became a stiff tail again, together with an increase in speed. This strange picture is explainable by assuming that some factor in the broth occasionally hardens the capsule. Investigations on the subject are continuing.

Independent Movement of Flagella

The observation that sometimes bacterial flagella move whilst their bodies lie still has been brought up by several authors (Ørskov 1947, Kauffmann 1948, Kingma Boltjes 1948 a, Johnson and Baker 1947), in the idea that this refutes my theory. But, although I maintain that normally bodily movements move the flagella, this does not imply that no other mechanical force can ever move them. Where these observations concern the large spirilla, they do not really apply, for I have shown (1949 a) that their flagella are in a different category and may perhaps be true motor organs. About Ørskov's experiment (particles of ink being whirled about in surface cultures of *Proteus vulgaris* by flagella he

could not see, attached to bodies that were supposed to lie still, although Kauffmann (1948) cautiously stated that they did 'move slowly or lie still'), it could be said that if the flagella move sufficiently to cause whirls, can the bodies lie quite still, and if the bodies do not lie quite still, which is the prime mover? There is, however, a much better way of answering the contention that because it sometimes happens that flagella show movement whilst the bodies lie still, the flagella must be live motor organs. That answer is that it would be very strange if capsular threads did not show movement under the conditions mentioned. It is an observational fact that all such structures under the microscope do show movement. Dying bacteria can often be seen forcibly extruding their contents through holes in the wall, in the shape of long threads, as in Pl. 8, fig. 6, where two such threads are coming out. These threads last for a long time and are never at rest, they perform all kinds of movements for a very long time. The phenomenon is similar to the movements exhibited by the fibrin threads that in scrapings from syphilitic sores can so successfully imitate spirochaetes. In a previous publication (1930) I have described how the granules of gum which result from the coating of bacterial flagella with gum move up and down the windings of their flagella for a very long time. Here, too, we have to do with forces that are not yet understood. In the microscopic flagellar structures dimensions are reached where sources of energy are available that are as mysterious as they are uncontrollable. We may speak of electric charges, of Brownian movement, of tensions in the material and of surface tensions, of anisotropy and of lack of homogeneity, but it is all rather aimless. The fact has to be faced that such forces and energies and movements exist, and that, same as in the soap-bubble, there is endless activity without equilibrium being reached. As d'Arcy Thompson (1942) has put it, a flagellum is a portion of matter *sui generis*.

General Considerations

The possibilities of visual microscopy are not yet exhausted. With direct sunlight properly used in a suitable microscope, the human retina can register things that are at the present time beyond recording by photographic means.

Acceptance of the theory proposed here might lead to a

phylogenetic classification of bacteria. The close relationship between blue-green algae and bacteria, which Fritsch (1945) thought should be ruled out on account of the frequent presence of flagella in bacteria, and their absence in the algae, would become quite feasible. The theory would abolish as unnecessary many laborious and uncertain efforts at flagella staining. It would make necessary a revision of the meaning and use of certain terms, such as flagellum, peritrichous and even bacterium and bacillus.

Discussion

Prof. Pijper showed and commented on a 400 feet 16 mm. cinemicrographic film, made with his sunlight darkground technique, which allows live microbes moving freely in a film of liquid to be filmed. The film started with typhoid bacteria, many of them exhibiting long thin tails, the object being to demonstrate that such tails never have sharp outlines and that when movement slows down, their point of attachment can shift from the pole to the side of the bacterium. The body of one bacterium performed a semi-somersault during fast forward movement whilst the tail remained where it was, and the body then moved on with the tail apparently attached to what a moment previously had been the front end. Further shots showed typhoid bacteria splitting their tails into two, and in one instance such a double tail was seen to untwist further into a number of wavy threads which appeared to be attached to the sides of the bacterial body. The film went on to show a culture after it had been vigorously shaken for 15 minutes. Motility appeared to be as good as before, but tails were conspicuously absent.

Borrelia recurrentis had been filmed in a drop of blood from an infected mouse. Movement was very lively, and the point made was that although these microbes possess a spiral structure, their movement appeared to be due to a superimposed spiral movement of the body, of much greater amplitude and length than the structural spiral.

The next subject was the motility of *Spirillum volutans*. Fast moving spirilla were shown, with their characteristic spiral bodily contortions, and usually exhibiting one flagellum at each pole. These flagella swung round very rapidly in a plane perpendicular

to the long axis of the spirillum. On slowing down, these flagella were seen to have a sharp outline, a definite pointed shape, and a fixed place of attachment to the body, with a thin neck between the cell wall and the flagellum proper. (In Prof. Pijper's view the flagella of the large spirilla were elongations of the tough cell wall, and not, as in typhoid bacteria, a product of the mucous covering.) Instances of 'freewheeling' were also shown, i.e. periods during which a spirillum just glides along with bodily and flagellar motility at rest. Some spirilla were shown to possess one flagellum only, and this evidently could occur either at the front or the hind end, but their motility was not any less than that of spirilla equipped with two flagella. A full field of frantically moving spirilla with fast turning flagella then illustrated that not all such movements lead to propulsion; many of these spirilla were not moving from the spot. Special attention was called to the next series of pictures where the bodies of spirilla were seen exhibiting spiral movements of which the rhythm was very much faster than the turning movements of their own flagella. The flagella just made an occasional turn whilst the bodies gyrated at a much higher speed. Further evidence that here too, as with typhoid bacteria, it is the body that moves the tail, and not the other way round, was brought by the next section, which showed that here also vigorous shaking of a culture could break off the flagella (a short portion of the thin neck sometimes remaining visible at the pole) without affecting motility.

Pictures of *Spirillum volutans* at high magnification demonstrated individuals whose bodies had become fixed to slide or coverglass, whilst the flagellum kept on waving about, in a curious whip-like fashion. In some instances such a flagellum split into four or five subflagella of exactly the same shape as the original one, all remaining attached to the same spot on the cell wall. These subflagella kept up their whip-like movement for a long time, each one following a tempo and frequency of its own. One of them might even keep still whilst the others performed their whip-like movement with different and varying speeds. This gave the impression that these movements were caused and conditioned by the structure of the flagella. The fact that lifeless fine microscopic threads of such nature, lying free in liquid, are

apt to show curious movements was then illustrated by the next portion of the film. Here dying bacteria of various kinds were seen to extrude their cellular content through a narrow hole in the cell wall. The resulting fine threads waved about very much like bacterial flagella.

The last section of the film was devoted to observations on the motility of *V. metschnikovii*. This vibrio was first shown exhibiting spiral bodily movement and carrying a 'snout' attached to its frontal end. In a few instances an individual was seen with such a snout in front and a somewhat similarly shaped tail at the back. The shape of the snout at higher magnification was seen to be a fine spiral, and that of the tail was the same. Their place of attachment during active movement was at or near the pole, but during slower movement it shifted somewhat to the side. At high speed the snout stood out very stiffly in front, very much like a bowsprit of a ship. At slower speeds it performed a movement as if it described the surface of a cone, the apex of the cone being the point of attachment to the body. It seemed as if there were more individuals with snouts than with tails, apart from the few that possessed both structures. The flagella of dead, fixed and stained vibrios seen in textbooks and electron photographs thus during life appeared as stiff snouts or tails of spiral shape. In the film these structures did not give the impression of having anything to do with propulsion. A very long individual was shown which performed undulating movements with the middle parts only of its body, in a region where no flagella could be present. The spiral bodily movements of the vibrios seemed quite sufficient to effect propulsion. For the sake of comparison at this stage a short section on typhoid bacteria was introduced in which these microbes showed sudden reversal of movement. In such cases the tail was always seen to 'flow' towards the new posterior pole, there to form a new tail. In the case of *V. metschnikovii* such sudden reversals were then seen to have a very different effect. Here a snout became a tail and a tail became a snout, there was no displacement of the material of which these structures consisted. Such happenings also explained the impression gained in the beginning, that there were more snouts than tails. When a snout became a tail, it became less easily visible, and vice versa. The obvious explanation was that the

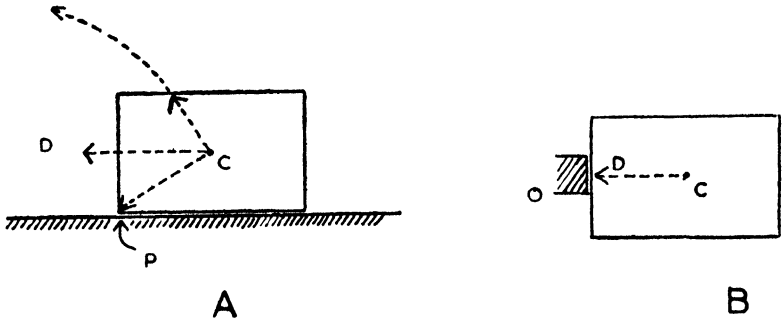
fine spiral thread constituting the snout or the tail became somewhat elongated and thus thinner when the forward movement of the body dragged it along as a tail, and became somewhat compressed and thicker when it was in front as a snout. If these structures played the part of motor organs, sometimes pushing the body as a tail, and at other times pulling it along as a snout, one would expect exactly the opposite changes in thickness and visibility. It was suggested that in *V. metschnikovii*, as in typhoid bacteria, these appendages are made of the mucous coat, but that in *V. metschnikovii*, for some reason or other, the mucous as a rule becomes rather hardened and a snout or tail once formed, therefore, does not shift its attachment so easily as in typhoid bacteria. Previous observations on typhoid bacteria were recalled where, through suddenly changing the pH of the film of liquid under the microscope, or through adding a *Vi* agglutinating serum, some bacteria evidently had their mucous hardened to such an extent that on sudden reversal of movement they swam 'with their tail in front'; in other words, that they exhibited a snout.

Dr. T. Y. KINGMA BOLTJES wished to bring forward a few objections to Prof. Pijper's views. In the first place Pijper's conclusion that the shaken bacteria must move without flagella, because no flagella are seen in the darkfield, is not justified. The darkfield, even with the sun as light source, is not able to reveal everything. The flagella may be too thin to be seen in the darkfield, as the following experiment showed. On a very clean coverglass a small drop of suspension of motile typhoid bacilli is dried. A small amount of silver ethylamine or a Peppler mordant solution is put on a clean slide, the coverglass put over this and the preparation is examined immediately. During the first five to ten minutes one observes only bacteria without flagella, but then the flagella become clearly visible because of the action of the silver solution or the mordant. That is, objects may be present in the darkfield without being visible.

Secondly, it is difficult to understand how outward forces, as, for instance, currents in the preparation, could rotate flagella in the same direction during as long a period as fifteen minutes at least, at a speed much too quick to be caused by currents. In a short time the opposing stress would become so great, that

either the flagella would come to a standstill or would be torn off. Neither happens, so one must conclude that the flagella move by their own forces.

Thirdly, how can an organism, moving by an undulating,



TEXT-FIG. 4.

C=centre of gravity; D=direction of movement of object; O=obstacle; P=point of friction on the road.

gyrating movement, make a somersault? When a cyclist puts on his front brake, he will topple over because there is a force that will lift up the centre of gravity (Text-fig. 4A). If, however, a bacterium suddenly reverses the spiral movement, no toppling over movement can occur. Nobody would expect a submarine, with screws moving at both ends, to turn over if the front screw suddenly reversed its movement. The effect would be like that with a moving body that hits an obstacle O just in front of the centre of gravity (Text-fig. 4B). This body will not topple over as there is no force that will lift up the centre of gravity.

Fourthly, it is difficult to understand how the bacteria could swim with a capsule, within which, as Pijper writes, the body can turn very easily. If this were really so, then it would be impossible that the gyrating undulating movement of the bacterium should be transferred to the capsule; nevertheless, the bacterium could not advance unless the capsule also made this movement. Moreover, the capsule has much resistance in the water, because of the flagella, so that it seems quite impossible that the bacteria could ever succeed in advancing, with the capsule hanging around it like a loose cloak. It is just as if, having two submarines, one inside the other, we were to expect that it would be possible to

move the outer submarine by rotating the screw of the inner one.

The facts show that bacteria move by their flagella and not by a gyrating movement of their body.

Sometimes bacteria move in ways that are easy to explain. It is always possible, however, that an invisible flagellum may be present, driving the bacterium in unexpected directions. Darkfield illumination is a trick illumination under which it is very difficult to analyse movements going on quickly. Siedentopf (1908, 1909, 1912) wrote some papers on the 'Azimutfehler' of the darkfield-illumination—which is also present in the darkfield of a cardioid condenser—to warn of this fallacy, by which some investigators have been led astray.

Prof. PIJPER commented on Dr. Boltjes' difficulties in understanding how a loose flagellum, or one that is attached to a motionless bacterium, can move. He could not explain it all, but within these submicroscopic dimensions a lot of things happen that we could not understand or explain. Some of his films had shown a spiral flagellum, very much thickened with the *H* antibody, that after several hours showed certain attached globules that were going up and down the spiral staircase for hours at a stretch. Could anybody tell him how that happened?

Dr. Boltjes was very much concerned about the cause of the somersault, or semi-somersault, and compared it to putting on the brake of a bicycle. Prof. Pijper said he would rather compare it to putting a motor car in reverse. Very few cars would stand up to that. If the car were going at a very high speed, it would go on through its own momentum, and those sitting in the car would not have to inquire about the centre of gravity; it would topple over and then go on in the same direction. If there were a tarpaulin over the car it would play the role of the tail, and would be in the same place notwithstanding the somersault of the car. When a bacterium that is going fairly slowly suddenly goes into reverse, it moves quietly backwards and the tail floats at the other end. When it suddenly reverses its spiral movement at high speed, naturally it topples over and goes on in the same direction, but the head becomes the tail and the tail the head. It would be very nice if this could be filmed in slow motion, but he had not

so far been able to do so. That was his explanation of the semi-somersault.

There were certainly limitations to the darkground method. There were lots of things he could not see, but after looking at darkground preparations for a matter of eighteen years, he still believed that what he saw repeatedly under varying conditions was really so. Dr. Boltjes had missed his most important point, which was his use of sunlight, which is several times stronger than the best arc lamp which present-day technique can produce.

Dr. KINGMA BOLTJES said that it did not make the least difference to the motor car whether the front wheels ceased to turn by putting on the brakes or by reversing the engine, and the point was irrelevant to the argument that a bacterium could not topple over by reversing the action of the body. The South African sun doubtless was really a magnificent source of light, but it was not necessarily good for darkfield illumination. Lord Raleigh computed that the amount of light scattered by particles in the darkfield is proportional to the sixth power of their radius. This meant, of course, that as the particles become smaller the light intensity has to be increased enormously to make small objects visible. But the difference between the arc lights and the sun is not so large that it greatly affects what we can and cannot see. He could only advise everybody who had a darkfield to use it and to look for himself at the flagella on the bacteria and decide what is going on. He had already set out his own views (Kingma Boltjes 1948 a, b).

Finally, Prof. Pijper's opinion that the flagella consist of polysaccharide material has been proved to be incorrect by Claes Weibull (1948), who showed in *Pr. vulgaris*, at least, that the flagella have the composition of a protein.

Sir ALEXANDER FLEMING said that Prof. Pijper's observations on flagella were of extraordinary interest and were almost convincing. He wished us to believe that the flagella we have been so proud of are merely capsular material thrown out by the gyrating, undulating movement of the bacterial bodies—completely passive and with no motor function.

For some time now he and Dr. Voureka had been making observations on *Pr. vulgaris* by phase-contrast microscopy, and

these had some interest in connection with the problem of flagella movement. Normally Proteus is too small and too active for the flagella to be seen clearly by phase contrast.

When grown on agar containing various concentrations of penicillin, they are transformed into long threads or irregular masses which are much less active. The culture had been spread on a coverslip, and when it had dried one or two drops of penicillin agar at 48° to 50° were allowed to fall on the coverslip and, as soon as the agar set, the culture was inverted on to a slide and was prevented from drying by paraffining or placing in a moist chamber. The most interesting forms were produced by growth at room temperature.

These cultures were watched undisturbed under the phase-contrast microscope with a 1/6th dry lens. An advantage of making the cultures in the way described, by drying the inoculum on the coverslip and the covering with agar, is that when the coverslip is removed, the agar comes with it and the culture can then be thrown in formalin in a Petri dish and, when fixed, the agar can be removed, leaving a beautiful impression of the culture on the coverslip which can then be appropriately stained.

Another great advantage of this method of slide culture is that the bacteria are all on the same plane—between the coverslip and the agar. In a fluid the flagella would be on different planes which would make them more difficult to see, but here they can only lie in the one level, so that it can easily be kept in focus during movement. The swollen-up bacteria are much less actively motile than normal and after twenty-four hours at room temperature most of them are motionless.

Types of motion. Proteus grown on penicillin agar in this way showed the most extraordinary morphological changes and often resembled a strange protozoon. There may be enormously long forms often with one or more bulbous enlargements, and these often curl themselves into spirals which rotate for hours in the same field of the microscope. Sometimes a bulb develops which seems to be compressed between the agar and the coverslip and the whole filament rotates with the bulb as the central pivot. Sometimes the bacterial body is wholly transformed into a large bulb which rotates without moving its position in the culture. There may be branched forms and one or more of the

branches may show movement at any one time. Some of the movements are such that it seemed hardly possible to imagine that any movements of the body (and frequently no movements of the body could be seen) could produce them.

Some of the long forms move straight forward or backward without any undulations of the body—others move with undulations exactly like a serpent. But they seldom move end on for long, they double up and then move with equal agility with the bend in front, and then flagellar 'ropes' are often seen coming from the inside of the bend. They often coil up and the whole coil will continue to turn round and round for hours in the same microscopic field. They may revolve once or twice per second, or they may take twenty or more seconds to make a single revolution. They may stop revolving, but still the flagella may be seen in active motion as if they were trying to push on the organism but were unable to overcome the resistance.

In these cultures the motion is not nearly as rapid as it is in fluid cultures. The organisms are partially anchored between the coverslip and the agar. The larger forms do not move so freely as the short forms and often the flagella can be seen distinctly.

He gave one or two examples of these observations.

1. A long bacillus had become bent like an S and was rotating slowly in a circular, counter-clockwise direction (Text-fig. 5). No tails were seen at the ends of the bacillus, but from the concavity of each of the bends of the S there were a number of flagellar 'ropes' in active motion. It was difficult to conceive of any undulating body movement (which could not be seen under the microscope) producing such a rotary motion, but it could easily be explained by the two groups of flagella simply pushing it round and round.

2. A circular form with two small projections had a simple rotary motion (Text-fig. 6). While it moved there were two bunches of flagella clearly seen attached to the angles between the projections and the bulk. When they stopped moving, the rotation stopped and started again when they started.

3. A large bulb which had developed in a long thread was rotating, taking with it the two parts of the thread which appeared to be merely flapping about in a spineless manner (Text-fig. 7). Two sets of flagella were easily seen starting from the angles

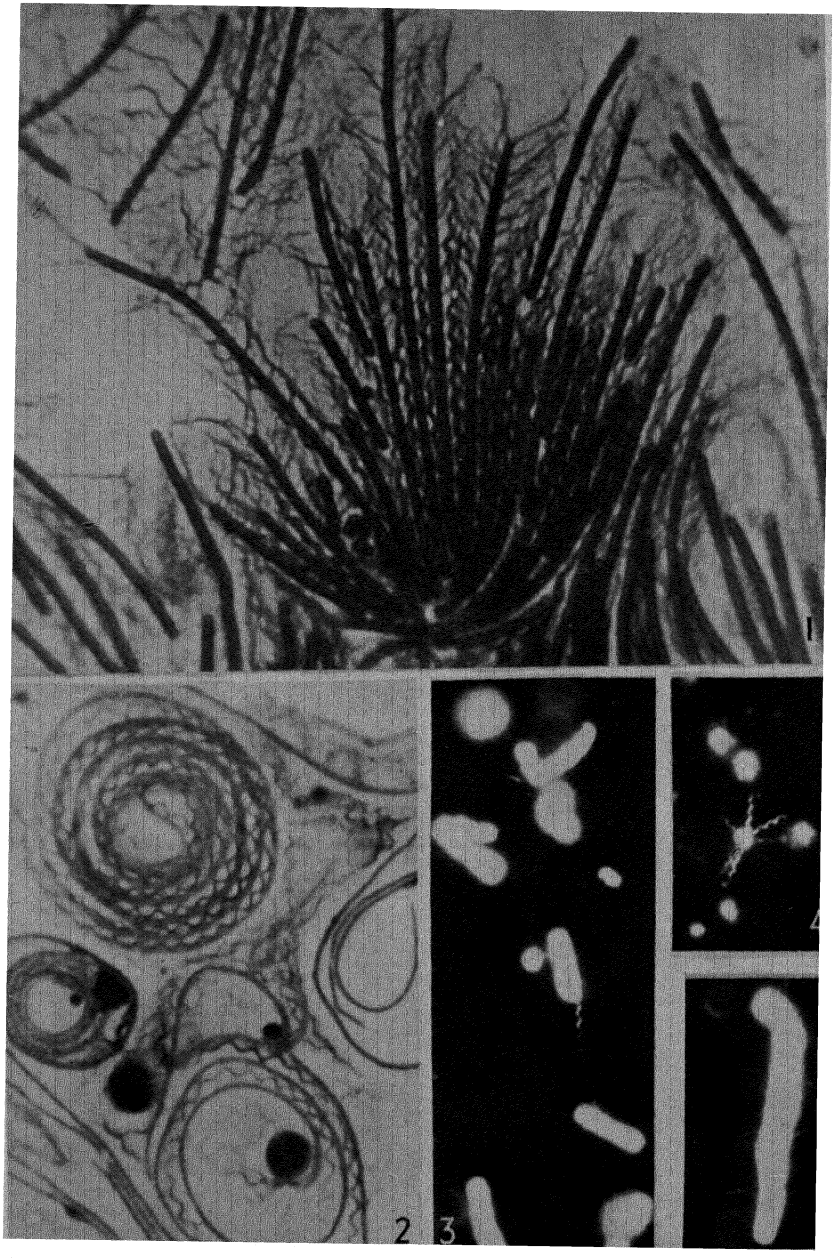
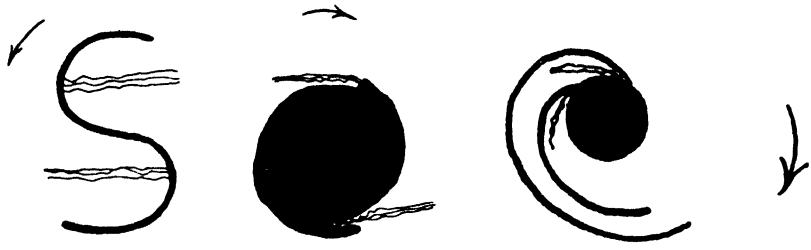


PLATE 9.

Figs. 1 and 2. Stained flagella of *Proteus vulgaris*. Note the thick 'ropes' of flagella where the coils of the bacterial body are close together, and the looser arrangement where they are more widely separated. (A. Fleming and A. Voureka.) Fig. 3. *Caryophanum latum*, rounded stationary segment of filament with spiral coils attached. Fig. 4. *C. latum*, young motile cell with tail. Fig. 5. *C. latum*, cell at rest showing peritrichous 'flagella.' (Figs. 3–4 by D. Erikson.)

between the bulb and the thread. These appeared to be pushing the bulb around.

Light seemed to have had a very definite stimulating effect on



TEXT-FIGS. 5, 6, 7.

flagellar movement. In these three cases, when a dark screen was put on the lamp, motion stopped. When this was removed there was a latent period of one or two seconds, then flagellar movement began. It was only after an interval—short but definite—that the organism moved. Sometimes it was possible to see that the flagella started to move before the mis-shapen body, sometimes they appeared to start simultaneously, but the body never started moving before the flagella.

Not infrequently bacillary bodies were found that were so packed around with other swollen organisms that they did not move although they had active flagella. These flagella stopped and started with the introduction or removal of a heat filter in exactly the same way as with the motile organisms. This stopping and starting of movement could be repeated time after time—always with the same result. It was not light which had this stimulating effect on movement, but heat. This could easily be shown by the introduction of a screen which absorbed the heat but affected the intensity of the light only a little. This was much more effective in slowing movement than a tinted screen of ordinary glass which gave a much lesser intensity of light. Stained specimens showing the arrangement of flagella on these bloated and exaggerated forms are shown in Pl. 9, figs. 1 and 2. In preparations in which flagella could be seen by phase contrast, fixed through the agar disk for two days in undiluted formalin, the flagella could be stained by a number of simple stains, of which the best was saturated watery solution of night

blue applied for about five minutes. This gives a remarkably clear preparation.

Observations of these undisturbed slide cultures left no doubt that flagella are active agents in the motility of bacteria. It would be impossible to prove that organisms cannot move without flagella. But most of the movements of these enlarged organisms cannot be explained without accepting flagella as a motive force.

Prof. PIJPER thanked Sir Alexander for saying that he had nearly been convinced and said he also was very nearly convinced, but not quite. The main strength of the argument was that these rather horrible structures went round and round for hours. He had looked at bacteria under similar conditions for many years, and knew that a live bacterium does not keep going round and round and round. The structures that Sir Alexander showed do that. He believed that there was a possibility that there were forces at work between the cover glasses and slides and agar surfaces—again, which we do not understand—which will keep such structures milling all the time. But the structures were not alive.

Dr. A. L. HOUWINK said that Prof. Pijper had said that he did not yet use the camera for stroboscopic observations. More than twenty years ago two German scientists, Metzner and Buder, used the stroboscopic method when studying the motility of *Spirillum* spp. Metzner found that the flagella turned round forty times a second, whereas the bacterium rotated only thirteen times a second—the flagella therefore turning three times as fast as the bacterium. He could not understand how this would be possible if the flagella were only slime, trailing behind the bacterium. Filming is a stroboscopic method; it should be possible, by changing the number of exposures per second, to slow down the rotating movement of the bacteria. According to Metzner's findings a larger number of exposures would be needed to slow down the movement of the tail. We might expect that this would yield interesting results.

If Prof. Pijper was right, the classification of bacteria according to flagellation could not be maintained. In a natural system no artefacts, caused by fixing and drying, should be used as a character. On the other hand, the established fact that some

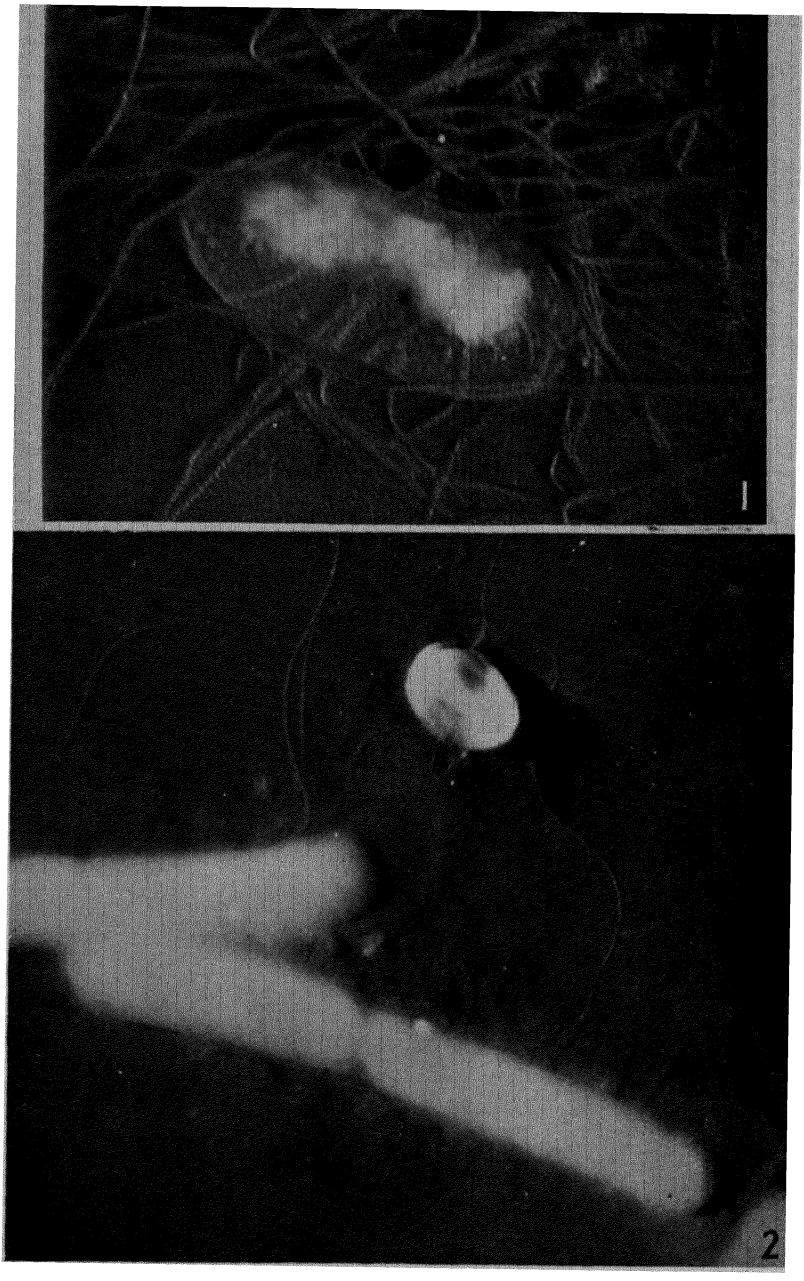


PLATE 10.

Fig. 1. Shadowcast electron micrograph of *Acetobacter xylinum*, showing long fibrils of extra-cellular cellulose. × 24,000. Fig. 2. Shadowcast electron micrograph of *Bacillus mesentericus*. × 12,800. Germinated from spores on a collodion membrane. Photograph taken at Princeton, N.J. (Dr. W. van Iterson.)

bacterial species are always seen to be cephalotrichous, which means with polar flagella, whereas other species are peritrichous, can be considered a valid argument against Prof. Pijper's hypothesis, as the latter cannot explain such ever-recurring differences in the mode of flagellation of different species. The reliability of this established fact has been disputed by Miss Pietschmann, who, using the darkfield technique, denied the existence of peritrichous bacteria and stated that all bacteria have one or two subpolar flagella near to the rear end of the bacterium. Seeming exceptions, such as a peritrichous appearance in long rods, she explained on the basis that such a rod represents an undivided group of several cells. Miss Pietschmann's papers are thus a stronghold in the defence of Prof. Pijper's ideas.

By electron micrography he and his colleagues had proved the occurrence of both peritrichous and cephalotrichous bacteria. That is, the bacterial species tested were exactly as they were supposed to be. Perhaps their technique of preparing the specimens was a little better than that which was used formerly. *Pr. mirabile* and *Bact. herbicola*, for instance, were in a fluid medium and fixed and mounted in such a way as to obtain a perfectly clean preparation. No loose flagella were present. In micrographs of various *Pseudomonas* spp. the flagella were restricted to the poles. Miss Pietschmann was mistaken in stating that flagella are subpolar in all species. The mode of flagellation is therefore valid for the purpose of classification.

It was exceedingly improbable that these flagella are disintegrated slime twirls. If they were, why should they be distributed at random along the sides of *Bacterium* spp. whereas they are polar in *Pseudomonas* spp.? Prof. Pijper had pointed out that in electron micrographs flagella are strikingly uniform in diameter. Although it seemed inconceivable to him that these filaments consist of dried slime, he nevertheless agreed with Prof. Pijper that the uniformity in width should not be used as an argument against the theory of slime twirls. However, micrographs of an unclassified bacterial strain from Dr. E. Gray showed both polar flagella and lateral flagella; the curious fact was that the polar flagella were thicker than the lateral. The difference was only about 25 per cent, but it was significant. This was not in favour of Prof. Pijper's hypothesis.

In a few micrographs Dr. Houwink had found loose flagella which ended in a little hook. Now, hooked flagella were never seen on intact cells and he felt justified in assuming that the hook was the basal part of the flagellum, and that normally it is situated inside the bacterial cell. These hooks were found only in peritrichous bacteria, and he did not know whether they also occurred at the base of a polar flagellum.

Prof. Pijper had stated that flagella are products of motility. Dr. Kingma Boltjes had already remarked in one of his papers that Sphaerotilis has got flagella before it comes out of its sheath. There was another argument against Prof. Pijper's view in a *Caulobacter* sp. This was a stalked bacterium isolated at Delft. When the organism is about to divide in two, one of the cells swims away with the polar flagellum. Later it attaches itself to some solid; for instance, a collodion membrane. At first it has no stalk, but one grows at the flagellated end; the free end of these young cells never bears a flagellum until shortly before fission, when a flagellum grows out of the anchored cell. This flagellum cannot be a product of motility. A characteristic of this organism is that it tends to grow in rosettes; the individual bacteria, however, are not connected to one another.

Dr. WOUTERA VAN ITERSON said that a student of artefacts (the present nickname of an electron-microscopist) could be expected to discuss whether flagella are organs of motility or not, especially in view of Prof. Pijper's studies of living bacteria in the light of the brilliant South African sun. In some respects her work on dead and desiccated cells with an artificial light source was overshadowed by methods that deal with living organisms. Nevertheless, the electron microscope has the advantage of very high resolving power and also the potentialities of new methods of specimen-preparation which are only at the beginning of their development.

Dr. Houwink had given examples of bacteria in which elementary filamentous flagella were inserted in a peritrichous and cephalotrichous manner, and had demonstrated on a *Caulobacter* sp. that a flagellum does not necessarily have to be a product of motility. There are those who characterize flagella as filamentous units (the old school founded on observations on stained prepara-

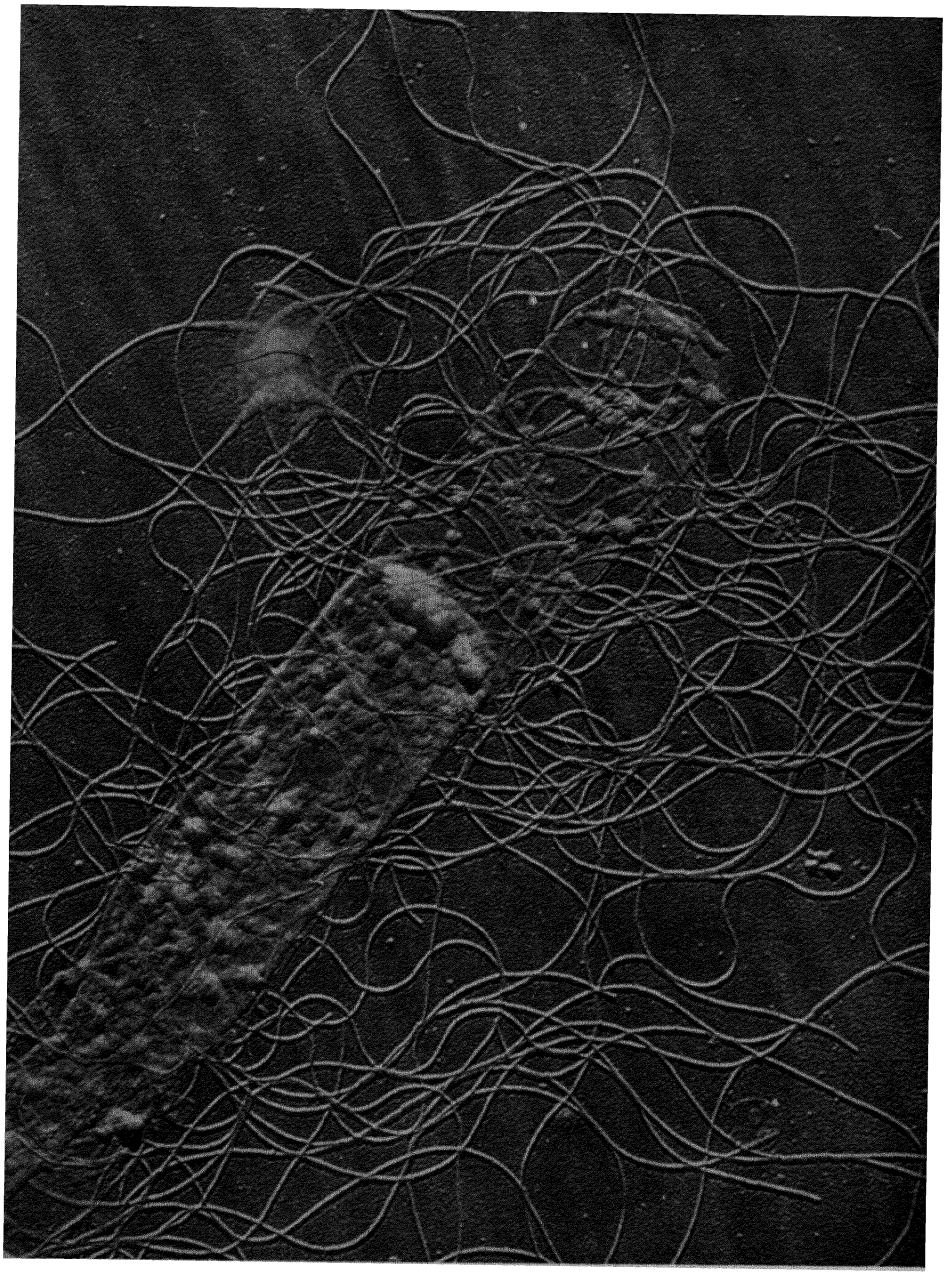


PLATE II.

Shadowcast electron micrograph of *Proteus vulgaris*. Cell is partly autolysed. ×23,860. Prepared in collaboration with Dr. C. F. Robinow. (Dr. W. van Iterson.)

(By permission of *Biochimica et Biophysica Acta*.)

tions) and those darkfield observers, with Prof. Pijper as a leader, who consider the tail of a fast moving bacterium to be an entity in itself.

The genesis of the flagellum may teach us something about this distinction. According to Prof. Pijper, the genesis of a tail is the peeling-off of the capsule or slime layer of a bacterium during fast movement; and he cited the following observation as bearing on the subject. *Acetobacter xylinum* produces very thick membranes consisting of cellulose, and Aschner and Hestrin (1946) observed in a preparation of such a membrane, disintegrated in a Waring blender, very long fibrils of the extracellular cellulose. Pl. 10, fig. 1, is an electron micrograph of the very same preparation and shows a bacterial cell within a mass of entangled fibrils of different diameter; the larger fibrils are composed of thinner ones. The production of cellulose by this organism is a very challenging problem in itself, but it seemed to have no connection with the formation of ordinary flagella, and for this reason only confused the present issue.

On the other hand, both slime layer and elementary flagella can be seen (Pl. 10, fig. 2) in *Bacillus mesentericus* germinated from spores on a collodion membrane. The flagella, as definitely linear structures, are well differentiated from the diffuse amorphous slime, which is against Prof. Pijper's hypothesis. The bacteria were grown directly on the collodion membrane, which was spread over nutrient agar during the incubation period. The only treatment to which the bacteria were finally submitted was drastic desiccation and shadow-casting. Because of the very limited amount of water present during growth, no important changes in original position of the bacteria and their appendages occurred during the desiccation. By this method it was also possible to get a faint glimpse of flagella genesis in *E. coli*. Cells of *E. coli* growing with little available water as part of a small colony had no opportunity to move freely over any distance and nevertheless developed peritrichous flagella. It is true that the flagella were much shorter than is usual in *E. coli*, and not as nicely undulating. The culture was only about four hours old, and at that time hardly any flagellated cells could be detected. She and her colleagues believed that the appearances were of juvenile flagella in the act of outgrowth.

Pr. vulgaris behaved in the same way (Pl. 11); flagella grew out peritrichously as separate structures. The same kind of structures were seen in cells of a swarming strain, fixed and centrifuged, and in cells on 'stripped-off' preparations made by Dr. Houwink. They had other examples of very short flagella in young cultures and longer ones in older cultures, and felt, therefore, that flagella originate by outgrowth.

With regard to their insertion (Pl. 12) the micrograph of *V. metchnikovii* shows an apparent connection with the protoplast either by a granule or by a protoplasmic extrusion.

It has been claimed that *Vibrio* and *Spirillum* spp. differ radically from *Salmonella typhi* or *Proteus* spp. Dr. Carl F. Robinow devised a method for making *Pr. vulgaris* transparent by storing a swarming culture for 36 hours at 5°. In the upper side of the resulting partly autolysed cell nothing of the original protoplast is left, except for a number of rather uniform spheres of about 100 mμ diameter (Pl. 11), which are at the base of the flagella. In other micrographs a few of the basal granules seemed to be situated between the cell wall and the retracted cytoplasmic surface. It was evident to them that, at least in *Pr. vulgaris*, flagella were really inserted inside the cell by means of basal granules. In the living state the granules would probably all be surrounded by the protoplasm. All this pointed to flagella being separate entities. With regard to the tails seen in moving bacteria, there were as yet no electron micrographs of such structures. But some micrographs gave a hint of how they might form. For example, a *Proteus* sp. grown on the collodion membrane developed flagella. When the bacillary cells grew in row-like configurations the flagella that were often seen escaping from the edges between neighbouring cells were aggregated in bundles. This case, of course, is not comparable with those of moving bacteria. Analogy goes only so far as there is in both instances aggregation of flagella into bundles. In this instance the flagella probably could not develop otherwise than close together by lack of space and moisture. Now we know from the work of Weibull and Astbury (1949) that flagella are made of protein and belong to the keratin-myosin-epidermis-fibrinogen group. In nature, myosin-, collagen-, nerve fibres, etc., occur in aggregates. Why should not the flagella fibres also possess some affinity for

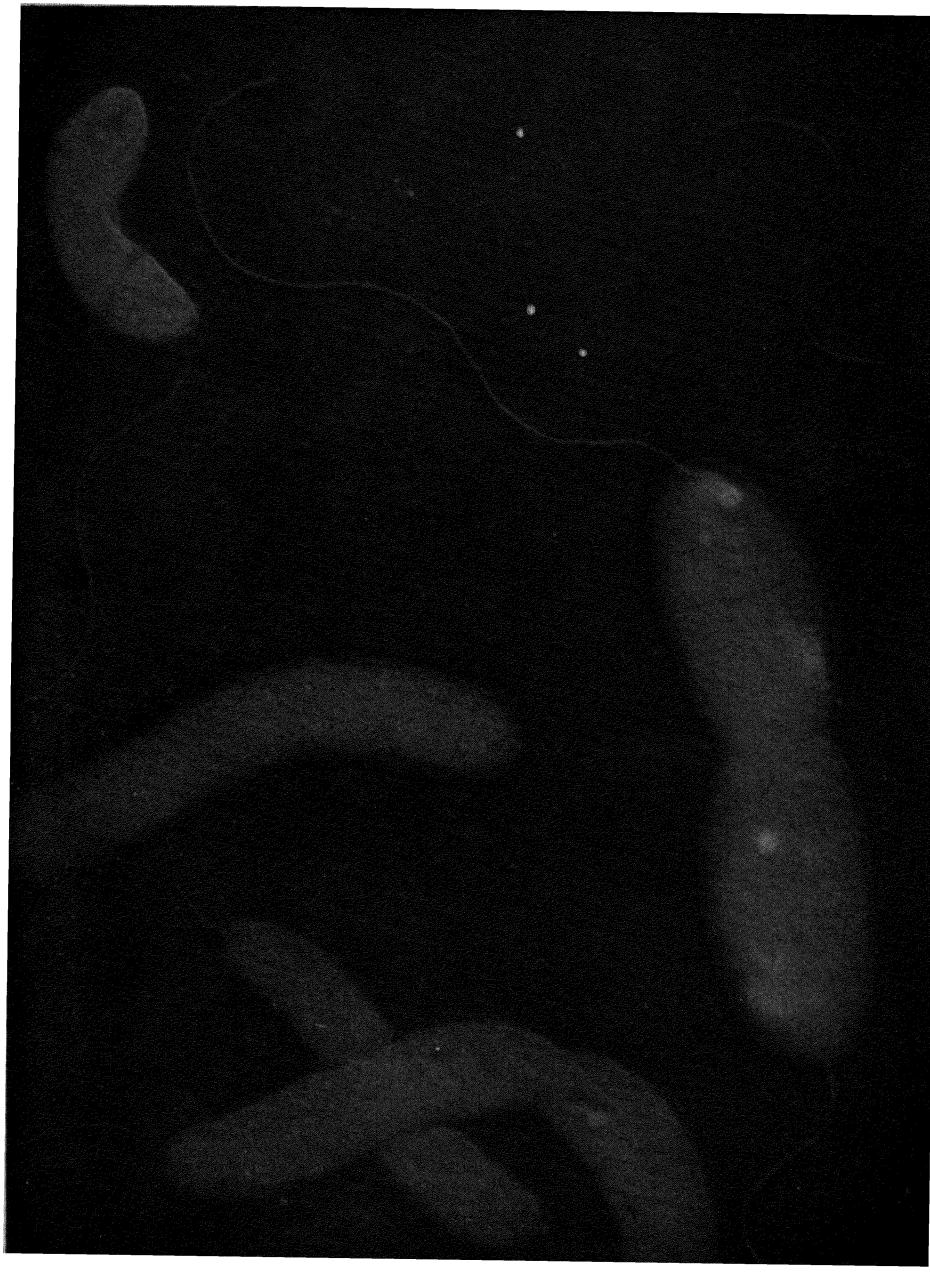


PLATE 12.

Shadowcast electron micrograph of *Vibrio metchnikovii*, showing an apparent connection of the polar flagellum with the protoplast. × 13,330. (Dr. W. van Iterson.)

each other? We know that pH and the presence of electrolytes influence the occurrence of tails. Of course, it was hazardous for an electron-microscopist to make such a suggestion, but it may be that bacteria possess some means of aggregating the elementary flagella into a tail, and reversibly disintegrating the tail into the flagella again. She left the suggestion with those who were familiar with the phenomena of life; it might serve as a bridge to link the two opposing points of view.

Dr. DAGNY ERIKSON said she had studied *Caryophanon latum* Peshkoff by Prof. Pijper's special sunlight-darkground technique because of its large size. As stated by Pringsheim and Robinow (1947), the dimensions of the filaments and component rods tended to diminish with cultivation on artificial media. When received from Dr. Pringsheim, the culture was composed of cells seldom exceeding 2 μ in diameter and 10–40 μ in length. It was found that growth, size and motility could be greatly enhanced by growing the organism on moist cellophan spread over sterile cow dung in Petri dishes. Under such conditions the organism grew in long filaments, 2·5–3·0 μ in diameter and sometimes 300 μ in length, which displayed a beautiful sinuous motion across two or more microscope fields, although the most actively motile cells were commonly arranged in pairs or singly. A simpler and less unpleasant medium, that also maintained size and motility, was prepared as follows. A single pellet of rabbit dung was sterilized in a tube, 5 ml. of non-nutrient, water agar poured over it and sloped so that the pellet was just covered by the agar, to which was added 1 ml. of the standard peptone-yeast extract broth (Pringsheim and Robinow 1947) seeded with cells of *C. latum*, and the tube incubated in an inclined position.

Many motile preparations were observed without seeing any tails or flagella-like appendages. Exposed to intense sunlight and subjected to surface-tension stresses in the narrow film between slide and coverslip, *C. latum* from a two-day culture was very sluggish in movement as compared with typhoid bacilli. The surface of the cells, however, was brilliantly defined, and also the internal structure. Spirally wound, flagella-like coils were first seen attached to intensely refractile, stationary, rounded bodies without defined cell-wall (Pl. 9, fig. 3), which appeared to

correspond with the dead, empty-looking, discoid compartments of the filaments that can occasionally be seen by means of the ordinary light microscope—the 'necridia' of Pringsheim and Robinow. In earlier stages of growth (6–18 hours) vigorous, young, motile cells and filaments were seen with one or two tails (Pl. 9, fig. 4), which were always of the same order of length, 6–12 μ. The characteristic peritrichous aspect (Pl. 9, fig. 5), such as has been described by staining and electron microscope methods, was noted only in cells that had come to rest.

All flagella-like appendages appeared to come from the surface of the cell. The difficulty of reproducing photographically all that the eye can register has already been stressed (Pijper 1946). Thus, the cells in Plate 9 have been over-exposed in order to demonstrate the appendages, and no longer show the clarity of outline and detail of internal structure which was visible to the observer. As a result of watching this very large bacterium, so brilliantly illumined that it was impossible at any time to confuse the appearance of one or two, or of a dense coat of flagella-like structures, she had become convinced that the phenomenon of motility must primarily be studied by techniques which deal with the living, moving cells. Whatever the ultimate verdict on the cause of motility, Prof. Pijper's studies had shown that the flagellated aspect of a cell at rest often does not coincide with its appearance in active life. The reproach of vitalism should not be levelled at those who held that microbiologists who attempt to explain the mechanism of motility solely by means of stained pictures or electron micrographs of dried bacteria are in danger of persuading themselves that in the midst of death we are in life.

Prof. PIJPER said that he felt it was almost a question of kissing and making up their quarrel. Dr. Houwink and Dr. van Iterson had almost communicated the contents of a whole book and it would be quite impossible for him to deal with it in the short time left to him. He was very glad to have had the discussion, and agreed completely with Dr. van Iterson that it should be possible to find some kind of compromise. At the moment it was impossible, but he suggested that Dr. Houwink and Dr. van Iterson go back to Delft and he to the sunshine, and then in five

or ten years they might come to some agreement. Prof. Pijper concluded:

'I think everybody should explain his own artefacts. I deal with living material, and that should be the leading principle. I get pictures from all over the world, and most of these things I regard as artefacts. I am quite prepared to discuss them with the people who make them, but on the basis that what I see in the living structure should be the fundamental position from which the artefacts should be interpreted.'

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