# *Macromolecules synthesised by Micro-organisms.*

THE TILDEN LECTURE, DELIVERED BEFORE THE CHEMICAL SOCIETY AT BURLINGTON HOUSE ON DECEMBER 6TH, 1946, AND IN CAMBRIDGE ON JANUARY 17TH, 1947.

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IN the past, the main effort dealing with the synthetic activities of bacteria has been directed towards solving the complex problems of their intermediate metabolism and in controlling fermentation reactions. In recent years a good deal of knowledge has been obtained regarding the growth requirements of bacteria particularly in respect to their essential amino-acids and " vitamins ". The synthetic activities of moulds (or the lower fungi), on the other hand, have been studied in a systematic way by H. Raistrick and his associates whose work has revealed the structures of many interesting crystalline metabolic compounds including pigments, quinones, bacteriostatic agents, carbohydrates, etc. This lecture deals with the chemical approach towards solving some of the problems of the architecture of the macromolecules of which the diverse components of the microbial cells are constituted.

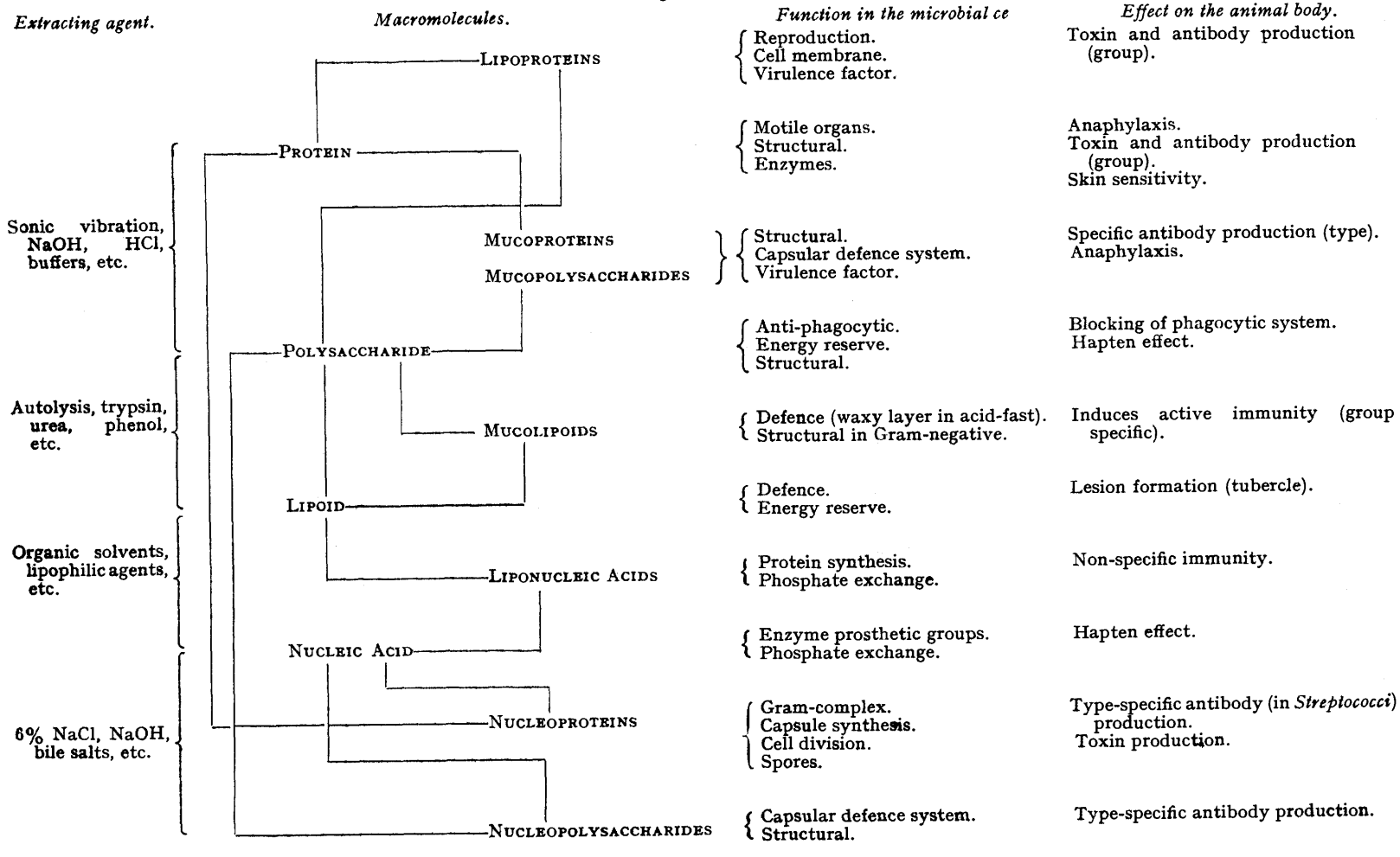
The nature of the reactions studied by biochemists has led them generally to regard the bacterial cell simply as a " bag of enzymes " carrying out respiratory, synthetic, and reproductive activities, etc., in the complex colloidal system of their metabolism media. Frequently these cells have been treated as some primitive form of life having a comparatively simple cellular structure. Far from this being so, however, one must now regard microbial cells as being highly complex and not differing in essentials in cell structure, function, or morphology from the highest evolved plant and animal cells. Most of us have seen evidence of the remarkable versatility and tenacity of life of the moulds, some of which can grow, for example, in organic solvents, on phenol, and on concentrated solutions of sulphuric acid. There is abundant evidence that almost all these cell systems can synthesise a wide variety of giant molecules, including their own autosynthetic molecules which behave like genes and chromosomes, from the simplest of carbon and nitrogen sources. We have the example of one class of micro-organisms, the " primitive " autotrophic bacteria, which can synthesise complex molecules from rocks, water, and carbon dioxide. This is good evidence of the possession of highly developed systems, and there is now a growing tendency to regard some micro-organisms as being derived from more highly organised forms of life. Viruses, for example, are undoubtedly parasitic upon certain plant and animal cells and may be degenerative forms descended from similar types of cells.

The splendid success of modern chemotherapeutic agents, particularly the antibiotics, has attracted the interest of many chemists who are now inquiring into the fundamental mode of action of many of these agents. This work involves a study of the growth requirements of micro-organisms, knowledge of the diverse variety of their enzyme systems, an investigation of their variation and of the mechanisms of their adaptation and reproduction. The kinetics of their complex cellular reactions is receiving particular attention (Hinshelwood, " The Chemical Kinetics of the Bacterial Cell," Oxford University Press, 1946).

Many aspects of the problems under consideration are set forth from the bacteriologist's point of view, in Dubos's book (" The Bacterial Cell ", Harvard University Press, Monograph No. 6, 1945). The intricate problems of immune phenomena (K. Landsteiner, " Specificity of Serological Reactions ", Harvard University Press, 1946) involve the behaviour of macromolecules of all biological types, and there is no doubt that these cellular constituents of agents of disease, which provide our present antigens or immunising agents, are in need of investigation from the detailed structural point of view. In considering the major components of all cells we find that there is a good deal of overlapping among animal and vegetable types. Thus glycogen is made by animals and plants (including yeasts and bacteria); hyaluronic acid is produced by animals and bacteria (cocci), while cellulose is synthesised by plants (including bacteria). Although we find among the micro-organisms an enormous variety of cells which show wide differences in morphology, yet the problems of their " bio-molecular " structure do not present difficulties which have not already been encountered in the fields of protein, carbohydrate, or nucleic acid chemistry. Indeed, one advantage is apparent inasmuch as with many microbial species one can work with cells of a single homogeneous type grown *in vitro* on a simple synthetic medium or at least on one of known composition.

The bacteriologist carries out his work on the living cell systems and from a vast experience bears in his mind a diagrammatic picture of the numerous components of each type of organism

*Figure* 1.


Extracting agent.
Macromolecules.
Function in the microbial ce
Effect on the animal body.
LIPOPROTEINS
Reproduction.
Cell membrane.
Virulence factor.
Toxin and antibody production (group).
PROTEIN
Motile organs.
Structural.
Enzymes.
Anaphylaxis.
Toxin and antibody production (group).
Skin sensitivity.
Sonic vibration, NaOH, HCl, buffers, etc.
MUCOPROTEINS
MUCOPOLYSACCHARIDES
Structural.
Capsular defence system.
Virulence factor.
Specific antibody production (type).
Anaphylaxis.
POLYSACCHARIDE
Anti-phagocytic.
Energy reserve.
Structural.
Blocking of phagocytic system.
Hapten effect.
Autolysis, trypsin, urea, phenol, etc.
MUCOLIPOIDS
Defence (waxy layer in acid-fast).
Structural in Gram-negative.
Induces active immunity (group specific).
LIPOID
Defence.
Energy reserve.
Lesion formation (tubercle).
Organic solvents, lipophilic agents, etc.
LIPONUCLEIC ACIDS
Protein synthesis.
Phosphate exchange.
Non-specific immunity.
NUCLEIC ACID
Enzyme prosthetic groups.
Phosphate exchange.
Hapten effect.
6% NaCl, NaOH, bile salts, etc.
NUCLEOPROTEINS
Gram-complex.
Capsule synthesis.
Cell division.
Spores.
Type-specific antibody (in *Streptococci*) production.
Toxin production.
NUCLEOPOLYSACCHARIDES
Capsular defence system.
Structural.
Type-specific antibody production.

as exemplified particularly by the coccus (see Gay, " Agents of Disease and Host Resistance ", Thomas, 1935, p. 527). The bacteriologist's representation has been gained by cytological and staining methods, and recent electron-micrographs (Professor W. T. Astbury, private communication) show that, in the coccus at any rate, the locations of some of the components such as the capsular substances are substantially correct.

In the present account of these macromolecules it will be convenient to consider separately the various components of the cell and to confine attention to those constituents on which we have some precise structural information. At present only in the carbohydrate group have any extensive studies been made.

From the chemist's point of view we can consider the nature of the main parts of the cell as deduced by qualitative and serological methods.

These different parts of the cell are by no means sharply differentiated, and in any colony or metabolism solution of such rapidly self-reproducing systems, where the cells must be of different ages, it will be apparent that cell composition is unlikely to be uniform. Nevertheless there is good evidence of a cell wall, so that we can usually distinguish between extra- and intra-cellular substances. In some organisms motile organs, or flagella, appear to exist, while in others there are well-defined regions outside the cell wall consisting of the capsular substance. Inside the cell membrane is the somatic part or protoplast in which nuclear structures are less clearly defined, though sound cytological evidence regarding the bacterial nucleus is now appearing (Robinov, Addendum in Dubos, *op. cit*; Dr. H. Henry, private communication).

There is no doubt that the macromolecular constituents are frequently in firm combination with one another and that the nature of any complex, such as a mucoprotein or a lipoprotein, is largely determined by the method of its extraction and isolation for which we need to choose a technique which has been found appropriate by past experience for some particular type of cell. Numerous processes for disintegrating cells and liberating their constituent macromolecules into solution are now available. We have, for example: freezing and thawing; autolysis; extraction with water, acids, alkalis, and buffers; sonic vibration; proteolytic hydrolysis; extraction with detergents, ethylene glycol, phenol, urea, guanidine, etc. When part or all of the components of the cells are solubilised, the usual methods for isolating proteins, nucleic acids, and polysaccharides can then be followed.

The nature of any complex molecule profoundly alters its biological function; this fact is of great importance in immunology, particularly from the point of view of vaccine and toxin production. In the past the main emphasis has been placed on the purification of the proteins, polysaccharides; etc., as essential entities, but the present trend is towards the production of complexes containing mainly one constituent combined with " prosthetic groups ". Such complexes may fulfil special biological functions. The numerous macromolecular types which can be isolated from micro-organisms are shown in Figure 1, in which are indicated also in a general way some of their functions and effects.

In the past, chemical researches on many micro-organisms have been difficult owing to lack of sufficient amounts of bacterial cells and the studies have thus, of necessity, been conducted largely by micro-methods. Mass production of cells such as *Mycobacterium tuberculosis* is now an established process so that chemical studies can go ahead much more rapidly. Many of the macromolecules are obtained in colloidal solution in water or in salt solutions, and we meet all the problems encountered in protein chemistry complicated by the added difficulties inherent in polysaccharide or nucleic acid chemistry. Future progress in the field will follow closely the advances in general protein, polysaccharide, and nucleic acid chemistry on both the physical and the structural side.

### *Extracellular Products.*

Great care is needed in deciding whether any product is truly extracellular, for large molecules may originate (*a*) by formation in the cell and then by proceeding by dialysis from within, outwards through the cell membrane; (*b*) by being synthesised in the metabolism solution by the agency of extracellular enzymes; and (*c*) by being broken away from the actual cellular structural components by the action of the autolytic cell enzymes such as the carbohydrases, nucleases, proteinases, etc.

One of the most widely known extracellular products is commercial " tuberculin " which is used for diagnosing tuberculosis infection especially in dairy cattle. The cell-free filtrates of numerous strains of *M. tuberculosis* have been intensively investigated by F. Seibert, who showed that tuberculin was composed essentially of proteins which were present in close association with nucleic acids and polysaccharides. It was shown by Seibert (*Chem. Reviews*,

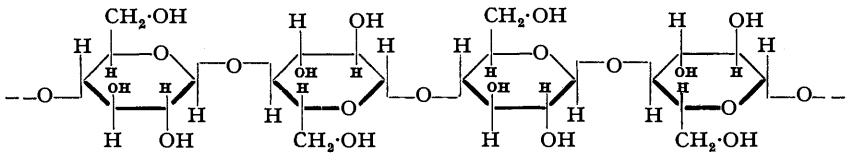
1944, **38**, 107) that the proteins such as the " Purified Protein Derivative " (or " P.P.D.") were highly serologically active and able to elicit skin sensitivity reactions in certain patients upon subcutaneous injection. By electrophoretic technique, Seibert and Munday (*Amer. Rev. Tuberc.*, 1932, **25**, 724; Seibert, Pederson, and Tiselius, *J. Exp. Med.*, 1938, **68**, 413) were able to separate from P.P.D. a crystalline protein and to show that this was the true tuberculin and that the deoxyribonucleic acid and polysaccharide components were serologically inactive.

Diphtheria toxin is an extracellular product of great importance since on injection into animals it gives diphtheria antitoxin used in the prophylaxis of diphtheria. It is the only bacterial extracellular toxin on which we have adequate criteria of purity and composition. The investigations described by Eaton (*Bact. Rev.*, 1938, **2**, 3) and Pappenheimer (*J. Bact.*, 1942, **63**, 373) show that diphtheria toxin is a well-defined protein molecule. During the war other remarkable bacterial toxins have been investigated and doubtless will be reported on in due course.

### *Bacterial Cellulose.*

This polysaccharide is probably one of the best examples of a true extracellular bacterial product. It is obtained in *ca.* 5% yield in the form of a tough membrane when *Acetobacter.* and other species are grown aerobically on hexoses, particularly fructose, and on the sugar alcohols. Hibbert and Barsha (*Canadian J. Res.*, 1934, **10**, 170) conducted a thorough investigation of bacterial cellulose produced in the form of a vast number of closely compacted membranes which appeared to give to the product a somewhat higher chemical stability than that of plant cellulose. Applications of the methods well known in cellulose chemistry, such as acetylation, acetolysis, methylation, hydrolysis, oxidation, *X*-rays, electron microscope, etc., have revealed that bacterial cellulose was chemically identical with cotton cellulose (Figure 2).

*Figure 2.*



*Cellulose.*

Interesting results were obtained by Franz and Schiebold (*J. Macromol. Chem.*, 1943, **1**, 4) in their *X*-ray and electron-microscope studies on bacterial cellulose. It was shown with some specimens that bacterial cells were connected by single fibres about 200 A. wide which became woven together. Recently, bacterial cellulose has been synthesised by resting cells of *B. xylinum* (Hestrin, Aschner, and Mager, *Nature*, 1947, **159**, 64).

### *Mould Polysaccharides.*

Mould polysaccharides appear to be true extracellular products, and those so far studied present interesting types of complex carbohydrate structure. When produced in a liquid medium, mould polysaccharides usually have relatively small molecular weights due, possibly, to the comparatively long period required for metabolism during which the lytic enzymes degrade the initial macromolecules. Most moulds produce a complex mixture of polysaccharides, the proportion of any one component depending upon the period required for growth. The biological function of the extracellular mould polysaccharide is unknown, it being generally assumed to act as a reserve carbohydrate. On solid medium it appears to behave as a mucilaginous " defensive colloid ".

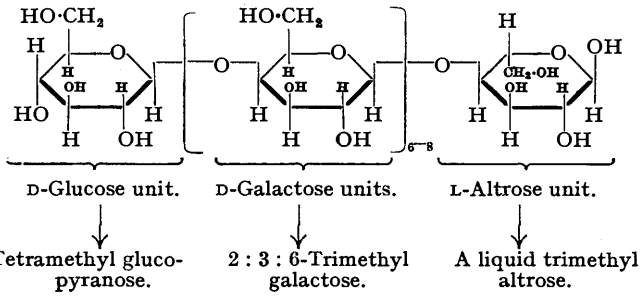
One can apply to the mould polysaccharides the well-tried methods known in polysaccharide chemistry. These methods involve fractionation with solvents, ultracentrifugation, electrophoresis, diffusion, etc., followed by hydrolysis and identification of the monosaccharide components by formation of characteristic crystalline derivatives or by paper chromatography. Acetylation, acetolysis, and methylation studies give an assay of the repeating units and of the side-chain and terminal residues. In neutral polysaccharides these also can often be measured by oxidation methods, particularly the periodic oxidation method of Brown, Dunstan, Halsall, Hirst, and Jones (*Nature*, 1945, **156**, 785).

Some examples of the types of structures of the repeating units in mould polysaccharides are shown in Figures 3—6.

*Varianose.*

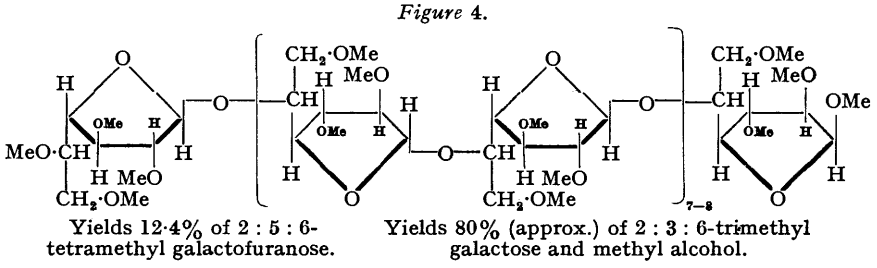
Varianose, produced from D-glucose by *Penicillium varians* G. Smith, consists of a chain of 6—8 β-D-galactopyranose units (Haworth, Raistrick, and Stacey, *Biochem. J.*, 1935, **29**, 2668)

*Figure* 3.




joined by 1 : 4 linkages with a D-glucopyranose residue at the non-reducing end and an L-altrose unit (Stacey, unpublished results) at the reducing end.

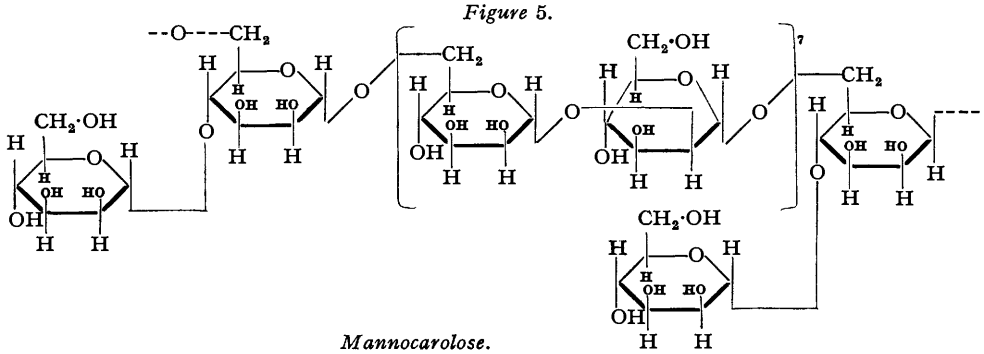
*Figure* 4.




*Methylated galactocarolose.*

Galactocarolose was produced from D-glucose by *P. Charlesii* G. Smith (Haworth, Raistrick, and Stacey, *Biochem. J.*, 1937, **31**, 640), and from a study of the methylated polysaccharide the minimum repeating unit was shown as a chain of 9—10 galactofuranose units linked through the 1 and 5 positions. This appears to be one of the rare cases where D-galactose is found to occur naturally in the furanose form (Figure 4).

*Figure* 5.

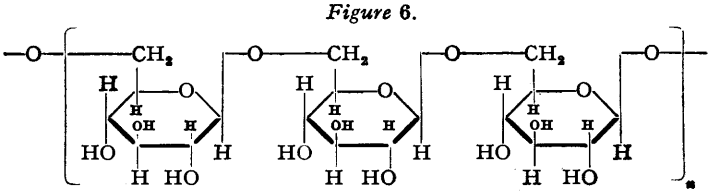


*Mannocarolose.*

Mannocarolose is a mannan produced along with galactocarolose from D-glucose by *P. Charlesii* G. Smith, and was reported (Haworth, Raistrick, and Stacey, *Biochem. J.*, 1935, **29**, 612) to consist of a straight chain of D-mannopyranose units linked through the 1 : 6-positions. The trimethyl mannose constituent of the methylated mannocarolose was noted by Haworth, Hirst, Isherwood, and Jones (*J.*, 1939, 1878) to differ from an authentic specimen of 2 : 3 : 4-trimethyl mannose. A recent re-investigation of the hydrolysed methylated

mannocarolose (Stacey, unpublished results) has revealed that the trimethyl mannose fraction consisted of equimolecular amounts of the 2 : 3 : 4- and the 3 : 4 : 6-trimethyl derivatives and also it was shown that the origin of the 2 : 3-dimethyl mannose component was not due to incomplete methylation. Accordingly, the branched chain structure (Figure 5) is now thought to represent more closely the repeating unit in this mould mannan.

*Figure 6.*



*Luteose.*

The unique acid-polysaccharide, luteic acid, discovered by Raistrick and Rintoul (*Phil. Trans.*, 1931, *B*, **220**, 225), is composed of a high molecular complex which was shown to be a polyglucose (" luteose "), esterified with malonic acid in the proportion of 2 molecules of glucose per molecule of malonic acid. Mild acid or alkaline hydrolysis readily destroyed the remarkable viscosity of aqueous solutions of luteic acid and split off the malonic acid residues. The structure of the luteose was shown to be represented (Figure 6) by a long chain of β-D-glucose residues joined by 1 : 6-linkages (Anderson, Haworth, Raistrick, and Stacey, *Biochem. J.*, 1939, **33**, 272) in which no terminal residues could be detected. This molecular pattern was therefore that of a " polymerised " gentiobiose. Another fungal polysaccharide was the specific carbohydrate of *Coccidioides immitis* which contained units of D-glucose, D-galacturonic acid, and an amino-sugar (Hassid, Baker, and McCready, *J. Amer. Chem. Soc.*, 1941, **149**, 303).

An interesting extracellular polysaccharide is that produced in high yield from glucose by *Phytomonas tumifaciens*, the crown gall organism, which produces an abnormal growth proliferation—a type of neoplasia or tumour—of plant cells of numerous types. The polysaccharide was shown by diffusion and sedimentation-velocity studies (McIntire, Peterson, and Riker, *J. Biol. Chem.*, 1942, **143**, 491) to consist of a chain of 22 glucose units.

Its optical behaviour in water and cuprammonium solution so closely resembled that of 2-methyl β-methylglucoside (Reeves, *J. Biol. Chem.*, 1944, **154**, 49) that it appeared that the D-glucopyranose residues were linked chiefly through the 1 : 2-positions.

Extracellular polysaccharides probably play an imporant rôle in soil and may assist in conservation of moisture.

### *Flagella.*

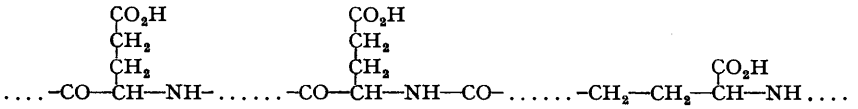
The motility of bacteria, particularly the *Salmonella*, is due to the presence of flagella of which a good deal is known immunologically since they constitute the characteristic " H " antigens. Beyond the fact that they resemble proteins in that they are digested with trypsin, are readily destroyed by heat and coagulate at pH 4·4, nothing is known of their molecular structure.

### *Capsular Substances.*

A good deal of interest centres in bacterial capsular substances because the formation of a capsule *in vivo* may be associated with the virulence of a pathogenic organism. In many virulent infections, such as pneumococcal and meningococcal, it can be shown that capsular substances act as the cell's defensive layer and on being released also circulate in the body and tend to decrease the resistance of the host by blocking the phagocytic system. Probably the most spectacular capsular substance is that of the anthrax bacillus. This is an unusual protein which was discovered by Tomcsik and Szongott (*Z. Immunforsch.*, 1933, **78**, 80) in the form of a copper precipitable substance obtained from anthrax broth cultures. It was later investigated by Ivanovics and his colleagues (*ibid.*, 1937, **90**, 304; 1940, **97**, 402, 442; **98**, 373), and was shown to be a polyglutamic acid composed solely of unusual *d*(+)-glutamic acid residues. None of the usual proteolytic enzymes will attack it. Hanby and Rydon (*Biochem. J.*, 1946, **40**, 297) described methods for the isolation and purification of the undegraded *B. anthracis* capsular protein. They showed that different strains of *B. anthracis* give an identical product which they confirmed was composed solely of *d*(+)-glutamic acid residues. It had a molecular weight of 50,000 and was a long chain molecule made up of α-peptide chains of 50—100 *d*(+)-glutamic acid

residues which were joined together by γ-peptide chains of *d*(+)-glutamic acid residues (Figure 7).

*Figure 7.*



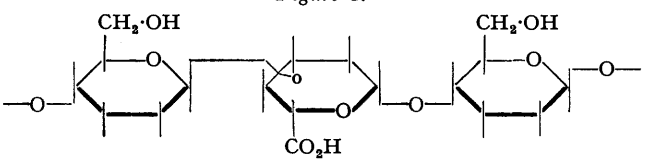

*Anthrax Capsular Protein.*

Other ærobic sporulating organisms such as *B. subtilis* and *B. mesentericus* produce a similar polyglutamic acid.

*Capsular Polysaccharides.*

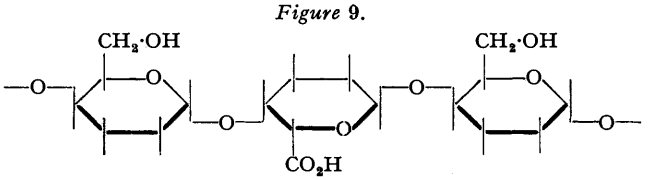
Most capsular substances are polysaccharides of high molecular weight, and problems of bacterial encapsulation in general, have been the most thoroughly studied in the pneumococcus group. The immunological significance of these polysaccharides was discovered by Avery and Heidelberger (*J. Exp. Med.*, 1923, **38**, 81) who showed that each pneumococcus type owed its sharp serological specificity to the special structural characteristics of the complex carbohydrate. More than forty types of pneumococcus have now been recognised, and the remarkable differences and similarities between the capsular polysaccharides are shown in Boyd's "Fundamentals of Immunology" (Interscience, New York, 1943). From the structural aspect, only the Type III specific polysaccharide has as yet been examined. Reeves and Goebel (*J. Biol. Chem.*, 1941, **139**, 511) showed that it is constituted of cellobiuronic acid residues linked through position 3 of the glucuronic acid moiety to position 1 in the next glucose unit giving a long chain structure which contains alternate 1 : 3-β-D- and 1 : 4-β-D-linkages (Figure 8).

*Figure 8.*



*Type III Polysaccharide.*

*Figure 9.*



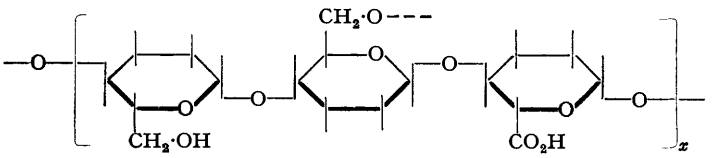
*Oxidised Cellulose.*

Hexuronic acids are found in other pneumococcus capsular polysaccharides, *e.g.* D-galacturonic acid in the Type I polysaccharide and D-glucuronic in those of Types II, VIII, etc., while Friedlander's bacillus—a Gram-negative bacillus—also synthesises uronic acid-containing polysaccharides closely related serologically and structurally to some of the pneumococcal polysaccharides. Another characteristic of pneumococcus capsular polysaccarides is the frequent occurrence in them of hexosamine constituents, the amino-groups of most of which are acetylated. Others, such as Type I, contain *O*-acetyl groups and some, such as Type IV, possess a high phosphorus content. Close structural and serological relationships have been established between oxidised cellulose (Figure 9), the polysaccharide of *Rhizobium radicicolum* (Figure 10), and the Pneumococcus Type III polysaccharide (Figure 8) (Schlüchterer and Stacey, *J.*, 1945, 776).

Dr. B. R. Record (private communication) has recently compared, in the ultracentrifuge and by diffusion measurements, some physical properties of relatively undegraded samples of the pneumococcus polysaccharides Types I, II, and III, prepared by the lecturer. Despite the big differences in chemical structure the physical constants and molecular shapes were very similar.

The great dependence of sedimentation velocity on concentration, typical of long chain molecules, was a characteristic of all three polysaccharides. A similar effect was found in the diffusion

*Figure* 10.




Rhizobium *Polysaccharide.*

constants though in the other direction, *i.e.*, of increasing rate of diffusion with increase in concentration, and it was important to extrapolate these two constants (sedimentation and diffusion) to zero concentration in the case of such abnormal behaviour in order to calculate molecular weights such as those shown in Figure 11.

*Figure* 11.

*Pneumococcus Polysaccharides. Sedimentation and Diffusion Data.* (Dr. B. R. Record.)

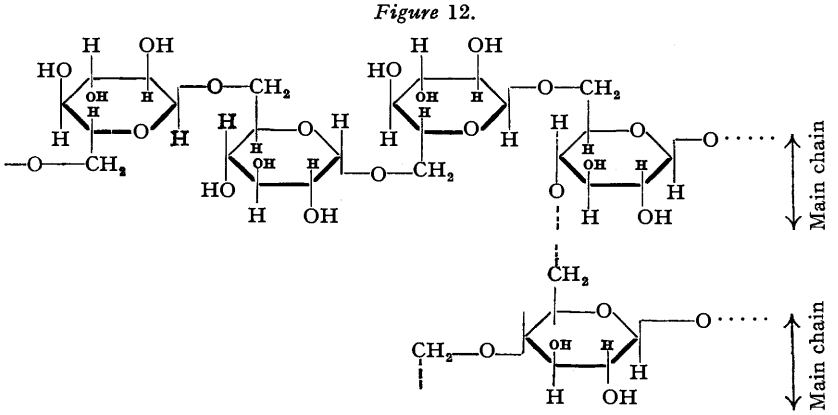
| Type. | $S_{20} \times 10^{13}$ ($c \to 0$). | $D_{20} \times 10^{7}$ ($c \to 0$). | Molecular Weight. | Frictional Ratio. |
|---|---|---|---|---|
| I. | 6·5 | 2·00 | 170,000 | 3·2 |
| II. | 7·2 | 0·75 | 500,000 | 6·0 |
| III. | 4·3 | 1·60 | 140,000 | 4·3 |

The large frictional ratios indicated a wide departure from linearity in the shape of the molecules which were probably very elongated.

*Bacterial Dextrans.*

These are striking capsular polysaccharides which are polyglucoses showing serological relationships with some of the pneumococcus polysaccharides (Sugg and Hehre, *J. Immunol.*, 1942, **43**, 119). They are produced from sucrose by a wide variety of species including the *Leuconostoc* and *Betabacteria* species. They are particularly characterised by having a high dextrorotation and structurally they contain a high proportion of 1 : 6 α-D-glucose linkages (*i.e.*, isogentiobiose " polymers ") and the chain may be either straight or branched. A type of the latter is shown for a *L. mesenteroides* dextran (cf. Evans and Hibbert, " Advances in Carbo-

*Figure* 12.




L. mesenteroides *Dextran.*

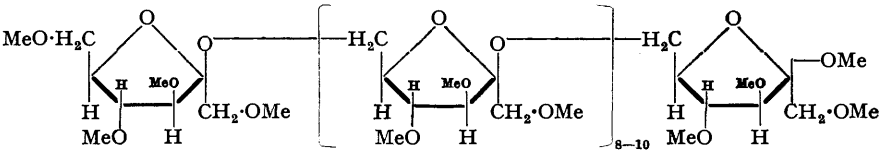
hydrate Chemistry ", Vol. II, p. 203) in Figure 12. Lively interest is attached to dextrans because they can be degraded to give a product which appears to be an excellent substitute for plasma in blood transfusion. Dextrans can be synthetised enzymically and it would appear that direct phosphorylation processes are not involved. Recently we have made the striking observation that the so-called " dextran synthesis factor " is *p*-aminobenzoic acid, a fact which is of interest concerning the mode of action of the sulphonamides.

*Levans.*

Bacterial levans form the mucilaginous capsules of numerous bacteria. The occurrence of fructosans in plants (Macdonald, " Advances in Carbohydrate Chemistry ", Vol. II., 1946) is widespread and it is well known that numerous groups of bacteria also can produce fructosans of the levan type.

Levans, as well as dextrans, can be produced only from sucrose or raffinose as substrate and they vary considerably in their physical properties. The most common levan-synthesising organisms belong to the non-pathogenic ærobic " potato " group, *e.g.*, *B. mesentericus* and *B. vulgatus*, in addition to *B. subtilis*, the hay bacillus. Early work on the structure of levans is due to Tipson and Hibbert, to Haworth, Hirst, and Challinor, and also to Peat, Stacey, and Lyne (Evans and Hibbert, *loc. cit.*). These workers discovered a previously unknown structure, similar to inulin in that it provides an example of the natural occurrence of fructose in the furanose form. In levans the fructo-furanoses are linked in the 2 : 6-positions in a chain of 10—12 contiguous units.

*Figure* 13.




*Methylated Levan.*

Notable also among the levan producers are the plant pathogens, *Ps. pruni*, *Ps. prunicola*, and *Ps. mors prunorum*, and in these the repeating units are identical. Physical differences appear to be due to the differences in the degree of aggregation of the repeating units, a view supported by the electron micrographs (Ingleman and Siegbahn, *Nature*, 1944, **154**, 237). Among the encapsulated pathogens which produce levans are *Streptococcus bovis* and *Str. viridans*. The levan type of complex molecule has been produced enzymically (Hestrin, Avineri-Shapiro, and Aschner, *Biochem. J.*, 1943, **37**, 450).

*Hyaluronic Acid.*

Certain so-called " mucoid " strains of *Streptococcus* (A and C strains) produce the polysaccharide-acid, named by Meyer and Palmer (*Amer. J. Ophthal.*, 1936, **19**, 859), " hyaluronic acid ". The substance is a normal component of the animal body in which it forms the ground substance of connective tissues and skin, and it is the main building constituent of the umbilical cord. It is claimed to be a linear polymer of glucuronic acid and *N*-acetyl glucosamine residues. It shows in aqueous colloidal solution a remarkable viscosity which can be broken either by simply shaking the solution in air, or much more dramatically by a spreading-factor enzyme " hyaluronidase " present in venoms, bacterial exudates, testes, etc. We find that it is a most labile carbohydrate and so far cannot be examined structurally by the usual methylation technique. The repeating unit seems to be small and to contain, perhaps, a stable " core " which is protected by other residues. Being a normal constituent of the animal body it does not stimulate antibody production, and streptococci possessing such a protective capsule are probably more pathogenic on that account. It presents many interesting problems for the physical chemist. For example, it forms apparent fibres which under the *X*-rays are not true oriented fibres (Professor W. T. Astbury, private communication). As in many natural complex acid polysaccharides the carboxyl groups are free and engage in salt linkage and hydrogen-bond formation.

*Acid-fast " Capsular " Substances.*

A further possible capsular substance is the so-called " waxy " layer of the acid-fast organisms. It is very doubtful whether this is a true capsule (Dubos, " The Bacterial Cell ", Harvard University Press, 1945) and it is almost certainly not true that all the fatty acids are located at the surface layer. On the other hand we ourselves have obtained evidence that the acid-fast fatty acids, *e.g.*, mycolic acid, are located mainly at the surface and that these are in close association with deoxyribonucleic acid and a polysaccharide (Haworth, this vol., p. 582). The lipoids can stimulate giant-cell formation, while the free fatty acids, natural and synthetic, which are of branched chain type, can produce tubercular lesions (Robinson, *Nature*, 1946, **158** 815). The polysaccharide is of a most unusual type and it is probably esterified with mycalic

acid to form a giant molecule of high chemical stability. The mycolic acid when freed from the carbohydrate appears to have some acid-fast properties. Related substances are found in *Mycobacterium phlei* and in the leprosy bacillus (*M. lepræ*).

At this stage we can make some generalisations regarding the nature of capsular substances. They are chiefly highly viscous complex " hapten " carbohydrates having a main structural architecture resembling that of the plant gums. The macromolecular state and the capacity to stimulate antibody production appear to depend on the presence in the molecule of a bound prosthetic group. Frequently they contain hexuronic acids (in which the carboxyl groups are free to engage in salt formation and hydrogen bonding) and amino-sugars in which the amino-group is usually substituted by an acetyl residue. A high degree of branching occurs in most types. The monosaccharide units may possess both pyranose and furanose forms and almost every kind of positional linkage, though invariably there is found a high proportion of glycosidic linkages.

It is clear that micro-organisms have evolved a mechanism for the synthesis of capsular structures which are not generally susceptible to enzymic cleavage. This is particularly true for the polyglutamic acid type of structure. In this connection it is of great interest to note that Dubos developed the antibiotic " gramicidin " by discovering the enzyme capable of destroying a pneumococcus polysaccharide capsule, and it is of further interest to point out that the powerful new antibiotic " streptomycin " contains *N*-methyl L-glucosamine among other constituents which include an aldehydo-carbohydrate.

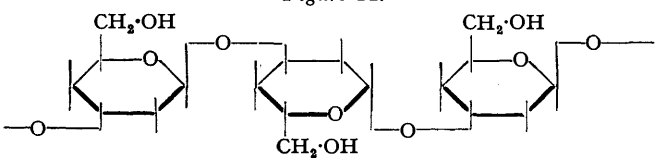
### *Somatic Constituents.*

The somatic parts of bacterial cells have been most studied in Gram-negative organisms which are not so generally encapsulated particularly in artificial culture. In the *Salmonella* group, of which there are now 110 distinct varieties, the serological relationships are well established. The *Salmonella* may possess the motile organs mentioned previously, while from the somatic part of the cell we can isolate the so-called " O " and " Vi " antigens. In the typhoid–paratyphoid vaccines these antigens possess the immunizing power of the vaccines. Studies by Raistrick and his colleagues, Boivin, Morgan, and others (Stacey, " Advances in Carbohydrate Chemistry ", 1946, Vol. II, 162) show that these O-antigens, which are the " endotoxins " of the cell, belong to the class of carbohydrate–phospholipoid complexes. In the *Salmonella* group structural studies have not yet been carried out, but the carbohydrate components appear to be very similar to the carbohydrate residue in some proteins.

The protein constituents of the somatic part of microbial cells have not been extensively studied, but some stimulation may now come from the modern developments of food yeast on which a knowledge of the amino-acid constituents is clearly desirable.

Information is available on the structure of some other types of somatic polysaccharides, *e.g.*, yeast mannan which forms a typical copper complex, and a highly insoluble yeast " cellulose " (a so-called yeast glucan). The latter is said to possess 1 : 3-β-linkages (Figure 14).

*Figure* 14.




*Yeast Glucan.*

We have recently studied a specific somatic polysaccharide (Haworth, *loc. cit.*) from the tubercle bacillus—obtained after removal of the waxy layer by means of urea. Goebel and his colleagues have made an important discovery regarding the " C " or group specific polysaccharide from the somatic part of the pneumococcus (Goebel and Adams, *J. Exp. Med.*, 1943, **77**, 435). They were able to differentiate between the " C " polysaccharide and the " F ", or heterophile, polysaccharide, inasmuch as the latter consists of the C substance combined with a fatty acid.

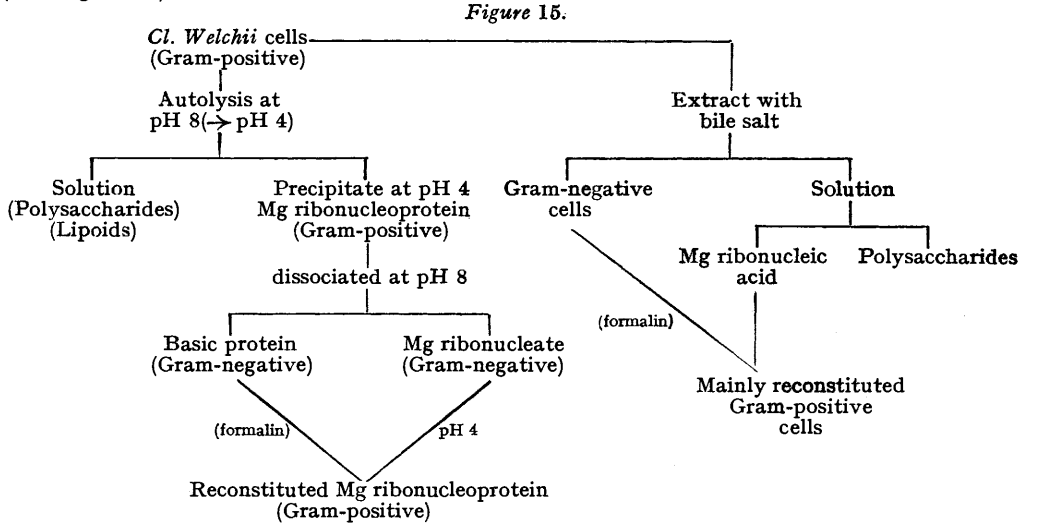
### *Bacterial Nucleoproteins.*

Although nucleic acids were thought to be somatic constituents, the ribonucleic acid of the Gram-complex in some organisms, *e.g.*, *Cl. welchii*, is certainly located on or near the surface.

In others, *e.g.*, staphylococci, it is diffused throughout the protoplast in the form of highly packed granules.

The " Gram method " of staining micro-organisms consists in staining heat-fixed smears with an aqueous solution of a dye of the pararosaniline series, mordanting with aqueous iodine, washing with alcohol until no more of the blue dye can be extracted, then counterstaining with a red dye of the acid fuchsin series. Those cells which retain firmly the blue dye are said to be Gram-positive, while those which are decolorised by the alcohol and take up the red dye are said to be Gram-negative. Many explanations, both physical and chemical, have been advanced to account for the differential staining (Henry and Stacey, *Proc. Roy. Soc.*, 1946, *B*, **133**, 391; Dubos, " The Bacterial Cell ", *loc. cit.*). It was apparent that there must be some fundamental chemical differences between Gram-positive and Gram-negative organisms to account for such diverse staining reactions. It was discovered (Henry and Stacey, 1943) that Gram-positive organisms could be made Gram-negative by various methods, *e.g.*, by enzymic attack and by chemical extraction methods. A process was developed whereby certain organisms, *e.g.*, *Cl. welchii*, could be stripped of an essential part their Gram-positive material and separated into a water-soluble Gram-negative extract and a Gram-negative cyto-skeleton. A technique was then developed whereby a constituent of the Gram-negative extract could be plated back on to the cyto-skeleton to reconstitute mainly a cell which could once more be stained Gram-positive (See Figure 15).

*Figure* 15.




The essential factor in the extract which could be " replated " was the magnesium salt of ribonucleic acid, and its presence at the surface of some Gram-positive organisms, and throughout the cell in others, differentiates them from some Gram-negative organisms.

The ribonucleic acids were not specific since, in the form of their magnesium salts, they could be " cross " plated back on to any receptive cell cyto-skeleton whatever their origin; for example, *Cl. welchii* magnesium ribonucleate could be plated on to yeast and *vice versa*. The Gram-complex of all microbial cells so far examined (except yeast) contained a small proportion of deoxyribonucleic acid the significance of which is not yet clear.

An interesting connection between the Gram-positive state and " smooth " and " rough " forms of colonies of organisms was demonstrated. Thus when *Streptococcus salivarius* was cultivated in a medium where the magnesium content was reduced to a minimum or where there was sufficient acid production to prevent the formation of magnesium ribonucleate, the cocci became essentially Gram-negative. Furthermore, these Gram-negative forms displayed good evidence of nucleation, and when subcultured on agar they gave rise to rough colonies whereas their normal Gram-negative relatives gave rise to colonies which were smooth. Since smoothness in a colony depends on the formation of relatively large amounts of polysaccharide it is apparent that there is a connection between the presence in the cell of the mucleic acid constituent of the Gram-positive complex and polysaccharide synthesis (cf. the work of Avery, Macleod, and McCarty, *J. Exp. Med.*, 1944, **79**, 137).

The cyto-skeletons from *Cl. welchii* and from yeast gave an intense Sakaguchi reaction

indicative of the presence of basic protein material, and it was considered that the Gram-positive complex (*i.e.*, the dye-retaining material) is a high molecular complex formed by the combination of magnesium ribonucleate with a reduced basic protein substrate. A successful search was then made for this protein and for the Gram-positive complex, and it was possible to extract the complex which had the essential characteristics of a nucleoprotein. The protein constituent of this appeared to be of a novel type and was not a simple histone or a protamine.

The complex was separated from Gram-positive organisms by an autolytic procedure, using a phosphate buffer. When the nucleoprotein in neutral aqueous solution was heated at 60°, the protein constituent came down in denatured form leaving the nucleic acid in solution. This behaviour closely resembled that of Stanley's tobacco mosaic virus ribonucleoprotein. The complex could be dissociated and parts re-combined as shown in Figure 15 (Henry, Stacey, and Teece, *Nature*, 1945, **156**, 720).

The autolysis procedure in the Gram-negative group did not result in the formation of nucleoprotein. It was possible, however, to extract proteins and the two types of nucleic acid from certain Gram-negative organisms. Moreover, these could be precipitated from aqueous solution at pH 5 in the form of protein nucleates which could not be stained Gram-positive although they had a certain amount of dye-retaining character.

The next step in the work on the Gram-complex involves a study of the lytic enzymes of the cell (see Dubos, *op. cit.*). Already our colleague, Dr. H. Henry, has identified specific nucleases, carbohydrases, and proteases, all of which play a part in the autolytic breakdown of the macromolecules of the cell and which may come into action between definite pH ranges at certain stages in the life of the cells.

Nucleoproteins containing both ribo- and deoxyribo-nucleic acids are probably involved in cell-division mechanisms and are responsible for many synthetic activities of the cell. The only bacterial deoxyribonucleic acid yet investigated appears to be the " transforming principle " of Avery *et al.* (*loc. cit.*) which converts the Rough Type II pneumococcus into the Smooth Type III pneumococcus. This factor extracted from a Smooth Type III pneumococcus is biologically active in minute quantities and is somehow responsible for type specific polysaccharide synthesis and is a deoxyribonucleic acid of high molecular weight. There is no doubt that this transformation of types is an authentic case of a specific mutation caused by essentially a chemical entity, and its importance cannot be over emphasised.

One considers (Stacey, " Bacterial Nucleic Acids and Nucleoproteins, *Symposium Soc. Exp. Biol. & Med.*, 1946, in the press) that Avery and his colleagues must have removed, together with their deoxyribonucleic acid, at least a unimolecular layer of the Type III specific polysaccharide in order to form the correct " pattern " for the carbohydrate synthesis. This may eventually be proved by reference to other specific polysaccharides, but so far it would appear that deoxyribonucleic acids themselves may have a sharply specific structure and function in this connection.

The vexed question as to the presence of a bacterial nucleus and chromosome system may be solved by isolation and chemical study of the cell nucleoproteins.

It is of interest to note that certain type specific " proteins " of streptococci (" M substances ") are probably nucleoproteins, and these are located near the surface of the cells. Of great potential significance is the work of Shear and his colleagues (*J. Nat. Amer. Inst.*, 1946, **6**, 488, and previous papers) who have shown that a complex polysaccharide, related to the somatic polysaccharides of other bacteria, which can be isolated from *B. prodigiosus*, may cause extensive necrosis in transplanted tumours.

It is hoped, by continuing these studies on the structure of cell macromolecules, to gain information which will be of fundamental value regarding the physics and chemistry of all cells and of practical importance in problems of chemotherapy and immunotherapy.