STUDIES ON THE POLYSACCHARIDES OF AEROBACTER

by

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ABSTRACT OF THESIS

Name of Candidate WILLIAM F. DUDMAN, B.Sc.

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Title of Thesis Studies on the polysaccharides of Aerobacter.

Part I. The composition of the extracellular polysaccharides of five strains of A. aerogenes, two strains of A. cloacae, three strains of Klebsiella and one strain of E. coli.

1. The exopolysaccharides of five strains of A. aerogenes, two of A. cloacae, three of Klebsiella and one of E. coli were isolated and examined.

2. They were found to be unusual polyuronides with the following compositions (expressed in molecular ratios.)

A. aerogenes

strain A1	glucose (8), uronic acid (4), fucose (2)
" A3	glucose (9), uronic acid (5), fucose (2)
" A3(S1)	glucose (9), uronic acid (5), fucose (2)
" A4	galactose (17), uronic acid (8), glucose (7), mannose (1)
" A29	mannose (2), galactose (1), uronic acid (1)

A. cloacae

NCTC 5920	galactose (3), fucose (3), glucose (2), uronic acid (2)
NCTC 5936	glucose, galactose, fucose, uronic acid.

Klebsiella

type K2	glucose (8), uronic acid (6), galactose (5), mannose (2).
" K26(S1)	galactose (4), glucose (3), mannose (3), uronic acid (2)
" K29(S1)	galactose (5), uronic acid (4), mannose (2)

E. coli

strain A102	uronic acid (10), glucose (8), galactose (8), fucose (8), rhamnose (1).

D-glucose and L-fucose were confirmed in the polysaccharide of A. aerogenes strain A3(S1) by isolation and preparation of derivatives. None of the uronic acid components were identified.

3. The slime and capsular polysaccharides of A. aerogenes strain A3 were isolated separately and shown to be identical.

4. The exopolysaccharide of a slime-forming variant (A. aerogenes strain A3(S1)) was shown to retain the composition of the exopolysaccharide of its parent capsulate strain (A3).

5. The immunological relationship of the exopolysaccharides of A. aerogenes strains A1, A3 and A3(S1) were examined and they were shown to be identical. The exopolysaccharide of strain A3(S1) in dilutions of 1,000,000 was found to react with homologous immune serum.

Part II. The influence of carbon substrate on the composition of the extracellular polysaccharide of A. aerogenes (Strain A3(S1)).

A non-capsulate slime-forming strain (A3(S1)) of A. aerogenes was

cultured in simple synthetic medium containing glucose, galactose, mannitol, xylose, fucose, rhamnose, glucurone and sucrose (used singly) as sole carbon and energy source. The exopolysaccharide produced by the organism from each sugar was examined and its composition found to be the same in each case: glucose (9 parts), uronic acid (5 parts) and fucose (2 parts). No levan was produced by the organism when grown on sucrose. The constant composition of the polysaccharide is interpreted to indicate that heteropolysaccharides are synthesised by some form of template mechanism.

Part III. The somatic and intracellular polysaccharides of A. aerogenes (strain A3(0)).

The somatic and intracellular polysaccharides of a smooth strain (A3(0)) of A. aerogenes were extracted by a series of increasingly drastic conditions: boiling water, boiling 10% potassium hydroxide and cold 60% sulphuric acid. The first and second stages remove 77% and 20% of the total polysaccharide respectively. The fractions appear to be mixtures of a galactan and a glucan in various proportions. The galactan comprises the bulk of the total polysaccharide of this strain, and is believed to account for the small amounts of galactose found in the exopolysaccharides of A. aerogenes strains A1, A3 and A3(S1). The identity of the galactose was confirmed by oxidation of some of the main fraction and isolation of mucic acid.

The main fraction of isolated polysaccharide reacted with A3(0) antiserum but could not completely absorb all the antibody against A3(0) cells.

CONTENTS

GENERAL INTRODUCTION

TO

BACTERIAL POLYSACCHARIDES

GENERAL INTRODUCTION TO BACTERIAL POLYSACCHARIDES

Carbohydrate compounds, predominantly in the form of polysaccharides, constitute 10 to 30% of the weight of dried bacteria,(Knaysi 1951). These polysaccharides which may occur free or combined with lipid and protein, are found chiefly in the "slime-layer" and cell wall, but are also found within the cell, where in some cases large quantities of carbohydrate inclusion bodies have been found. Mainly on the evidence of staining reactions these inclusion bodies are believed to be glycogen or starch.

Most of the interest in bacterial carbohydrates has centered on the polysaccharides outside the cell wall. This is mainly because it has been found that the immunological properties of capsulated organisms are determined by the nature of their capsules, and that a relationship exists between virulence and capsulation in many pathogenic species. Furthermore these materials are among the most easily isolated of all bacterial products. Except for a few cases all capsules contain large amounts of polysaccharide, and it has been shown that the immunological specificity of capsulated organisms depends on the polysaccharide moiety acting as hapten or as the complete antigen. Thus bacterial extracellular polysaccharides, especially those of certain pathogens have been the subject of much study.

2. Slime and capsule. The extracellular polysaccharides can be considered to be of two forms - slime and capsule. When the polysaccharide material is detached from the cell and is present as an amorphous mass that disperses freely it is called slime; it is described as a capsule when the polysaccharide adheres to the cell and has a distinct shape and edge.

Much confusion has arisen through the indiscriminate use of ambiguous terms to describe these structures, e.g. gum, mucus, sheath, slime, slime-layer, slime-envelope etc. Recently the need for greater precision of description has been stressed, (Duguid,1951; Mudd, 1953). Duguid has suggested avoiding the term slime-layer in order to prevent confusion with slime(as defined above). The same author proposes that the term capsule be used only for any microscopically demonstrable covering layer external to the cell-wall which is firm enough to remain adherent when the cells are suspended in water.

There is yet need for a term to describe extra-cellular polysaccharide in general, without reference to slime or capsule. It is here suggested that the term "exopolysaccharide" be used for this purpose, by analogy with exotoxin and exoenzyme. It will be used with this sense in the rest of the thesis.

3. Demonstration of slime and capsule, Bacterial slime and capsule are difficult to demonstrate microscopically on dried and fixed films because of their

low affinity for dyes, and their liability to gross shrinkage and distortion on drying and fixing. After a thorough investigation of the problem and assessment of the available methods Duguid(1951) reported that the wet-film India-ink method is the most reliable for general application. The important advantage of this method is that, with care, there is no distortion; the capsule and slime are seen as clear bright zones against the dark background.

Another important method is the "specific swelling reaction" or "quellung reaction" of Neufeld(1902), in which the capsules are rendered visible by the formation of insoluble antigen-antibody complexes at the capsule surfaces. This method is of less general application than the india-ink method because it is necessary to treat the cells with homologous antiserum, or with an antiserum capable of cross-reacting with the capsular substance before the capsules are rendered visible, but the method is of obvious value in the typing of organisms. Non-specific quellung reactions, not depending on the formation of antigen-antibody complexes, have been described by Neufeld and Etinger-Tulczynska(1931), and more recently by Jacox(1947) and Tomcsik and Guex-Holzer(1954). The name of the method is misleading as it is now known that swelling does not in fact occur.(Kleineberger-Nobel 1948, Duguid 1951). The appearance of capsules in place of the smaller apparently bare cells give the illusion of swelling.

Occurrence of bacterial exopolysaccharides

Exopolysaccharide production is a highly variable property and cannot be considered without reference to cultural conditions. The cell may be able to manufacture an exopolysaccharide, but whether it produces it or not depends on the environment and on the presence of suitable raw material. The exopolysaccharide characteristics of strains may change on repeated subculture, leading to reduction, loss, increase or sudden appearance of exopolysaccharide. (For example see Maassen, Knothe and von Krohn, 1952). Mucoid variants of normally non-mucoid species, and vice-versa, are of relatively common occurrence.

According to Etinger-Tulczynska (1933) bacteria may be arranged into three groups; bacteria producing no exopolysaccharide, bacteria producing either capsules or slime, and bacteria producing both capsules and slime. The second group must be amended, because it is now known that although many organisms produce slime without capsules, bacteria never produce capsules without slime. In every instance when cell-free culture filtrates of capsulated species have been examined, microscopically invisible but chemically and immunologically detectable slime has been found. Duguid (1951) showed that to demonstrate slime microscopically it is necessary to prepare films from surface cultures on solid media. If films are prepared from liquid suspensions of solid cultures. or from

liquid cultures, only the capsules are seen; the slime is dispersed to give a uniform solution in the liquid and as such remains invisible, giving the appearance of capsule formation without slime. In the light of present knowledge therefore, a classification on the basis of exopolysaccharide production would show the following types:

1. Bacteria producing no exopolysaccharide
2. Bacteria producing slime alone
3. Bacteria producing both capsules and slime

It is emphasised that any consideration of exopolysaccharide production must be referred to defined environmental conditions, otherwise the presence or absence of exopolysaccharide would be meaningless as a criterion of classification. An example of this is the organism Leuconostoc mesenteroides which when cultured on sucrose-containing medium produces large quantities of slime, but which produces none when grown on media containing monosaccharides. Besides affecting the total exopolysaccharide production, environmental conditions can influence the relative amounts of slime and capsule. (Duguid and Wilkinson, 1953).

Despite the marked influence of cultural conditions, certain species produce capsules in most circumstances, e.g. Diplococcus pneumoniae, the Klebsiella -Aerobacter group, Bacillus anthracis, Clostridium welchii and Micrococcus tetragenus. Slime-forming bacteria that

do not produce capsules are often variants of capsular species and nearly all of them are motile. Some species are known to have strains corresponding to all three of the above exopolysaccharide types. Duguid (1951) examined 52 type I Escherichia coli strains and found 8 slime-forming non-capsular strains (6 motile 2 non-motile), 2 capsulate strains (both non-motile), and the remaining 42 strains producing neither capsules or slime in detectable amounts. Wilkinson, Duguid,and Edmunds (1954) described variant strains of Aerobacter aerogenes which included all three exopolysaccharide types:- strain A3(0) produced no exopolysaccharide, strain A3(Sl) produced slime only, and strain A3 produced both capsules and slime.

Colony appearance and exopolysaccharide distribution

The appearance of a bacterial colony is affected by the morphology of its constituent cells. Slime and capsules, being exterior to the cells and capable of being produced in very large quantities, have a decisive influence on colony appearance; and as a consequence, colonial morphology is subject to the same influences as exopolysaccharide production. Essentially, there are three types of colonies, mucoid (M), smooth(S) and rough (R), but intermediate types exist. Unfortunately, these adjectives have been defined inadequately, causing confusion between descriptions applied by different authors, especially

when colony appearance is applied as an index of antigenic structure of different species and strains.

Wilkinson, Duguid and Edmunds (1954) have correlated colonial with microscopic appearance, in the case of A.aerogenes, A.cloacae and E.coli strains, and have introduced a quantitative approach to the problem. They found that all strains producing large amounts of slime and capsule, or slime alone, formed mucoid colonies. Smooth strains produced much less polysaccharide, most of it intracellular, while rough strains produced least polysaccharide. They found that the factor controlling the degree of mucoidness appeared to be the extent by which the total volume of surface cultures (including exopolysaccharide) exceeded the total volume of the bare cells in the culture. In non-muccid cultures (S and R) these two terms were approximately equal, and the bacterial dry-weight was found to make up 22 - 37% of the wet-weight of the cultures. In slightly mucoid cultures the cells occupied 13 - 44% of the total volume of the cultures, and the bacterial dry-weight was 10-14% of the cultural wet-weight. With highly mucoid cultures the cells occupied only 1- 5% of the total volume of the cultures, and the bacterial dry-weight was only 3 - 6% of the cultural wet-weight.

Influence of environmental conditions on exopolysaccharide production

Although it has long been known that exopolysaccharide

production depends very much on environmental conditions, comparatively little work has been done to investigate this aspect of bacterial physiology. Many authors, in studying the exopolysaccharides of particular organisms, have experimented with cultural conditions in order to obtain optimum yields of exopolysaccharide, but there have been few systematic investigations. Recently thorough quantitative studies have been made on the influence of cultural conditions on polysaccharide production by A.aerogenes (Duguid and Wilkinson, 1953) and pneumococcus type III (Bernheimer, 1953). In correlating the available data some uncertainty arises, for prior to the studies just cited, investigations were made mostly by methods which estimated only part of the total polysaccharide. In the sections that follow, an attempt is made to present the results found for the influence of various factors on exopolysaccharide production.

(1) Influence of carbon source

Non-exacting heterotrophic organisms may utilise a single carbon compound as source of both energy and assimilated carbon; part of the carbon substrate is oxidised to provide energy for growth and the assimilatory processes, and the remainder is assmilated to provide building units for the synthetic processes. Seigel and Clifton (1950) found that the ratio of carbon assimilated to carbon utilised by E.coli in a glucose medium was constant, between 0.56 and 0.60, but no

information was given by the authors concerning the fate of the assimilated carbon. Working with washed cells of A.aerogenes, Stark and Wilkinson (unpublished results) found that the ratio of glucose assimilated to glucose utilised ranged between 0.60 and 0.82 and that about half of the assimilated glucose was converted to polysaccharide; i.e., A.aerogenes was found to convert about 20% of the glucose substrate into polysaccharide.

All workers have found that to obtain abundant exopolysaccharide it is necessary to provide an excess of utilisable carbon substrate, normally carbohydrate. When the level of the other components in a suitable growth medium are fixed, it is found that over a short range, increasing sugar concentrations lead to increased polysaccharide production, but an upper limit is reached beyond which increasing sugar in the medium becomes inhibitory. Hoogerheide (1939) observed that when grown in glucose peptone broth, polysaccharide production by Klebsiella pneumoniae was maximal with 0.5 - 1% glucose; concentrations of glucose greater than 10% were inhibitory.

It is now known that the absolute concentration of carbohydrate substrate in a medium is less important than its concentation relative to that of other essential substrates. This effect was observed by Hoogerheide, who reported that exopolysaccharide production by Kl. pneumoniae was increased when growth was limited by phosphorus deficiency; he postulated that exopoly-

saccharides are formed in conditions unfavourable for growth rather than during active proliferation. Duguid (1948) during a study of capsule sizes of A.aerogenes grown on defined solid media, made the significant observations that the largest capsules were obtained when growth was carried out on media in which the carbohydrate/nitrogen or carbohydrate/phosphorus ratios were high. These results suggested that exo-polysaccharide synthesis is favoured when growth is limited by deficiency of an essential nutrient other than the carbohydrate source of energy; under such conditions the energy required for growth is limited and the excess carbohydrate can be used for polysaccharide synthesis. Bunting, Robinow and Bunting (1949) confirmed Duguid's findings with Serratia marcescens and A.aerogenes. The earlier results were confirmed by Duguid and Wilkinson (1953) in a quantitative study carried out on a single capsulate strain (A3) of A.aerogenes in which the slime and capsular poly-saccharides were estimated chemically, and the intra-cellular polysaccharide by staining, so that the total polysaccharide was measured. It was confirmed that deficiency of nitrogen and phosphorus in the presence of excess sugar greatly increased polysaccharide production, and it was discovered that sulphate deficiency had a similar effect. An upper limit was found for the amount of polysaccharide that can be produced, even in cultures deficient in nitrogen; polysaccharide production

ceased while sugar and oxygen were unexhausted, the pH favourable and most of the cells alive. Duguid and Wilkinson also observed that exopolysaccharide was formed under all conditions; a small amount was produced even when the sugar supply was very limited.

Wilkinson, Duguid and Edmunds (1954) investigated strains of E.coli and A.cloacae as well as other strains of A.aerogenes using the method developed by Duguid and Wilkinson, and found that the same behaviour was shown by these organisms. In contrast to the effect of nitrogen, phosphorus and sulphur deficiency in the presence of excess carbohydrate, it was observed that potassium deficiency caused a decrease both in growth and exopolysaccharide production. Hoogerheide (1939) reported that calcium deficiency had a similar effect on Kl.pneumoniae. Bernheimer (1953) found that the absence (singly) of magnesium, potassium and phosphorus caused a decrease in the exopolysaccharide produced by a suspension of enzymatically-decapsulated pneumococci.

Future work over a wider range of organisms may show that exopolysaccharide production in all bacteria is influenced in a similar manner by nutritional conditions, i.e. that maximum polysaccharide production is attained when the ratio of sugar to nitrogen, phosphorus or sulphur in the medium is large. It may be found too, that deficiency of other essential nutrients such as an essential amino acid, in the presence of excess sugar, may be as effective in causing maximal exopolysaccharide

production.

The nature of the carbon substrate can have an important influence on the course of exopolysaccharide production. Most organisms can produce their exopolysaccharides from any utilisable carbon source, the amount formed depending on the nature of the substrate; i.e. with most species the exopolysaccharide is a normal concomitant of growth, and does not require a specific carbon substrate for its elaboration. But exceptions are known where the organism can produce its exopolysaccharide only from one carbon source, even though it can utilise a large number of substrates for growth. These exceptions are found among the dextran and levan-producing species. Levan-producers (e.g. Bacillus Subtilis, Bacillus mesentericus, Aerobacter levanicum and Streptococcus salivarius, etc.) form levans only from sucrose and raffinose; other common sugars, like glucose, fructose and maltose are not suitable substrates. Dextran-forming species like L.mesenteroides, Leuconostoc dextranicum and Betabacterium vermiforme require sucrose for the production of their exopolysaccharides. Other dextran-formers, Acetobacter viscosum and Acetobacter capsulatum, require dextrin as specific substrate for dextran synthesis.

Morgan and Beckwith (1939) in a study of the effect of different carbohydrates upon mucoid growth of Escherichia and Salmonella strains, found the following substrates effective in various degrees; glucose,

fructose, galactose, L-rhamnose, sucrose, lactose, L-arabinose, xylose, sorbitol, dulcitol, trehalose, raffinose, dextrin and salicin. They found that fermentation reactions paralleled the mucoidness of growth on solid media incorporating the sugars. Hoogerheide (1939) studied the effect of using different sugars as carbon source for Kl. pneumoniae, by adding 1% of the sugar to 4% peptone broth, and measuring the size of capsule produced. When glucose, sucrose and maltose were used, the capsules were practically the same size; with mannitol the capsules were somewhat smaller, while with pyruvate, glycerol and rhamnose they were considerably smaller. Bernheimer (1953) found that no other sugar was as effective as glucose for high yields of exopolysaccharide by decapsulated type III pneumococci, as shown in the following table.

Effect of various sugars and related compounds on exopolysaccharide formation by type III pneumococcus

Substrate		Exopolysaccharide-forming activity. (glucose = 100)*
fructose	M/100	72
galactose	"	50
maltose	M/200	63
sucrose	"	38
lactose	"	65
cellobiose	"	0
glucosamine	M/100	47
glucose 1-phosphate (di K salt)	M/100	11
K 3-phosphoglycerate	M/100	19
glycerol	M/100	3
Na glucuronate	M/100	3
Na cellobiuronate		0
menthol glucuronide		0
glycogen	(1.8 mg/ml.)	13

*(exopolysaccharide production measured after 60 minutes incubation at 37°)

Assimilation studies with E.coli (Siegel and Clifton, 1950) have given the following ratios, for substrate assimilated to substrate oxidised;- arabinose 0.64, lactose 0.55, succinate 0.41, fumarate 0.43, lactate 0.40, pyruvate 0.44, glycerol 0.70 and glucose 0.58,. No information was given regarding the extent of conversion to polysaccharide. The results led the authors to conclude that the molecular structure of the substrate and of the intermediate degradation products, is of greater importance to the cell than the free energy of its oxidation, in determining the extent of synthesis to cell material.

(ii) Influence of nitrogen source

The nitrogen source plays a less important role in exopolysaccharide production than the carbon source. Its main influence appears to be in determining the relative surplus of carbon that will be available for exopolysaccharide synthesis after growth energy-requirements, if controlled by the amount available, have been met.

Hoogerheide (1939) examined the influence of different nitrogen sources (proteins and peptones) on the capsule size of Kl.pneumoniae and obtained results which suggested that the nitrogen substrate had some influence on capsule size. The results are of doubtful value, however, because it is unlikely that the sugar/nitrogen ratio was the same in all the media. Duguid and Wilkinson (1953) found that the exopolysaccharide production of A.aerogenes was unaffected if bactopeptone

was used in place of ammonium sulphate, while maintaining the same sugar/nitrogen ratio.

(iii) Influence of temperature of incubation

The influence of temperature of incubation on exopolysaccharide production appears to depend on the organism. It has been found that exopolysaccharide production by Salmonella and Escherichia organisms is favoured by low temperatures (10 - 15°) (Birch-Hirschfeld, 1936; Morgan and Beckwith, 1939; Beiser and Davis, 1953; Wilkinson, Duguid and Edmunds, 1954), while with Klebsiella and Aerobacter species the reverse is true, more polysaccharide being produced at 37° than at lower temperatures (Etinger-Tulcznska, 1933; Hoogerheide, 1939; Maassen, Knothe and von Krohn, 1952).

Duguid and Wilkinson (1953) obtained interesting results with A.aerogenes strain A3,. They found that in nitrogen-rich conditions exopolysaccharide production was greater at 15 - 20° than at 37°, but in nitrogen-poor conditions temperature had no effect on the exopolysaccharide yield; the same amount of growth took place under both sets of conditions. Wilkinson, Duguid and Edmunds (1954) found that in one strain (NCTC 5920) of A.cloacae exopolysaccharide production was increased by incubation at 15 - 20° only in conditions favourable for growth (i.e. in nitrogen-rich media); in nitrogen-poor conditions, exopolysaccharide production is slightly less at lower incubation temperatures. The same

authors found that A.cloacae strain NCTC 5936 produced more exopolysaccharide at 37° than at 15 - 20°, both in nitrogen-rich and nitrogen-poor media.

(iv) Influence of oxygen

Anaerobic conditions have been found to decrease exopolysaccharide production; the most immediate cause is probably the much smaller amounts of energy available under anaerobic conditions. Bernheimer (1953) found that the yield of exopolysaccharide produced by pneumococcus type III under anaerobic conditions was about 20-25% of the aerobic value. Duguid and Wilkinson (1953) also found a marked decrease in exopolysaccharide yield when A.aerogenes was cultured anaerobically, and suggest that anaerobiosis may be specifically unfavourable for polysaccharide synthesis apart from its effect in decreasing the amount of energy available from the sugar supply.

(v) Influence of pH

Even the earlier investigators of bacterial polysaccharides were aware of the necessity of maintaining the pH of cultures near neutrality in order to obtain maximal yields of polysaccharide. Emmerling (1900) and Schardinger (1902) working with Aerobacter strains added chalk to their cultures to prevent the pH from becoming too low when growth was to be continued over long periods.

Hoogerheide (1939) reported that exopolysaccharide

production by Kl.pneumoniae was independant of pH over the range 5 to 9. Duguid and Wilkinson (1953) found that polysaccharide production by A.aerogenes was inhibited by low pH values, even when these were not low enough to inhibit growth. Bernheimer (1953) found that decapsulated type III pneumococci were able to synthesise appreciable amounts of exopolysaccharide over the range of pH 5.0 to 8.0, but that synthesis was greatest in the region of 6.0-6.7,. Here almost twice as much polysaccharide was produced as at the extreme values of pH. It was observed that the tendency of the polysaccharide to diffuse away from the cell is a function of pH, and is minimal in the region of 6.3,. Stark and Wilkinson (unpublished results) have found that pH was a critical factor in polysaccharide production by A.aerogenes; the rate of production by washed cells was greatest at pH 6.0, a sharp fall in production occurring on either side of this value.

(vi) Influence of serum

In the course of many investigations it has been reported that the presence of serum in the medium is necessary for good capsule formation and for good yields of exopolysaccharide, (e.g. Knolle, 1953). In all the studies in which serum is advocated as being necessary for maximal exopolysaccharide production, the polysaccharides were estimated by microscopic examination and measurement of capsule size. It is most likely that the serum acts passively by improving the

demonstrability of the capsules, rather than by actively influencing exopolysaccharide synthesis. (Toenniessen, 1921; Kleineberger-Nobel, 1948).

(vii) Influence of growth in vivo

It has been claimed for a long time that pathogenic capsulated bacteria show their maximal capsule production when grown in animal tissues, mainly on the evidence of microscopic examination of dried, fixed stained films. There can be no doubt that large quantities of exopolysaccharide are produced by organisms grown in, or freshly isolated from, animal tissues, but until quantitative chemically-estimated comparisons have been made, it is not proven that growth in artificial media under favourable conditions cannot give rise to even greater yields of exopolysaccharide.

The high yields of exopolysaccharide obtained by growth in vivo may arise through the influence of the following factors operating simultaneously or singly:

(a) In animal tissues only capsulated cells have good chances of survival; the others are soon removed by the body defences, so that there is a selection within the body favouring capsulated cells.

(b) The environmental conditions in animal tissues may be favourable toward polysaccharide synthesis; it may be that the relative sugar substrate concentration is favourable. In the case of a non-exacting species in the blood, the determining factor may be the ratio of sugar to utilisable sources of nitrogen, sulphur or phosphorus. The composition data for normal human

blood shows that the following are present (mg./100 ml.): glucose 70 - 100, total non-protein nitrogen 25 - 35, inorganic sulphate 0.9 - 1.1, inorganic phosphorus 3 - 4. It will be seen that if the growth of an organism were limited by the sulphur or phosphorus concentration, the relative glucose concentration would favour polysaccharide synthesis. In the case of an exacting organism the critical nutrient to compare with the glucose may be some growth factor present in very small amounts, making the ratio of glucose/ essential growth-limiting nutrient even more favourable for exopolysaccharide production.

(c) Capsules of organisms isolated from animal tissues will be rendered more demonstrable by the serum present in the body fluids.

The course of exopolysaccharide production during growth

The small number of organisms which have been studied to reveal the course of their exopolysaccharide production during growth all show the same general pattern of behaviour. In the cases of Kl.pneumoniae (Hoogerheide, 1939), pneumococcus type III (Bukantz, 1940; Bukantz, Cooper and Bullowa, 1941), and A.aerogenes (Duguid and Wilkinson, 1953) it has been shown that polysaccharide production takes place throughout the period of growth, but reaches its maximum value only after the logarithmic growth period. The rate of production per cell, however, is greatest during the logarithmic period and diminishes progressively thereafter.

Duguid and Wilkinson found that the main production of slime polysaccharide by A.aerogenes occurred later than the main production of intracellular and capsular polysaccharides. After the first 24 hours slime accounted for only approximately 20% of the total polysaccharide, while after 96 hours it accounted for about 50%

Physical properties of bacterial exopolysaccharides

When isolated and purified, bacterial exopolysaccharides possess the same general physical characteristics as other polysaccharides. They are amorphous white solids, which can be isolated in forms varying from powders to stringy threads, depending on the method of isolation and final precipitation. These solids are highly hygroscopic, and when moistened swell up to form gels. They are generally soluble in water in varying degrees to give viscous solutions, but are completely insoluble in neutral organic solvents such as alcohol, acetone, ether, etc. The polysaccharides that contain uronic acid residues behave in solution as highly charged anions, with high equivalent conductances and electrophoretic mobilities; they can be titrated with alkalies. All the polysaccharides are optically active, often with high rotation values.

Molecular weight determinations have not been carried out on many bacterial polysaccharides (see review by Greenwood, 1952), but it is evident that the homopolysaccharides like the dextrans and levans have

much larger molecules than the heteropolysaccharides that have yet been examined. In many cases this may be because the heteropolysaccharides were isolated by procedures sufficiently drastic to cause some degradation. Dextran fractions of L.mesenteroides have been found to have weights ranging from 14,000 to 38,000,000 (Ingelman and Halling, 1949), while the levan produced by Bacillus vulgatus has been shown to have a molecular weight of the order of 50 to 100 million (Ingelman and Seigbahn, 1944). The levan from Aerobacter levanicum has a molecular weight estimated to be 40 million (Hestrin, 1953). The molecular weights of the exopolysaccharides of pneumococcus types I, II and III were found to be 171,000, 540,000, and 141,000 respectively (Record and Stacey, 1948); these are the largest heteropolysaccharide molecules yet found in bacteria.

The chemical composition of bacterial polysaccharides

The exopolysaccharides of bacteria are a very diverse group, and include polysaccharides of every type of composition and structure. The polysaccharides produced by different organisms may vary enormously in composition and structure, or they may be very similar. Taxonomic relationships are no guide to polysaccharide composition; closely related organisms, even the different strains of a species may produce polysaccharides completely different from each other - e.g. the various exopolysaccharides produced by the different pneumococcus types. On the other hand, completely unrelated organisms

may produce very similar polysaccharides. Immunological cross-reactions between completely unrelated organisms are found frequently, but this in itself shows no more than that similarity exists in the steric arrangement of the immunologically dominant groups present in the cross-reacting polysaccharides.

Recent investigations of bacterial exopolysaccharides have led to the discovery of three new monosaccharides, which had never previously been found in polysaccharides. They are the 3,6-dideoxysugars, tyvelose (from Salmonella typhi O-901 and Salmonella enteritidis-Gärtner) and abequose (from Salmonella abortus equi and Salmonella paratyphi-Kröger B) reported by Westphal, Luderitz, Fromme and Joseph (1953), and the N-acetylamino-hexuronic acid found to be the sole component of the Vi antigen of a strain of E.coli (Webster, Clark and Freeman, 1954).

Like the polysaccharides in general, bacterial polysaccharides may be divided into two main groups, the homo- and the hetero- polysaccharides.

(1) Bacterial homopolysaccharides

The polysaccharides of this group are characterised by giving rise to only one component sugar on hydrolysis. With the exception of the Vi antigen of E.coli strain 5936/38 which is a polysaccharide built up of aminouronic acid residues, all bacterial homopolysaccharides have been found to be glucosans and fructosans, and to be less antigenic than the heteropolysaccharides. The

homopolysaccharide-producing bacteria are of much current biochemical interest in connection with the problems of polysaccharide synthesis.

The main facts relating to the bacterial homo-exopolysaccharides are given in table 1. It must be remembered that polysaccharides of the same general type with the same component sugar and the same linkages, need not necessarily have identical structures; e.g. L.mesenteroides, L.dextranicum and Betabacterium vermiforme all produce dexrans with mainly 1,6-linked D-glucopyranose units, but the polysaccharide of the first organism is much more highly branched than those of the others.

Table 1. Bacterial homoexopolysaccharides

(i) Polysaccharides containing D-glucose

Organisms	Substrate required for polysaccharide production.	Polysaccharide	Linkages	References
Acetobacter acetigenum	not restricted	cellulose	1,4-β	Kaushal and Walker (1951) Bourne and Weigel (1954) Barclay, Bourne, Stacey and Webb (1954)
Aceto. acidum-mucosum	dextrin	starch-like		Tosic & Walker (1950): Hehre (1951)
Aceto. capsulatum	"	dextran		Hehre & Hamilton (1949)
Aceto. viscosum	"	"		" " "
Aceto. xylinum	not restricted	cellulose	1,4-β	Tarr and Hibbert (1931) Hibbert and Barsha (1931) Mühlethaler (1949) Shirk and Greathouse (1952)
Betabacterium vermiforme	sucrose	dextran	mainly 1,6-α some 1,4 -	Baker and Stacey (1939)
Leuconostoc dextranicum	"	"	"	Fairhead, Hunter and Hibbert (1938) Peat, Schlückterer & Stacey (1939)
L. mesenteroides	"	"	"	Hassid and Barker (1940) Levi, Hawkins & Hibbert (1942) Stacey and Swift (1948)
Neisseria perflava	Sucrose glucose-1-phosphate	amylopectin-glycogen type	mainly 1,4-α	Hehre and Hamilton (1948) Barker, Bourne and Stacey (1950)
Strept. bovis		dextran-like		Niven, Smiley & Sherman (1941)
Strept. Group H.	sucrose	dextran		Neill, Sugg, Hehre & Jaffe (1941)
Strept. salivarius		small amount of dextran (also levan)		Niven, Smiley & Sherman (1941)
Phytomonas tumefaciens	sucrose D-glucose D-fructose	dextran-like	1,2-β	Reeves (1944) Hodgson, Riker & Peterson (1945) Putnam, Potter, Hodgson & Hassid (1950)

(ii) Polysaccharides containing D-fructose (believed linked 2,6-)

Organism	Substrate required for polysaccharide production	Polysaccharide	References
Aerobacter levanicum	sucrose, raffinose	levan	Hestrin, Avineri, Shapiro and Aschner (1943)
Bacillus cereus	" "	"	Forsyth and Webley (1949)
B. megatherium	" "	levan(also polyuronide)	Lyne,Peat & Stacey(1940): Forsyth & Webley(1940)
B. mesentericus	" "	levan	Haworth, Challinor and Hirst (1934)
B. polymyxa	" "	levan(also polyuronide)	Forsyth & Webley (1949): Murphy (1952)
B. pumilus	" "	levan	Forsyth and Webley (1949)
B. subtilis	" "	"	Hibbert & Brauns (1931) : Hibbert, Tipson & Brauns (1931) : Mitchell & Hibbert (1932)
Phytomonas pruni	" "	"	Lyne, Peat and Stacey (1940)
Phyto. prunicola	" "	"	" " " "
Pseudomonas mors-pruni	" "	"	Gilbert and Stacey (1948)
Strept. salivarius	" "	levan (also dextran)	Niven, Smiley & Sherman(1941)

Recently a number of intracellular polysaccharides have been examined and found to be homopolysaccharides. A glycogen-like intracellular polysaccharide has been isolated from B.megatherium and shown to be similar in all respects to glycogen isolated from other sources. (Aubert, 1951; Barry, Gavard, Milhaud and Aubert, 1952; 1953). An intracellular polysaccharide from Clostridium butyricum has been studied by Gavard and Milhaud (1952) and found to be a type intermediate between amylose and amylopectin. These results confirm, at least for these species, the correctness of the staining reactions by means of which many intracellular granules have been identified as being starches or glycogen.

(ii) Bacterial heteropolysaccharides

The polysaccharides of this group are characterised by giving rise to more than one component sugar on hydrolysis. The majority of polysaccharides from animal pathogens that have been examined belong to this group. The heteropolysaccharides exhibit marked antigenicity and immunological specificity, in contrast to the much lower activity of the homopolysaccharides. Many of them are highly complex for which reason only a few of them have been studied in detail; with two exceptions (B.pyocyaneus and H.pertussis) which may have arisen through incomplete analysis, all bacterial heteropolysaccharides have been found to contain uronic acid or hexosamine components, and sometimes both together.

The pneumococcus polysaccharides have been the most thoroughly investigated of bacterial heteropolysaccharides. The most complex bacterial polysaccharides appear to be those of Mycobacterium tuberculosis, from which organisms a large number have been isolated and partially characterised. They are all highly complex in structure and composition and are some of the few natural products known to contain the rare sugar D-arabinose (Haworth, Kent and Stacey, 1948). The tubercle polysaccharides have been reviewed by Stacey and Kent (1948), and will not be included in the table of bacterial heteropolysaccharides. In table 2 a list is given of the bacterial heteropolysaccharides that have been at least partially characterised.

Table 2: Bacterial Heteropolysaccharides

Organism	Component sugars	References
Azotobacter chroococcum	D-galactose	Lawson & Stacey (1950)
	D-glucose	" " (1954)
	D-glucuronic acid (4%) unidentified component	
Bacillus alvei	glucose, uronic acid	Forsyth & Webley (1949)
B.anthracis	D-galactose, N-acetyl-D-glucosamine (1:1)	Ivanovics (1940)
	D-galactose (43%) D-glucosamine (37%) acetyl (15%)	Smith & Zwartouw (1954)
B.brevis	glucose, uronic acid	Forsyth & Webley (1949)
B.circulans		
(ATCC type 294)	glucose, mannose, uronic acid	" "
(ATCC type 295)	glucose, mannose, uronic acid, xylose	" "
B.circulans-macerans	glucose, mannose, uronic acid	" "
B.krzemieniewski	glucose, mannose uronic acid (ratio 3:2:2)	" "
B.megatherium	glucose, uronic acid	Aubert (1949)
B.pyocyaneus	glucose, fructose unidentified component	Akiya, Takahashi Kuriyama & Ogawa (1952)
Clostridium bifermantans	glucose, hexosamine, uronic acid	Tardieux & Nisman (1952)
Cl.sordelli	"	" "

Table 2: Bacterial Heteropolysaccharides(contd.).

Organism	Component sugars	References
Diplococcus pneumoniae type I	D-galacturonic acid(28%) hexosamine, acetic acid	Heidelberger, Goebel & Avery (1925a)
II	D-glucose, D-glucuronic acid, L-rhamnose,(ratio 2:2:8)	Kent (1952)
III	D-glucose, D-glucuronic acid (ratio 1:1)	Goebel (1935)
IV	D-glucose, N-acetyl-hexosamine	Heidelberger & Kendall (1931)
VIII	D-glucose, D-glucuronic acid (ratio 7:2)	Goebel (1935)
XIV	D-galactose, N-acetyl-hexosamine	Goebel, Beeson & Hoagland (1939)
"C" polysaccharide	hexosamine, acetic acid phosphoric acid	Goebel, Shedlovsky, Lavin & Adams (1943)
Escherichia coli type "Kroger O-8"	rhamnose (39%), xylose (16%) glucose (10%),galactose (3%) N-acetyl-hexosamine(7%)	Luderitz & Westphal (1952)
type"Kauffman O-18"	galactose,glucose,rhamnose (hexosamine?)	Westphal, Luderitz, Fromme & Joseph (1953)
para colon intermediate type	D-glucose(55%), D-glucosamine(17%) galactose	Ikawa, Koepfli, Mudd & Niemann (1952)
Haemophilus pertussis	galactose, ketose,mannose	Akiya, Takahashi, Kuriyama & Ogawa (1951
Klebsiella pneumonia type A	D-glucose, D-glucuronic acid.	Goebel (1927) Goebel & Avery (1927)
B	D-glucose, aldobiuronic acid	Heidelberger, Goebel & Avery (1925b)
C	" "	Goebel & Avery (1927)
Lactobacillus bifidus	galactose, glucose,fucose uronic acid, unidentified component	Norris, de Sipin, Zilliken, Harvey & Gyorgy (1954)

Table 2: Bacterial Heteropolysaccharides (Contd.)

Organism	Component sugars	References
Rhizobium radicicolum	D-glucose, D-glucuronic acid (ratio 2:1)	Schluchterer & Stacey (1945)
Salmonella abortus -equi	galactose, glucose, mannose, rhamnose, abequose (hexosamine?)	Westphal, Luderitz, Fromme & Joseph (1953)
Salmonella enterititis	glucose(44%), galactose(8%) mannose(19%), hexosamine(5½%)	Elkin (1951)
Salm. enterititis-Gärtner	galactose, glucose, mannose, rhamnose, tyvelose (hexosamine?)	Westphal, Luderitz, Fromme & Joseph (1953)
Salm. gallinarum	glucose(37%), galactose(15%) mannose(23%), hexosamine(8%)	Elkin (1951)
Salm. typhi	glucose(33%), galactose(21%) mannose(23%), hexosamine(6%)	" "
strain O-901	glucose(40%), galactose(17%), mannose(21%), hexosamine(0-1.5%) rhamnose(20%)	Pon & Staub (1952)
strain O-901	galactose, glucose, mannose, rhamnose, tyvelose (hexosamine?)	Westphal, Luderitz, Fromme & Joseph (1953)
Salm. paratyphi Strain Kroger B	galactose, glucose, mannose, rhamnose, abequose (hexosamine?)	" "
Shigella dysenteriae	D-galactose, L-rhamnose N-acetyl-D-glucosamine	Partridge (1948a)
Sh. flexnerii	" "	Slein & Schnell (1953)
Staphylococcus aureus	D-glucosamine, pentose	Fellowes & Routh (1944)
Streptococcus sp.	galactose, rhamnose(18%) uronic acid (22%), glucose	Hobson & Macpherson (1954)
Strept. pyogenes group A	D-glucuronic acid, N-acetyl-D-glucosamine (ratio 1:1)	Kendall, Heidelberger & Dawson (1937)
	rhamnose, glucosamine (ratio 5:2)	Schmidt (1952)
group C	D-glucuronic acid, N.acetyl-D-glucosamine (ratio 1:1)	Kendall, Heidelberger & Dawson (1937)
Vibrio cholerae type I	galactose, glucuronic acid	Linton & Mitra (1934)
II	galactose, glucuronic acid arabinose	" " "
III	galactose, glucuronic acid arabinose	" " "

Influence of carbon source on polysaccharide composition.

Little is known of the influence of carbon substrate on the composition of bacterial heteropolysaccharides. It is a commonly observed fact that apart from type-transformations and antigenic variations of the smooth-rough type the immunological specificity of organisms remains constant, regardless of the medium on which they are cultured. This would suggest that the composition of the polysaccharide antigens remain unchanged, but immunological methods would not detect small changes in the relative proportions of the components and such methods are open to the objection that the apparent identity may be the result of cross-reactions between polysaccharides in which the pattern of the immunologically dominant groups are unchanged, while the rest of the molecules may be different.

Levan- and dextran-producing species require specific sugar substrates for polysaccharide production (see table 1), in the absence of which no levan or dextran is produced. With these organisms the nature of substrate merely controls the presence or absence of the polysaccharide. The carbon substrate also appears to have no influence on the composition of bacterial cellulose; Barclay, Bourne, Stacey and Webb (1954) examined the cellulose synthesised by Aceto.acetigenum cells from mannose and found it to contain only glucose.

Interesting results have been obtained with species that produce two exopolysaccharides. Hestrin, Avineri-Shapiro and Aschner (1943) found that B.polymyxa

produced a levan when grown on sucrose medium, and an unidentified polysaccharide, not a levan, when grown on lactose in place of sucrose. Similar results were reported for other strains of B.polymyxa and for B.megatherium by Forsyth and Webley (1949), who also examined other species of the genus Bacillus but found that they produced no levans, only polyuronides the component sugars of which were unaffected by the carbon substrate (sucrose, D-fructose, D-glucose, D-galactose and L-arabinose - used singly) in the medium. Forsyth and Webley realised that the unusual behaviour of B.polymyxa and B.megatherium was the result of these organisms being able to produce two different polysaccharides each, one being a levan and the other a non-fructose-containing polyuronide (see table 2). It was found that when grown on monosaccharide-containing media both of these organisms produced only their polyuronide exopolysaccharides; the complete absence of levan was not unexpected since levan production in the absence of sucrose or raffinose has never been reported. When B.polymyxa and B.megatherium were grown on sucrose media both the polyuronides and levans were produced, the relative amounts varying according to the cultural conditions. Forsyth and Webley found that levan production was optimal when the sucrose and phosphate concentrations in the medium were high. This finding is explicable in the light of present knowledge of polysaccharide synthesis. It is very likely that the conditions found by Duguid and Wilkinson

(1953) to favour optimal exopolysaccharide production by the coliform group, i.e. limitation of growth by exhaustion of nitrogen, phosphorus or sulphur in the medium in the presence of relative excess of sugar, may also apply to polyuronide production by B.polymyxa and B.megatherium. Thus the conditions reported to favour optimal levan production by these species, high sugar and phosphorus concentrations, would be unfavourable for polyuronide production. On the other hand, levan synthesis from sucrose is an exergonic process (Hehre, 1951) and does not compete for metabolically-produced energy, thus it would proceed independantly of growth. In these circumstances levan production would predominate not because it is enhanced, but because polyuronide production is selectively diminished.

The immunological properties of bacterial polysaccharides

Bacterial polysaccharides are multiple antigens; each cell contains numbers of antigenic substances which are postulated to occur at various levels about the surface. The outermost antigen is believed to dominate the antigenic pattern and to be responsible for the immunological specificity of the cell. As the outermost structures of most cells are of polysaccharide nature the immunological specificities of most species of organisms have been found to be determined by their exopolysaccharides, either in the form of slime and capsule ("K" antigens) or as somatic "O" antigens. For this reason these polysaccharides are often

referred to as specific or immuno- polysaccharides.

From examination of the structure of polysaccharides isolated from species of known immunological relationship it has been established that they exert a structural specificity. This was confirmed by experiments with "synthetic" antigens containing simple sugar and other haptens attached to proteins. It was observed that polar groups play a particularly important part in determining specificity; this explains the marked immunological specificity and antigenicity of the bacterial heteropolysaccharides, all of which contain polar groups, either in the form of uronic acid or hexosamine, or both together. It also explains the comparatively low specificity of the homopolysaccharides, which do not contain polar groups, except the polyaminouronide present in the Vi antigen of E.coli strain 5936/38, which possesses a high degree of specificity and antigenic activity (Webster, Landy and Freeman 1952). The large number of immunologically-distinct types that exist in some species were found to arise as a result of the variety of different polysaccharide antigens produced by these organisms. At present 75 immunological types of pneumococcus are known (Raffel 1953) indicating that this organism is capable of producing at least this number of different polysaccharides.

An important consequence of immunological specificity being determined by polysaccharide structure is that immunological cross-reactions occur when micro-organisms produce antigenic polysaccharides of similar composition.

For cross-reactions to occur it is not necessary for the polysaccharides to be identical, only that they possess similar molecular surfaces and configurations, especially of their polar groups. The following are a few of the very large number of cross-reactions known, given to illustrate the widely different species that can give polysaccharides capable of cross-reaction. In each instance there is a higher degree of reactivity of antibody with its homologous substance, probably based on differences in the spacing of the groups.

Cross-reacting organisms	References
Kl.pneumoniae and pneumococcus type II	Avery, Heidelberger & Goebel (1925).
B.anthracis, pneumococcus type XIV (and blood group A specific substance)	Ivanovics (1940_b)
Azoto.chroococcum, R.radicicolum and pneumococcus types III and VIII	Lawson and Stacey (1950)
Streptococcus group H, L.mesentericus pneumococcus types II and XX	Neill, Sugg, Hehre & Jaffe (1941)

Little is known regarding the relationship of capsules to the capsular antigens as they occur in vivo; but since antigenic function depends, inter alia, on molecular size and shape, and since bacterial polysaccharides cover the whole range of sizes, from giant molecules with weights of the order of millions, to comparatively small molecules, it would not be unexpected if the composition of antigens varied, depending on the size and molecular weight of the exopolysaccharides produced by the organism. In species with very large molecules the polysaccharide alone might be the complete antigen

(e.g. polysaccharides of pneumococcus type I (Avery & Goebel, 1933) and type III (Heidelberger, MacLeod, Markowitz & diLapi, 1951), while in other species with polysaccharides of smaller molecular weight the antigens may consist of these polysaccharides attached to proteins and other substances (e.g. the O-antigen of Sh.dysenteriae which was found to be a polysaccharide-protein-phospholipid complex (Morgan and Partridge, 1941).

Antigenic changes

(i) S-R variation. Because bacterial cells normally contain many antigens, it is possible for cells to change their immunological specificity by the loss or gain of surface antigens. These antigenic changes are often related to morphological and pathogenic changes, the commonest manifestation of these changes being in colony appearance.

The early work was done with Salmonella and Shigella species in which it was found that parent smooth colonies, with full antigenic make-up, gave rise to daughter rough colonies, the cells of which had lost their outermost, dominating O-antigens. (Arkwright, 1920; 1921). Similar changes were observed in many other species, including Friedländer's bacillus (Julianelle, 1928) and the pneumococcus (Griffith, 1923; Reimann 1925, 1927). Dawson (1934) showed that what had been described as smooth-rough (S → R) variations for these capsulate organisms were in fact mucoid-smooth (M → S) changes, and that true (S → R) variations took place as a second step, each step being accompanied by successive loss of antigen.

Since then, it has been found that many other capsulate species (e.g.haemolytic streptocci (Dawson, Hobby and Olmstead, 1938)) exist in three main phases (M, S, R) each with different antigenic specificity and colonial morphology. Variations from (M → S) are accompanied by loss of capsules, and in some species (e.g. pneumococcus) with loss of virulence and type specificity; (S → R) variations are not detectable in the microscopic appearance of the cells. Organisms which have virulent S phases (e.g. Salmonella and Shigella species) undergo loss of virulence as well as antigenic and colony changes in (S → R) variations.

In most cases the cells with complete antigenic structure form mucoid or smooth colonies; loss of antigens is accompanied by increased roughness of the colonies. The change in colony appearance is not always in the same direction for all species. Some are known, e.g., the anthrax bacillus, which have rough colonies when possessing complete antigenic structure, and smooth colonies when the surface antigens are lost. Obviously also the important influences of cultural conditions on colony appearance cannot be overlooked. Thus the designations "smooth" and "rough" cannot be applied in a general sense to describe antigenic condition; immunological tests are the only reliable guide to antigenic structure. To reduce the prevailing confusion in nomenclature of antigenic condition, it has been suggested that the letters R and S be used as symbols of the incomplete and complete antigenic variants without

referring to colony appearance, thus making it possible for example to refer to rough colonies of S-forms.(Topley and Wilson, 1946). Unfortunately much confusion remains because many authors still use the S → R notation for what are in reality M → S changes.

Generally, S-forms are type specific (there are exceptions, with type specificity only in the M-phase) while the corresponding R-forms are species or group specific. The circumstances in which M → S and S → R dissociations may occur are often simply by continued culture in laboratory media, when changes may occur spontaneously; but the processes may be deliberately induced by incorporating either antibodies prepared against the appropriate M - or S-phase, or by including a bacteriophage prepared against the M- or S- antigen, in the culture medium,. These conditions provide an environment selectively favourable to the S- or R-form. The reverse R → S and S→ M variations may also occur spontaneously but much less frequently than the changes in the M → S → R direction. These reverse processes can be induced likewise, either by animal passage, or by culture on media containing anti-R or anti-S antibodies.

(ii) <u>Type transformation</u> (Note:- The notation in general use for describing the antigenic phases of the pneumococcus differs from the M, S, R system proposed by Dawson (1934). The capsulated phase with mucoid colonies is described as smooth (S), the non-capsulate phase with smooth colonies is described as rough (R), and the non-capsulate phase with rough colonies as extremely rough (ER).)

In the S - R dissociations the various forms retain their antigenic heritage; when a given S strain is converted to its corresponding R form and then is changed back to S, it is found to be identical with the original strain. In the normal course of events then, the antigenicity of the S form is stable and can be regained after S → R → S dissociations.

Another type of transformation is known, called type transformation, in which the final S form can be obtained antigenically different from the original S phase, when passed through the S → R → S route. This phenomenon was first observed by Griffith (1928) who injected mice with a living, avirulent and non-capsulated R strain of pneumococcus type II together with a heat-killed capsulate S strain of pneumococcus type III. He found that the mice had succumbed to the infection and yielded type III S cells identical with the original type III S; Griffith correctly concluded that the living R. cells had been transformed by material from the killed S cells into the same S type as had been inoculated into the mice. Furthermore the new cells could be killed and used to provide fresh transformations.

These observations were confirmed by other groups of workers and soon it was found that transformations could be induced in vitro, by growing non-capsulated variants of one type of pneumococcus on medium containing a suspension of heat-killed pneumococci of another type. If purified capsular polysaccharide was used in place of the suspension, transformation did not take place.(Dawson

and Sia 1931). The nature of the active transforming principle has been elucidated by the work of Avery and his co-workers, who isolated it in a pure state from pneumococcus type III in a form so highly active that it would act even at dilutions of the order of 1:600,000,000. It was found to be a highly polymerised form of deoxyribose-nucleic acid (DNA). (Avery, Macleod and McCarty 1944, McCarty 1946). The substance is visualised as reacting with R cells to initiate a series of reactions culminating in the synthesis of type III exopolysaccharide. Once the transformation has occurred it is permanent, and both the capsular material and the specific DNA are reproduced in successive generations. Similar specific transforming principles have been isolated from type II and VI pneumococci (McCarty and Avery 1946).

Type transformation is not a phenomenon restricted to the pneumococcus. Similar transformations have also been achieved with several other organisms - E.coli (Boivin, Delaunay, Vendrely and Lehoult 1945, 1946; Boivin 1947), Sh.paradysenteriae (Weil and Binder,1947) and H.influenzae (Alexander and Leidy, 1950; 1951). The transforming principles of E.coli and H.influenzae have been found to be deoxyribonucleic acid, but analyses on the principles of these species are not as advanced as in the pneumococcus. Type transformations with all four species only occur with certain "reactive" strains, and all transformations are carried out in complex environments. Little is known of the causes underlying susceptibility to transformation, or of the

factors involved in the enviromnental conditions required. (See the review by Austrian, 1952).

The synthesis of bacterial polysaccharides

The mechanisms of polysaccharide synthesis so far known relate only to the homopolysaccharides; nothing is yet known of the synthesis of heteropolysaccharides. Bacteria and bacterial enzyme preparations have been a fruitful source of study yielding much valuable information concerning the pathways of homopolysaccharide synthesis.

Although this aspect of polysaccharide studies is only 15 years old, beginning with the investigations into the reversibility of phosphorylases, a large volume of work has been done, and from the results important general principles, governing all the known synthetic routes, have emerged.

(1) All the known instances of polysaccharide synthesis appear to be energetically self-sufficient, not requiring any external source of energy. That is, they all start from substrates which contain the structural unit of the polysaccharide at a higher energy level than that at which it occurs in the polysaccharide. The enzyme responsible for polysaccharide synthesis cannot directly utilise unsubstituted monosaccharides; they all require substrates with either preformed glycosidic bonds or ester-linked phosphate bonds, both of which types of compounds contain the sugar units in reactive high energy states. In practice these substrates are sucrose, maltose, dextrins and glucose-1-phosphate.

Unless provided with these substrates preformed the organism must naturally synthesise them from the carbon sources available, before polysaccharide synthesis can begin.

(ii) All the polysaccharide-synthesising enzymes can be regarded as transferring enzymes. At first this concept of transferring enzymes was evolved to explain dextran and levan synthesis, but the theory has been expanded to include all polysaccharides. (Hestrin 1953). Now it would appear that all enzymes involved in polysaccharide reactions may be regarded as transferring enzymes, transferring glycosyl units from suitable donors (see above) to suitable acceptors, the function of the enzyme being determined by the nature of the acceptor. If the enzyme transfers glycosyl units to a saccharide it is a synthetic enzyme, if to phosphate it is phosphorolytic and if to water it is hydrolytic. Such enzymes are known as, specific transglycosidases, phosphorylases and hydrolases respectively.

(iii). All polysaccharide syntheses proceed by repetition of a unit step, and all syntheses may be expressed by the same fundamental equation:

$$\text{R-glycoside} + \text{S} \rightleftharpoons \text{S-glycoside} + \text{R}$$

where R- and S-glycoside are donors of high energy glycosyl units and R and S are the acceptors. The unit synthetic reaction is merely the transfer of glycosidically or sugar-phosphate-bound anhydro-sugar units from one compound to another, but the repetition of this unit reaction will result in the formation of a polysaccharide.

The transfer is carried out in such a manner that the sugar unit is attached to the non-reducing end of the growing saccharide chain.

Theoretically all the synthetic reations must be regarded as reversible, but in practice most of the transglycosidic reactions are almost irreversible, being reversed only in forced conditions in vitro, in circumstances unlikely to be met with in nature. The degree of reversibility depends on the thermodynamic considerations the most important of which is the free energy change involved in the reaction. The law of mass action, and pH sometimes intervene to affect reversibility.

The various synthetic pathways known at present are briefly appended here, classified according to the enzyme responsible for the synthesis.

Phosphorylases

Starch-glycogen phosphorylase,.

This pathway of polysaccharide synthesis was the first to be found, following the discovery by the Cori group of the phosphorolytic route of starch and glycogen breakdown involving phosphorylase and glucose-1-phosphate. It was found that, unlike hydrolytic breakdown of polysaccharides, phosphorolysis was reversible; by functioning in reverse, starch-glycogen phosphorylase synthesised starch (actually the amylose fraction) from glucose-1-phosphate. The reversible reaction can be expressed:

$$\text{Starch} + n(HPO_4)^{--} \rightleftharpoons n \text{ glucose-1-phosphate}$$

It was found that pure phosphorylase and glucose-1-phosphate would not react; a small amount of starch, glycogen or dextrin was found necessary as a "priming" agent, to act as glucose acceptor, because the enzyme acts not as a condensing agent but as a transferring agent, and requires a preformed saccharide chain to which to add.

The product formed by the repetition of the unit reaction is a long unbranched molecule, and is believed to be what occurs when starch-glycogen phosphorylase is the only enzyme present. But in the presence of an accessory "branching factor" a branched polysaccharide is obtained. Much less is known of this branching factor but it is believed to be different from potato "Q" enzyme in having no effect on preformed amylose.

Nearly all the work on the synthesis of starch-like polysaccharides by phosphorylase action was done with plant and animal material, but this pathway appears to operate in some bacteria. Hehre and his co-workers found that C.diphtheriae, N.perflava and some streptococci are able to form starch-like polysaccharides, in vivo, form glucose-1-phosphate, but not from unphosphorylated sugars. (Carlson & Hehre 1949; Hehre, Hamilton and Carlson 1949).

Transglycosidases

With the exception of phosphorylase, all the mechanisms of polysaccharide synthesis involve transglycosidases, most of which are found in microorganisms. Only one enzyme is known, sucrose phosphorylase first found in

Pseudomonas saccharophila, which can function both as a phosphorylase and a transglycosidase. As it is concerned with the synthesis of disaccharides and not polysaccharides, it will be omitted from this discussion.

(i) Dextransucrase. This enzyme converts sucrose into dextran by polymerative degradation. The reaction can be represented by the following equation:

$$\underset{\text{sucrose}}{nC_{12}H_{22}O_{11}} \longrightarrow \underset{\text{dextran}}{(C_6H_{10}O_5)_n} + \underset{\text{fructose}}{nC_6H_{10}O_6}$$

The enzyme is highly specific and will react only with sucrose, and slightly with raffinose. It has no action on glucose, fructose or any other sugar tested. The reaction appears to be irreversible, and at present it is unknown whether a primer is needed to initiate the reaction. All the enzyme preparations so far studied have been contaminated with dextran. Hehre (1951) has provided results to indicate that dextran may actually be contained as an integral part of the dextransucrase molecule. If is possible that sucrose itself may also act as a receptor to initiate dextran synthesis.(Stacey 1954).

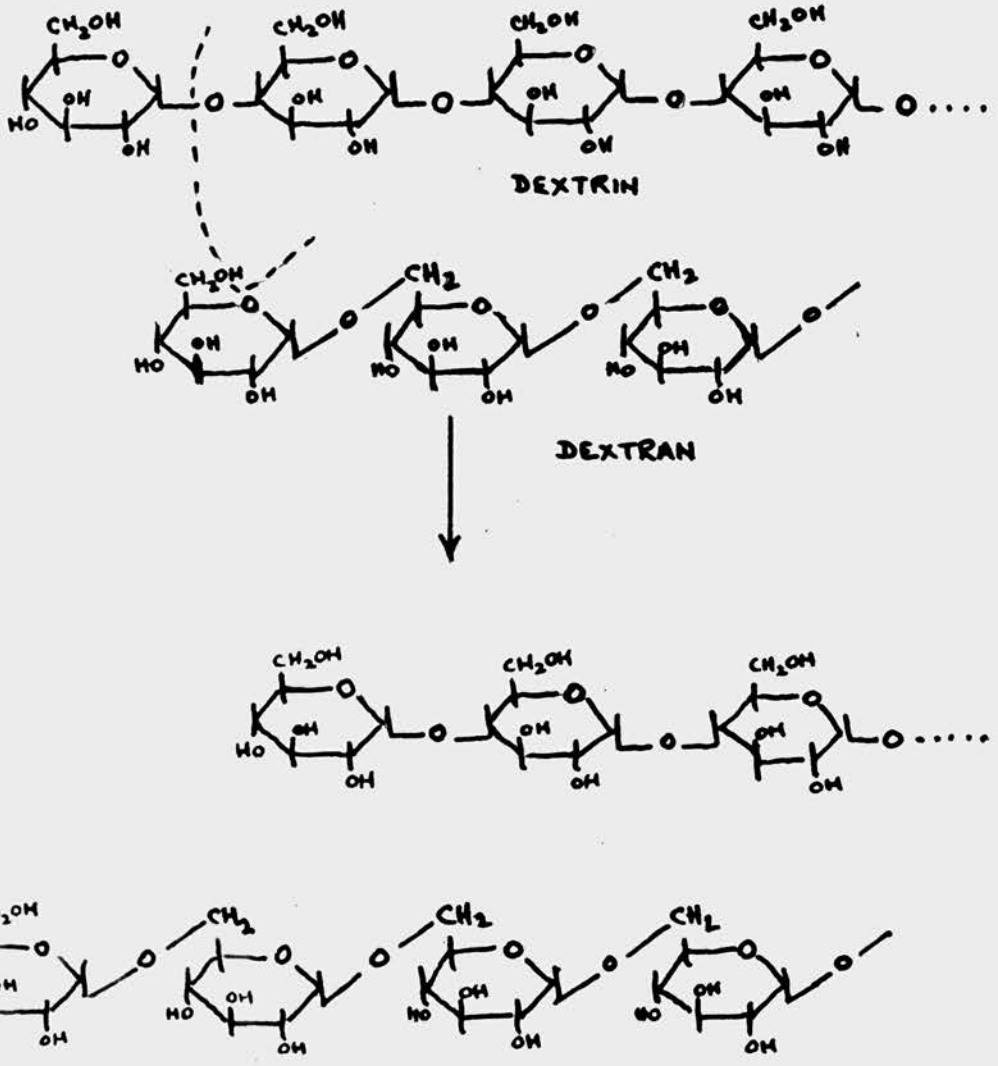
It is known that different bacteria produce dextrans that differ in structural detail, with varying degree of branching. As each enzyme is believed to form only one type of linkage these structural differences suggest the participation of other enzymes. At present nothing is known of a dextran branching factor, and unfortunately dextrans synthesised in vitro have not been subjected to structural studies. Barker and Bourne (1953) suggest

that the branched polysaccharide is formed directly from the unbranched one by the action of a second enzyme, as is the case with amylose and Q enzyme.

The production of dextransucrase seems to be restricted to one Family of bacteria, the Lactobacteriaceae, to which the dextran-producing species belong: L.mesenteroides (Betacoccus arabinosaceous), L.dextranicum, Betabacterium vermiforme, Streptobacterium dextranicum and various non-haemolytic streptococci, e.g.,Streptococcus group H, Strept.salivarius, Strept.bovis and Strept. viridans. (Hehre 1951).

(ii) Dextrandextrinase. This enzyme also synthesises dextran but differs from dextransucrase in requiring dextrin as its specific substrate. The dextran produced closely resembles that of L.mesenteroides in structure (Barker, Bourne,Bruce and Stacey) and immunological properties (Hehre 1951). The enzyme cannot utilise glucose, fructose, sucrose, maltose, or raffinose, nor amylose, amylopectin or glycogen. It appears to require open-chain dextrins containing 4-10 glucose residues, such as are obtained by α-amylase or acid hydrolysis of starch; dextrandextrinase has no action on cyclic dextrins.

The reaction mechanism is believed to be the transfer of glucose units (linked 1:4-α) from non-reducing terminal positions in amylodextrin to corresponding positions (linked 1:6-α) in the growing dextran molecule. (Hehre and Hamilton 1951). This reaction may be represented as follows:-



No information is available regarding the need for primers.

The transformation of dextrin into dextran by this enzyme is the first example of the biological conversion of one polymer into another by complete rearrangement of all the units. Dextrandextrinase is found only in certain species of the acetic acid bacteria, Acetobacter viscosum, A.capsulatum and A.acidum-mucosum. (Hehre 1951).

(iii) Levansucrase. This enzyme synthesises levans from sucrose and raffinose by polymerative degradation. It is highly specific and does not act on any other sugars. The enzyme converts sucrose into levan while releasing free glucose; with raffinose as substrate levan is produced while melibiose is released. The reactions being respectively:

n sucrose $\longrightarrow$ levan + n glucose

n raffinose $\longrightarrow$ levan + n melibiose

However, fructose is often found free after levansucrase action. This is generally interpreted as the result of the presence of hydrolytic enzymes in levansucrase preparations. Recent work (Hestrin 1953) indicates that levan producing organisms, with the exception of Aerobacter levanicum possess enzymes, levanpolyase and levanoligase, which hydrolyse levans to levan oligosaccharides and fructose respectively, so that the presence of fructose does not necessarily indicate sucrase action. Another possible explanation, but one which has no supporting evidence except analogy to invertase (see Bacon and Edelman, 1950; Blanchard and Albon, 1950; Fischer, Kohtes and Fellig, 1951,*) is that levansucrase may transfer fructose residues to water as well as to saccharide acceptors.

The nature of the fructose acceptor at the start of the reaction has not been established, and it is not known whether a primer is required. It

* Bealing and Bacon(1951); Edelman and Bacon(1951); Pazur(1952); Edelman(1954))

is possible that fructose and sucrose act as initial acceptors, giving di- and trisaccharides respectively, and that as levan synthesis continues the levan chain acts as acceptor. If sucrose acts as an initial acceptor the levan molecule would be expected to be terminated by a non-reducing glucose unit linked as in sucrose to the penultimate fractose residue. That this is the case has been found in one instance: the levan of B. subtilis has been shown to contain a trace of glucose which is believed to be part of the levan molecule, and not to arise from impurity (Palmer 1951).

Levansucrase has been shown to be a reversible enzyme. When incubated with glucose in the presence of invertase to destroy any sucrose as it is formed, and thus force the reaction leftward, levan is broken down by levansucrase.

Levansucrase is a widespread enzyme and exists in the many microorganisms known to synthesise levan from sucrose. (See table 1).

(iv) Amylosucrase. This enzyme converts sucrose into fructose and an amylopectin type polysaccharide. It is believed to act in a manner analogous to dextransucrase, the reaction being represented by

$$n\,C_{12}H_{22}O_{11} \longrightarrow (C_6H_{10}O_5)_n + C_6H_{12}O_6$$

sucrose — amylopectin — fructose

The enzyme is highly specific for sucrose, and was shown not to react with maltose, lactose, trehalose,

melibiose, raffinose, melizitose or methyl α-D-glucoside. It is not known whether a primer is needed to initiate the reaction. The polysaccharide produced by amylosucrase in vivo has been shown by Barker, Bourne and Stacey (1950) to be a member of the glycogen-amylopectin group, with a highly branched molecule, and evidence is now available that a branching enzyme, similar to Q enzyme is associated with amylosucrase.

Amylosucrase has been shown to be difficultly reversible. The forward reaction is accompanied by 98% conversion to polysaccharide, but it has been shown that when amylosucrase is incubated with starch or glycogen and dextransucrase, amylosucrase appears to convert the starch to sucrose, detected by the appearance of small amounts of dextran(Hehre and Hamilton 1949).

The presence of amylosucrase has only been shown with certainty in Neisseria perflava (Hehre and Hamilton 1948). It has been suggested this enzyme may be responsible for the synthesis of the amylopectin type polysaccharide by Cl. butyricum, but this is not proven. N. perflava is a remarkable organism, in that it has been shown that it can produce amylopolysaccharide from glucose-1-phosphate as well as sucrose. This was found to be caused by a phosphorylase and not the amylosucrase which is independent of phosphorylation. This organism

thus possesses two metabolic pathways for the synthesis of its amylopolysaccharide, one starting from glucose-1-phosphate and not acting on sucrose, and the other starting from sucrose and not having any action on glucose-1-phosphate.

(v) Amylomaltase. This enzyme converts maltose into amylose-type polysaccharides. It is specific for maltose and has no action on glucose-1-phosphate, lactose, melibiose, cellobiose, α- or β-methyl glycoside. The reaction is represented as follows:

$$n \text{ maltose} \rightleftharpoons (\text{glucose})_n + n \text{ glucose}$$

The product has not been subjected to structural studies. When no steps are taken to remove the free glucose formed the polymeric product consist chiefly of short dextrins of 4 - 6 glucose units length; but when notatin (glucose oxidase) is added to remove the glucose as it is formed the product is a much larger molecule, with starch-like behaviour, e.g., staining blue with iodine.

The most remarkable feature of this enzyme is its reversibility. In the absence of glucose oxidase the conversion of maltose to polysaccharide stops when the reaction is only two-thirds completed. Barker and Bourne(1952) fractionated the products formed from maltose by Monod's strain of E. coli and found that of the glucose residues initially present in the maltose, 29% appeared as glucose, 24% as unchanged maltose and 35% as higher saccharides.

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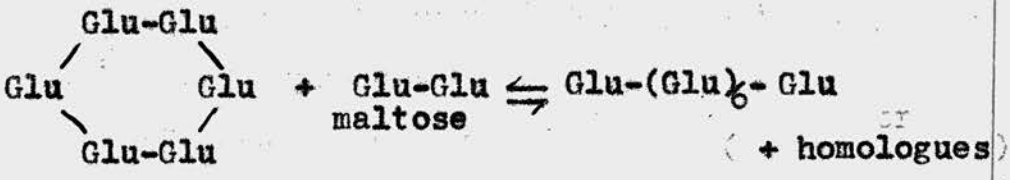
In the presence of glucose oxidase the reaction goes to completion.

Amylomaltase has been found only in special variants of B. coli (Monod and Torriani 1948, Torriani and Monod 1949, Duodoroff, Hassid, Putnam, Potter and Lederberg 1949).

(vi) Bacillus macerans amylase. This is an enzyme present in B. macerans capable of acting reversibly, either converting starch into non-reducing crystalline dextrins (Schardinger dextrins), or by acting on these dextrins to convert them into longer oligosaccharides. The Schardinger dextrins are cyclic dextrins of 6,7 or 8 units. It has been shown that in the presence of glucose, maltose, cellobiose, sucrose or maltobionic acid the enzyme opens the dextrin rings and attaches whatever sugar is present to form a larger oligosaccharide. In the presence of maltose, for example, the reaction would be



cyclic dextrin

(glu=glucose)

This enzyme differs from the preceding ones in several respects. First, it is less specific towards its acceptors, being able to use a variety of sugars, Secondly it apparently transfers in one step a whole chain of glucose units (if the cyclic dextrin is regarded as the donor). Thirdly

is the absence of a by-product caused by the scission of a cyclic rather than terminated substrates. Lastly, and most important, is the fact that B. macerans amylase action in reverse has not been found to lead to polysaccharide synthesis. Unless evidence is brought forward for polysaccharide synthesis, this reaction must be regarded as only a potential pathway of polysaccharide synthesis.

This enzyme has been shown capable of reacting with smaller, non-cyclic saccharides, as part of a more general reaction. Norberg and French(1950) found B. macerans amylase to catalyse a re-distribution of glucose residues in linear amylopolysaccharides. There is a possibility that future work may show close resemblances between B. macerans amylase and amylomaltase.

Hehre (1951) has summerised the present knowledge of polysaccharide synthesis in a concise manner - "it is now evident that multiple and alternative enzymic pathways exist for the synthesis of polysaccharides of a particular type (amylopolysaccharides, dextrans), that important carbohydrates (sucrose, glucose-1-phosphate, maltose, amylopolysaccharides, dextrans) occupy a definite relationship to one another on a scale of (decreasing) glucoside bond energy or condensing power, and that structurally, these carbohydrates possess in common a transferable terminal α-D-glucopyransido radical (the polysaccharides

having in addition, many other α-D-glucopyranose residues.)".

Origin of bacterial capsule and slime

The origin, or physiology of formation, of bacterial capsules and slime is still uncertain. It was a commonly held opinion, at one time, that these structures were formed by cell-wall swelling, and that they were therefore firmly-attached integral parts of the cells by which they were produced. This view was opposed by many authors who believed that capsules and slime were products of secretion (Meyer 1912; Zettnow, 1918). At the present time the latter hypothesis is very widely accepted as being correct (see Dubos, 1945, Knaysi 1951, Lamanna and Mallette, 1953). Capsule and slime are visualised as being built up by accumulation of exopolysaccharide or other extra-cellular polymeric material (protein) around the cell.

The evidence now available, although strongly in favour of the secretion hypothesis, is of indirect rather than direct nature. The view that capsules were the result of cell-wall swelling or thickening was shown to be untenable when specific depolymerising enzymes were found which could remove capsules without impairing the viability of the cells; when the decapsulated cells were transferred to fresh media they formed new capsules rapidly (e.g. see Bernheimer, 1953)

showing that the action of the enzyme was only to destroy the capsule and not to affect any of the cellular functions, Such decapsulating enzymes have been found for haemolytic streptococci and some pneumococcus types, the best known being the one specific for pneumococcus type III (Dubos and Avery, 1931). Further evidence against the concept that capsules were caused by cell-wall thickening was provided by the discovery of exopolysaccharide synthesis by cell-free extracts, first demonstrated with cell-free preparations of L. mesenteroides (Hehre and Sugg, 1942). It was thus shown that cell walls are not essential for the production of exopolysaccharides.

The question next arises as to the site of synthesis of slime and capsular polysaccharide material. As it is difficult to visualise how a polysaccharide can pass through the cytoplasmic membrane, it is postulated that exopolysaccharides are synthesised at or beyond the cell-wall (Stacey, 1949). In some homopolysaccharide producing species (e.g. L. mesenteroides) extracellular enzymes also participate in exopolysaccharide synthesis; Stacey (1949) believes that the dextransucrase of L. mesenteroides is part of the cell-wall, being attached to it by relatively labile linkages which permit some of the enzyme to pass into the medium.

It is postulated that the secreted or cell-

surface-synthesised polysaccharides accumulate around the cell-walls and thus form capsules, and that when capsular material passes into solution it becomes slime. There are different views concerning the accumulation of exopolysaccharides to form discrete capsules. According to one viewpoint it is merely a matter of low solubility and high viscosity and that the rate of synthesis being greater than the rate of solution of the polysaccharide, a capsule is formed; according to this hypothesis the capsule is constantly passing into solution to become slime. A difficulty arising from this simple explanation of the origin of slime and capsule is the observation frequently made, that capsules remain firm and discrete even when cells are washed vigorously or left in liquid medium for long periods, conditions which would be expected to cause the capsules to dissolve if it were only a case of rate of passing into solution.

Another hypothesis to account for capsule formation is one which may shortly be proved to be correct. It is possible that capsules are not structureless layers, but that they are given shape and rigidity by a network or framework. There is evidence for the existence of such frameworks in the capsules of several species. It is reported that a mucopolysaccharide basket-like network is present in pneumococcus capsules (Stacey, 1949); polypeptide

fibrils have been observed emerging from the surface of B. megatherium, the interseptal spaces of which are filled with polysaccharide material (Ivanovics and Horvath, 1953). Tomcsik (1951) has reported evidence of a framework of polysaccharide in the capsule of Bacillus M in which the interstices are filled with polypeptide. It may be found that in general, capsules are formed by the filling of interstitial spaces of the structural frameworks that are postulated to be present in the capsule-forming species. As the exopolysaccharide is synthesised it fills the framework and diffuses outwards through it; once beyond the framework it becomes amorphous slime. This hypothesis is in keeping with the observation that the main production of slime polysaccharide occurs later than the main production of capsular polysaccharide (Duguid and Wilkinson, 1953). On the basis of this hypothesis, dissociation of capsular into purely slime-forming variants could be explained simply by the loss of the ability to form the framework. It is possible that the problem of capsule formation may be finally elucidated when observations are made to discover what happens when a capsular strain becomes a non-capsular slime-forming variant.

The relationship of capsule and slime.

Although all that has been said above about the mechanism of capsule formation depends on it,

very little work has actually been done to establish that the slime and capsular polysaccharides of an organism are in fact identical.

In 1933 Etinger-Tulczynska proposed that slime and capsule were of different origin, that slime was a secretion while capsules were manifestations of cell-wall swelling. This work was based, in part, on the misinterpretation of some of the microscopic evidence (Duguid 1951) and the hypothesis is of course valueless in the light of present knowledge presented in the previous section. It is surprising that recently a claim was made by Kleineberger-Nobel (1948) to have obtained positive evidence that the slime and capsules of an organism are biochemically distinct. This claim was based on staining methods involving special fixatives and mordants, by the use of which it was claimed that slime and capsules stain differently, that therefore this constituted evidence for chemical differences. It is difficult to believe that a stain can be indicative of chemical nature when it stains all capsules irrespective of their composition and structure. At most these differential stains of Kleineberger-Nobel are probably specific for particular physico-chemical states, perhaps reacting preferentially with gels of certain degrees of hydration.

The only reliable evidence about the relationship of slime and capsular polysaccharides has been serological tests with Klebsiella (Edwards and Fife 1952) and A. aerogenes

(Wilkinson, Duguid and Edmunds 1954). In each investigation it was shown for one strain of the species that the slime and capsular polysaccharides were serologically identical, but these findings are subject to the limitions of serological proofs of identity, ie. that differences in composition that do not affect the location of the immunologically-dominant groups may be present but undected. The identity of slime and capsular polysaccharides have yet to be shown by chemical methods.

The function of bacterial capsules and slime.

Very little is known with certainty of the functions of capsules and slime. It has been suggested that the main biological function of these structures is in the control of the physical properties, in the widest sense, of the external milieu in which the organisms find themselves (Hestrin 1953). Perhaps capsules may be regarded as buffers protecting their cells from harmful influences.

Mudd (1953) suggests that capsules may be regarded as secondarily acquired protective devices, especially as adaptations to parasitism. It has been demonstrated that in the body capsules exert their protective effect by preventing phagocytosis; in the soil it is believed that capsules protect organisms against attack by protozoa and phages. Because the exopolysaccharides are highly hygroscopic it is believed that capsules and slime function as

moisture conservers, a factor which may be of major importance to soil bacteria. (e.g. Revis, 1913).

A fresh approach to the problem of function was encouraged by the discovery, reported by Hestrin (1953) that as a group, levan-producing species are able to utilise the levan they form, thus indicating that in these species levan is not an end product but an intermediate in the metabolism of sucrose. It has been suggested too that exopolysaccharides are the products of metabolic shunts (Lamanna and Mallette 1953). The intracellular polysaccharides are probably food reserves (Knaysi 1951).

It is possible that the main function of capsule and slime varies with the type of habitat and characteristics of the organisms.

PART 1.

THE COMPOSITION OF THE EXOPOLYSACCHARIDES OF FIVE STRAINS OF A.AEROGENES, TWO STRAINS OF A.CLOACAE THREE STRAINS OF KLEBSIELLA AND ONE STRAIN OF E.COLI.

THE COMPOSITION OF THE EXOPOLYSACCHARIDES OF FIVE STRAINS OF A.AEROGENES, TWO STRAINS OF A.CLOACAE, THREE STRAINS OF KLEBSIELLA AND ONE STRAIN OF E.COLI

Although it has long been known that organisms of the Aerobacter-Klebsiella group produce very mucoid growths, few attempts have been made to investigate the nature of their capsules and slime. Recently, attention has been drawn to the exopolysaccharides of these organisms by the work of Edwards and Fife (1952) who showed that these bacteria exist in a large number of immunologically-distinct types of high specificity, evidenced by a very small number of cross-reactions. Edwards and Fife identified 57 types, a number of which has since been increased to over 100 (Edmunds 1954 ; Henriksen 1954). These results indicate that, like the pneumococcus, the Aerobacter and Klebsiella species are able to synthesise large numbers of immunologically-different antigenically-active polysaccharides.

In the present study the exopolysaccharides of five strains of Aerobacter aerogenes and three strains of Klebsiella species, representing six immunologically-different types in the Edwards and Fife classification, have been examined, together with the exopolysaccharides of two strains of Aerobacter cloacae and one strain of Escherichia coli, to observe their nature and to find whether those produced by the different immunological types differ

markedly in composition.

Note : The relationship of Klebsiella and Aerobacter organisms is a controversial matter, and in the opinion of many authors they should be combined in one genus, (see Kauffmann 1951). Edwards and Fife (1952) in their definite paper have included A.aerogenes (but not A.cloacae) within the genus Klebsiella, and have discarded species names within the genus because they consider the grounds for differentiation to be insufficient. Instead they have described all Klebsiella organisms with reference to their capsule types; e.g. Kl. pneumoniae types A and B are called Klebsiella types 1 and 2. This classification has the merit of unambiguity and may become universally accepted, but in the present work the name Aerobacter is used.

Previous studies of Aerobacter aerogenes exopolysaccharides.

Emmerling (1900) isolated the "gum" from a lactose-grown liquid culture of A.aerogenes and obtained it as a nitrogen-free white powder with no reducing properties until after acid hydrolysis. Analysis for carbon and hydrogen indicated an empirical formula $C_6H_{10}O_5$. On the evidence that nitric acid oxidation of the polysaccharide gave rise to mucic acid Emmerling claimed it to be a galactan; he was unaware that galacturonic acid would also have given rise to mucic acid.

A more careful study was carried out by Schardinger (1902), of the slime produced by an organism identified by him as B.lactis pituitosi, the characteristics of which suggest that it was probably a strain of A.aerogenes. To isolate enough polysaccharide for the investigation Schardinger grew 50 litres of culture in simple synthetic liquid medium of the following composition : 5-8% sucrose, 0.5% NH_4 (chloride or sulphate), 0.1% KH_2PO_4 and 0.05% $MgSO_4$. Chalk (1-1.5%) was added to maintain neutral conditions. Growth was carried out in 2-litre flasks which were inoculated with 2-3 day old broth cultures and then allowed to stand for 5-6 weeks at 25-30°. After about a month most of the chalk had dissolved and the cultures were very turbid and viscous. The polysaccharide was isolated and purified by a long series of precipitations with alcohol under various conditions, and a final yield of 6.5 g. was obtained from the 50 litres of cultures. The product was found to be difficultly soluble in water, often requiring the addition of acid or alkali to dissolve it completely. The aqueous solutions were reported to be optically inactive. The analysis figures for carbon and hydrogen agreed closely with the values for $C_6H_{10}O_5$. Emmerling's findings were repeated when it was shown that nitric acid oxidation gave mucic acid, but Schardinger showed that the

presence of galactose unambiguously by hydrolysing the polysaccharide and preparing galactosazone. By measuring the reducing power (Allinn and Fehling's Method) it was estimated that the polysaccharide contained 79.6% galactose or 71.6% galactan. Furfural was detected when the polysaccharide was distilled with hydrochloric acid, which Schardinger interpreted as evidence for the presence of pentose, but which of course could also have come from a uronic acid.

Tomcsik (1927) isolated the exopolysaccharide of a capsular strain of A.aerogenes. He attempted to isolate the polysaccharide from culture filtrates, but found that the capsules would not dissolve into the medium, even after standing for 30 days, whereupon more drastic treatment was employed. Two-day growths from solid cultures were suspended in distilled water and precipitated with alcohol. The cells were re-suspended in 2 litres of 10% KOH solution and incubated at 37°; samples were removed at intervals and examined under the microscope to follow the course of decapsulation. It was found that 3-4 hours were required, after which the cells were removed by centrifugation and the polysaccharide precipitated from the supernatant with alcohol. Owing to the relatively drastic isolation procedure the product appears to have been contaminated with the cytoplasmic contents of disrupted cells, shown by the high nitrogen content, 1.4%, which on purification fell to 0.9%. Acid hydrolysis of the polysaccharide have a reducing value equivalent to

66% glucose. Tomcsik made no attempt to identify any of the component sugars of the polysaccharide.

Warren (1950) demonstrated that the exopolysaccharide from a capsular strain of A.aerogenes was susceptible to depolymerisation by hyaluronidase action. The polysaccharide was isolated from organisms grown for 5 days at 37° in liquid medium (0.3% beef-extract, 1% Wilson SM peptone and 0.5 NaCl). The cultures were freed of cells by centrifugation and the polysaccharide precipitated from the viscous supernatant with acetone. It was purified by reprecipitation several times, dialysed, and then finally freeze-dried. The yield was 75-100 mg of polysaccharide per litre of broth. Analysis of the polysaccharide gave the following results: nitrogen 7.9%, organic phosphate 1.27%, ash 10.5%, sulphur not detectable. No attempt was made to identify the component sugars. Despite the high nitrogen, protein tests gave negative results (the 7.9% N would be equivalent to approximately 48% protein which should have been readily detectable if it were present); the disproportionately low P value indicates that the high N is not due to nucleic acid. It was not recognised by Warren that the high nitrogen content therefore strongly suggests a hexosamine component, which would help to explain the susceptibility of the exopolysaccharide from this strain to hyaluronidase activity.

All these investigations were carried out with untyped strains.

Previous studies of Klebsiella polysaccharides.

Preliminary studies by Toenniessen (1921) and Kramar (1922) indicated the presence of galactose in the exopolysaccharides of unidentified strains of Friedländer's bacillus (Kl.pneumoniae) by the preparation of galactosazone from the polysaccharide hydrolysates. The main body of knowledge about Klebsiella polysaccharides derives from the investigations made a few years later by Avery, Heidelberger and Goebel on the polysaccharides of Friedländer's bacillus, types A, B and C.

The type A polysaccharide was isolated and purified in the following manner (Goebel and Avery 1927) : washings from 72 hour cultures on glucose agar medium (pH 7.6) were autoclaved and treated with trypsin until the turbid suspension was clarified. After adding sodium acetate the polysaccharide was precipitated with 2 volumes of ethanol. The crude polysaccharide was freed from protein by repeatedly dissolving the polysaccharide in water, acidifying the solution with acetic acid, removing the protein precipitate and then precipitating the polysaccharide with ethanol. This process was repeated until the polysaccharide ceased to be opalescent in solution. It was dissolved in water acidified with HCl and dialysed against distilled water until free from ions. The solution was then concentrated in

vacuo to low volume, and the last traces of protein removed by a last acetic acid treatment and the polysaccharide finally precipitated with 10 volumes of acetone. The yield from 72 Blake bottles was 5-6 grams of polysaccharide.

The polysaccharides of Friedlander's bacillus types B and C were prepared in a similar way, except that in the case of type B the trypsin digestion was omitted and the autoclaved washings were centrifuged to remove cells and debris. (Heidelberger, Goebel and Avery, 1925). A drawback of the above preparative method which the authors were unaware of at the time, was that the drastic preliminaries - autoclaving and trypsin digestion - will have caused disruption of the cells and therefore mixture of extra - with intracellular polysaccharides. However, as the amount of extracellular polysaccharide would be much greater than the intracellular the results obtained using these polysaccharide preparations may be considered to be more or less correct for the exopolysaccharides.

The characteristics of each polysaccharide were determined and are shown in the table. All the polysaccharides were found to contain D-glucose, identified as the osazone and by oxidation to saccharic acid, and each polysaccharide was found to contain a uronic acid component, which in the case of the type A polysaccharide was identified as D-glucuronic acid (Goebel 1927)

PROPERTIES OF KL. PNEUMONIAE POLYSACCHARIDES.

Type	$[\alpha]_D^{20}$	N	Acid equivalent	Reducing power after hydrolysis	Component sugars.
A	-102°	0	430	66% as glucose	Glucose Glucuronic acid
B	+100°	0	694	73% " "	Glucose Uronic acid
C	+ 95°	0	645	74% " "	Glucose Uronic acid

Although the polysaccharides of types B and C appear to be very similar, they were shown by immunological tests to be different; they would not cross-react with antisera prepared against each other.

Previous studies of E.coli polysaccharides.

Within the past few years several E.coli polysaccharides have been examined and their compositions elucidated with the aid of modern techniques. In each case they were found to be components of "Boivin antigens", i.e. complexes of polysaccharide, phospholipid and protein, normally found in many gram negative organisms. These complexes are of great interest because of their marked biological (other than immunological) activity. One of the E.coli complexes has been found to possess tumour-regressing properties (Ikawa, Koepfli, Mudd and Niemann 1952), while others have been shown to possess marked pyrogenic activity and are being intensively studied by Westphal and his school with a view to elucidating the mechanism of bacterial inflammation and irritation in the animal body.

Westphal, Lüderitz, Eichenberger and Keiderling (1952) isolated a highly active pyrogen from E.coli strain Kröger O-8, by using the phenol-water extraction method described by Westphal, Lüderitz and Bister (1952). The bacteria were grown in glucose-nutrient broth liquid medium with constant aeration for 24-36 hours and were harvested by centrifugation. The cells were washed with saline and dried with acetone and desiccated, before extraction with hot (65-68°) phenol-water homogenous mixture. After cooling, the aqueous phase was separated and found to contain a protein-free mixture of nucleic acid (40-50%) and the polysaccharide-phospholipid complex (50-60%). The lipopolysaccharide was partially purified from nucleic acid by ethanol fractionation but was finally isolated in a pure state by centrifugation in a preparative ultracentrifuge. The product was obtained free from protein and nucleic acid, and was found to contain 75% polysaccharide and 25% lipid. The yield of lipopolysaccharide was 1-2% of the initial bacterial dry weight.

The lipopolysaccharide was analysed for its component sugars by hydrolysis and paper chromatography both qualitative and quantitative (Lüderitz and Westphal 1952). The sugars found to be present in the polysaccharide were rhamnose, xylose, glucose, galactose and glucosamine; the last component was detected and estimated by the Morgan and Elson method. A sugar component moving faster than rhamnose was observed on the chromatograms

but was not identified. The presence of rhamnose was confirmed by isolating it from multiple chromatograms and preparing the osazone. The sugars other than glucosamine, were estimated by a novel technique, using the intense red colour produced when triphenyl tetrazolium chloride (TTC) is reduced. The paper chromatograms were sprayed with TTC solution, and on warming, intense red spots were produced in the areas where the reagent had been reduced by the sugars. The papers were cut to isolate the spots and the red colour (a triphenyl formazan compound) eluted with pyridine and the colours measured and compared with standards. The following results were obtained using this method :

COMPOSITION OF THE POLYSACCHARIDE FRACTION OF E.COLI (KRÖGER "8) LIPOPOLYSACCHARIDE PYROGEN.

(Results corrected for 100% reducing sugar).

Rhamnose	56.9 ± 4.5%
Xylose	14.6 ± 1.1%
Glucose	14.1 ± 0.2%
Galactose	3.6 ± 1.0%
	89.2 ± 6.8%
Glucosamine	8.7
TOTAL	97.9 ± 6.8%

The rhamnose content was checked by estimating the C-methyl groups present in the polysaccharide and a value obtained equivalent to 59% rhamnose, confirming the above result. Physico-chemical measurements

indicated that the lipoprotein complex had a molecular weight of about 1 million, and that the molecule had a spheroid shape (Schramm, Westphal and Lüderitz 1952).

Lüderitz and Westphal also examined the pyrogen of E.coli (strain Kauffmann O_{18}) by the same methods and found it to have the same component sugars as above, but having mannose in place of xylose; the sugars appear to be present in different proportions, (Westphal, Lüderitz , Fromme and Joseph 1953).

From the culture filtrates of an unidentified atypical strain of E.coli (described as being a para colon intermediate type) Ikawa, Koepfli, Mudd and Niemann (1952) isolated an agent causing regression and haemorrhage of experimentally-induced mouse tumours. The agent was found to be a phospholipid - polysaccharide-polypeptide complex which could be separated by trichloroacetic acid treatment into polypeptide and phospholipid-polysaccharide fractions. The latter was shown to contain 21 - 25% bound lipid, and to have a reducing power equivalent to 52 - 55% glucose.

Its glucosamine content was estimated to be 15-17 %
By examining the ultra-violet absorption spectrum of the phospholipo -polysaccharide in 79% sulphuric acid solution, Ikawa et al (1952) reported the absence of mannose, ketoses, 6-deoxyaldohexes, aldopentoses and uronic acids. D-glucose, D-galactose and D-glucosamine were isolated from the hydrolysed phospholipo-polysaccharide and identified by the preparation of derivatives, but their relative proportions were not determined.

Webster, Landy and Freeman (1952) reported the extraction with 0.9%saline solution of the Vi antigen of E.coli strain 5936/38 as a complex. Mild acid hydrolis of the complex followed by electrodialysis led to the isolationof the pure polysaccharide which was found to possess a striking resistance to hydrolysis: it gave less than 3% reducing sugar after hydrolysis with 6N hydrochloric acid at 100° for an hour under 50 lbs. pressure. It was later shown that the polysaccharide was built up from a single component recognised to be a N-acetylaminohexuronic acid (Webster, Clark and Freeman, 1954). No other polysaccharide of similar composition is known.

MATERIALS AND METHODS.

Organisms.

The following organisms were used for polysaccharide production.

Aerobacter aerogenes.

Strains: A1, A3 A4 & A29. (A1 and A3 correspond to Klebsiella type 54) (A4 corresponds to Klebsiella type 8) (A29 corresponds to Klebsiella type 57)	Capsulated strains from the collection of Dr. J.P. Duguid (Bacteriology Dept. Edinburgh University) isolated from faeces, infected urine, wounds and milk, whose individual sources are unknown. These strains have been typed by Dr. P.N. Edmunds (Bacteriology Dept., Edinburgh University) and found to belong to the Edwards and Fife scheme (Edmunds 1954).
Strain: A3(S1)	A purely slime-forming variant of A3 isolated by Dr. J.F. Wilkinson (Edinburgh University) by a chance variation upon repeated subculture of A3. Immunologically A3 and A3(S1) are identical (Wilkinson, Duguid and Edmunds 1954).

Aerobacter cloacae.

Strain: NCTC 5920	A slime-forming non-capsulate strain obtained from the National Collection of Type Cultures.
Strain: NCTC 5936	" " " "

Escherichia coli.

Strain: A102	A purely slime-forming strain isolated from faeces by Dr. J.P. Duguid, and found to belong to Wilson's biochemical type 1.

Klebsiella.

Type 2 (NCTC 5055). A capsulated strain obtained from the National Collection of Type Cultures.

Types 26(S1) and 29(S1). Non-capsulated slime-forming variants obtained by chance from Edwards' types 26 and 29 by Dr. P.N. Edmunds.

[redacted]

STRAIN CHARACTERISTICS OF ORGANISMS USED.

Strain.	Motility	Methyl red	Voges-Proskauer	Koser citrate utilisation	Indole production	Eijkman	Urio acid utilisation	Growth in Std NH_3 synthetic medium	Gelatin liquefaction	Fermentation Reactions: Glycerol	Glucose	Lactose	Sucrose	Mannitol	Dulcitol	Inositol
A. aerogenes.																
A1	-	-	+	+	-	-	+	+	-	+	+	+	+	+	-	+
A3	-	-	+	+	-	-	+	+	-	+	+	+	+	+	-	+
A3(S1)	-	-	+	+	-				-	+	+	+	+	+	-	+
A4	-	-	+	+	-	-		+	-	+	+	+	+	+	-	+
A29	-	-	+	+	-	-	+	±	-	+	+	+	+	+	-	+
A. cloacae.																
NCTC 5920	+	-	+	+	-	±	-	(+)	+	+	+	+	+	+	-	+
NCTC 5936	+	-	+	+	-	-	+	+	-	+	+	+	+	+	-	+
E. coli.																
A102	+	+	-	-	+	+		-	-	+	+	+	+	+	+	-
Klebsiella.																
NCTC 5055	-	-	+	+	-	-	-	-		+	+	+	+	+	+	+

Cultural conditions.

All the organisms were grown on a simple synthetic medium which had the following composition : 1% glucose, 0.03% $(NH_4)_2SO_4$, 1.0% Na_2HPO_4(anhydrous) 0.3% KH_2PO_4, 0.1% NaCl, 0.1% K_2SO_4, 0.02% $MgSO_4$ and traces of the following salts, $CaCl_2$, $Co(NO_3)_2$, $MnSO_4$ and $FeSO_4$. The medium was made up with distilled water, and contained the relatively high sugar and low nitrogen concentration found by Duguid and Wilkinson (1953) to give maximal polysaccharide production. The medium was sterilised by steaming for 30 minutes on three consecutive days.

In the earlier experiments growth was carried out in Roux bottles on solid medium obtained by adding 2% agar to the above medium (50 ml. per bottle). The bottles were inoculated by pouring in 24 hr. peptone-water cultures and then incubated horizontally at 37°. The use of solid medium was discontinued however, because with liquid medium under the conditions described below the relative yields of polysaccharide were greater, and because there was always danger of contamination of the polysaccharide with agar. The latter became especially important when small amounts of galactose were detected in some of the polysaccharide hydrolysates.

Growth in liquid medium was carried out in 1000 ml. screw-capped bottles (200 ml. of medium per bottle). After sterilisation the bottles were inoculated with loopfuls of the required organisms grown on agar plates, so that each loopful was a heavy inoculum.

Each bottle after inoculation was oxygenated by passing oxygen through sterile plugged tubing into it for one or two minutes and then replacing the cap.

To maintain adequate oxygenation of the liquid during growth the bottles were fitted horizontally onto a rotating drum of horizontal axis, capable of holding 6 bottles at a time, and mounted in a 37° incubator. The cultures were grown for 2-3 days by which time they were usually highly viscous. The viscosity of one culture was measured and found to be 22 times greater than that of water.

Isolation of slime polysaccharide.

The procedure for isolating slime polysaccharide was the same irrespective of whether capsular or purely slime-forming strains were used. If the culture had been grown on solid medium the cells were suspended in physiological saline (0.85% NaCl solution) by gently scraping the growth with rubber-enclosed glass rods, taking care not to break the agar surface. The washings were then stirred briskly with a mechanical stirrer for about 30 minutes to ensure that the suspension was homogenous, and then centrifuged at high speed. A MSE high-speed angle-head centrifuge capable of 13,000 rpm was used. If the culture had been grown in liquid medium it was centrifuged directly. The centrifugation was much more quickly completed with purely slime-forming cells which were found to be nearly all sedimented after about 30 minutes, than with capsular cells which were difficult to sediment, even

after an hour at the highest speed. Owing to the viscosity of the liquids, however, it was found impossible to remove all the cells at this stage, whether they were non-capsulate or not.

The supernatant liquid was poured off and the slime polysaccharide precipitated from it by the addition of 2 volumes of acetone or 2 volumes of ethanol. By stirring, the polysaccharide gel adhered to the stirring rod and could be removed to another beaker and washed with fresh acetone thereby dehydrating the highly aqueous gel. If large volumes of supernatant were available, the precipitation was carried out using no more than about 250 ml. at a time, and the polysaccharide accumulated at this stage. After several washings with acetone, the gel was converted into a stringy greyish-white solid. The crude polysaccharide was dissolved in a small volume of water and de-proteinised by the Sevag method. To each 250 ml. of polysaccharide solution, 5 ml. of glacial acetic acid and 10 g. of sodium acetate was added, and this was shaken with 60 ml. of chloroform-butanol mixture (50/10). After shaking for about 30 minutes the emulsion was centrifuged, and the proteins found as a gel-like emulsion at the chloroform water interface. The aqueous layer was withdrawn and shaken with fresh chloroform-butanol mixture, and the process was repeated until little or no protein was found at the interface. In the earlier preparations it was found that high viscosities of the solutions made

shaking and centrifugation difficult; when it was shown that the viscosity could be reduced by boiling the polysaccharide solution for 15-30 minutes at pH 7 without causing any apparent hydrolysis, this step was inserted in the isolation precedure, to be done before attempting the de-proteinisation process. The polysaccharide was precipitated with 2 volumes of acetone and re-dissolved in about 200 ml. water, and the solution then dialysed for 40-48 hours against running tap water. After dialysis, in the earliest preparations the solutions were treated with Amberlite ion-exchange resins, butthis practice was later discontinued as unnecessary. The dialysed solution was centrifuged at high speed and no longer being viscous, all the remaining cells were removed.

On one occasion, the polysaccharide was finally isolated from the dialysate by freeze-drying but all the others were isolated by precipitation with acetone. In the absence of dissolved salts the polysaccharides were difficult to precipitate, but when 1% of sodium acetate was dissolved in the solution 3-4 volumes of acetone gave complete precipitation almost immediately. For ease of manipulation it was found convenient to use no more than about 200 ml. of solution for each precipitation. The polysaccharide gel was washed first with 70% aqueous acetone and then with dry acetone while being broken up into small fibres or flakes to facilitate drying, and then dried with ether. The grey-white strings or flakes

were kept in a vacuum desiccator over phosphorus pentoxide. The physical state of the final product was found to vary with the conditions of the last precipitation and the manipulation during washing and drying.

Isolation of capsular polysaccharide.

The residues of cells from the initial centrifugation, described above in the procedure for isolating slime polysaccharides, were used for the extraction of capsular polysaccharide, if the strain was capsular. First the residues were washed twice with equal volumes of water to remove adhering slime before the capsules were stripped from the cells. An attempt was made to remove the capsules of A.aerogenes (A3) by shaking with sodium chloride solution, but with concentrations of 1%, 5%, 10% and 20% NaCl no effect was observed, making it necessary to use more drastic conditions. Two different ways of removing capsules were found to give good results- boiling and soda treatment.

Boiling - The cells were suspended in several volumes of water and the pH checked, and adjusted to 7.0 if the suspension was not neutral. Boiling for 15 minutes was sufficient to remove the capsules from the cells.

Soda treatment - The cells were suspended in 2 or 3 volumes of water, and the suspension made 1% wrt. NaOH and stirred mechanically at room temperature for

15 minutes, after which it was neutralised with N HCl. When it was observed that addition of 1% formalin to the suspension about 15 minutes before the start of the soda treatment reduced cell disruption during the stirring with NaOH, this additional step was introduced in the procedure.

The boiled or soda-treated suspension was then centrifuged and the sediment of decapsulated cells discarded. The capsular polysaccharide was precipitated from the supernatant by addition of 2-3 volumes of acetone, and then purified in the same way as the slime polysaccharide.

Optical Rotation.

The optical rotation of the polysaccharides was found by measuring the rotation of 0.1% polysaccharide solutions contained in 1 dm. cell. The turbidity of the polysaccharide solutions made it necessary to use such dilute solutions.

Ash.

The ash content of the polysaccharides was estimated by ignition of weighed samples (5-10 mg) in a platinum crucible to constant weight. At first there was difficulty in arriving at constant weight, even after a dozen ignitions. This is believed to be because/of the ash was in the form of volatile much sodium salts. The difficulty was overcome by adding a drop of 60% perchloric acid to the crucible after each initial ignition and carefully heating until all the liquid had evaporated off. In this way

constant weight was reached very quickly. The weighing was done with a Paul Bunge microbalance.

Nitrogen.

The nitrogen content of the polysaccharides was determined by the Kjeldahl method, using 5-10 mg. samples. Digestion was carried out for 14-17 hours with 1 ml. N-free comcentrated sulphuric acid using a selenium catalyst, after which the ammonia was distilled in a Markham micro-distillation apparatus into 2% boric acid. The solution was then quantitatively transferred to a 25 ml. standard flask and 2 ml. of Nessler reagent added, and the colour measured with a Hilger "Spekker" absorptiometer, using Ilford No. 601 (violet) filters. A straight line calibration curve was obtained for the range 0-100 µg nitrogen.

Phosphorus.

The phosphorus content of the polysaccharides was determined by the Fiske and Subbarow method, using 5-10 mg. samples. The colour was measured with a Hilger "Spekker" absorptiometer, using Ilford No. 608 (red) filters. A straight line calibration curve was obtained over the range 0-100 µg phosphorus.

Sulphur.

The sulphur content of one polysaccharide (Aerobacter aerogenes - strain A3(S1)), was determined, by the barium sulphate turbidmetric method (Snell and Snell, "Colorimetric Methods of Analysis", Vol. II p. 766-768) scaled down to deal with less than 100 µg

sulphur. The procedure used was as follows :-

The sample (about 10 mg. polysaccharide) was weighed into a 50 ml. Kjeldahl flask; 3 ml. of conc. nitric acid was added and the sample allowed to dissolve at room temperature. The flask was then warmed gently until brown fumes ceased to be evolved, and 1.5 ml. of 60% perchloric acid added. The flask was boiled until white fumes appeared and the solution was colourless. It was allowed to cool and 8 ml. of 12% sodium chloride solution added, and the liberated chlorine was boiled off. Concentrated ammonia (0.880) was added dropwise until the solution was alkaline and the solution was then evaporated to dryness, after which the residue was strongly heated to decompose ammonium salts. The residue was dissolved in 10 ml. of water and transferred to a 25 ml. standard flask, and conc. hydrochloric acid added dropwise until the pH was at or below 3. Finally 1 ml. of 10% barium chloride was added and the solution diluted to the mark and shaken. After standing for 5 minutes the flask was vigorously shaken and the turbidity of the solution measured in a 4 cm. cell with a Hilger "Spekker" absorptiometer, using the colourless Ilford heat filters (H508)

It was thought possible that the phosphate present in the polysaccharide might interfere by precipitating out as barium phosphate, but it was found that barium ions do not give a precipitate with phosphate at

such low concentrations (50-100µg/25 ml). The method may be subject to considerable errors with such small quantities, but reasonably concordant results were obtained with the polysaccharide examined. A straight line calibration curve was obtained over the range 0-50µg sulphur.

Anthrone value.

The anthrone value of the polysaccharides was determined, at first by the procedure described by Seifter, Dayton, Novic and Muntwyler (1950) using 0.2% anthrone in 95% sulphuric acid, but later the modification of Fairbairn(1953), using 0.1% anthrone in 72% sulphuric acid, was adopted. The latter procedure was found to posses a number of advantages:- the reagent can be added to the polysaccharide solution with less danger of unwanted heating ; because of the smaller final volume (12 ml. as opposed to 15 ml.) a stronger colour is obtained for a given weight of material and the reagent does not require daily preparation.

The method used was as follows :- 2 ml. of polysaccharide solution (containing 0.1 - 0.2 mg) contained in a 6 x 1" boiling tube, was cooled in running water while 10 ml. of anthrone reagent was added from a burette. The first 3 ml. of reagent were added in 1 ml. amounts, and then the remainder was added without stopping: the tube was vigorously shaken throughout. When all the tubes of the batch

were prepared, they were heated in a vigorously boiling water bath for 8 minutes (Fairbairn reported that glucose required 12 minutes heating for maximum colour development, but in the present study a sharp maximum was found after 8 minutes.) The tubes were cooled rapidly in cold water, and the colours measured in a Hilger "Spekker" absorptiometer, using Ilford No. 607 (Orange) filters. A straight line calibration curve was obtained for 0-300 µg. glucose, but it is necessary to include glucose standards (usually 100 µg. was used) with each batch of determinations. The results were expressed in terms of % glucose.

Reducing value.

The reducing power of unhydrolysed and hydrolysed polysaccharides was determined by Nelson's (1944) colorimetric modification of Somogyi's reduction method, as described by Somogyi (1945.) The colours were measured in a Hilger "Spekker" absorptiometer using Ilford No. 608 (red) filters. Glucose standards were included with each batch of determinations.

Identification of component sugars.

The identification of the component sugars present in the polysaccharides was mainly based on the evidence of paper chromatograms. With the exception of the uronic acids all the components were identified unambiguously by the rates of movement parallel with sugar standards in several solvent systems, and by

the colours produced with spray reagents. In one instance (Aerobacter aerogenes, strain A3(S1) the component sugars were isolated after separation on a cellulose column and identified by the preparation of derivatives.

The identification of the uronic acids presented great difficulties and none were identified with certainty. Attempts were made with allegedly specific colour methods (Dische 1947, Dische 1948) and with chromatograms run with glucuronic acid, galacturonic acid, and mannuronic acid standards in acidic solvents (butanol-acetic-water 4:1:5: and ethyl acetate-acetic water 3:1:3) but without success, The uronic acid of A.aerogenes A3(S1) was isolated but no definite identification obtained.

Determination of the component sugars.

The component sugars were estimated in two different general ways; some (uronic acids and 6-deoxysugars) were estimated by specific methods directly on the unhydrolysed polysaccharide, while all the components (except the uronic acids) were estimated after hydrolysis by chromatographic separation on paper, according to the general principles described by Flood, Hirst and Jones (1948) using the periodate oxidation method (Hirst and Jones) 1949 for measuring the amounts of each sugar fraction. The details of the chromatographic method will be described first.

(1) Hydrolysis. All the polysaccharides were found to be polyuronides and to be resistant to hydrolysis. Trial experiments described more fully in the results section below, indicated that 24 hours hydrolysis in N sulphuric acid at 100°, or even 7 hours in 96% formic acid followed by 6 hours in N sulphuric acid, (both hydrolyses at 100°) were not sufficient for complete hydrolysis. The conditions finally adopted as the standard method of hydrolysis of these polysaccharides for the purpose of quantitative analysis, were 24 hours in 96% formic acid at 100° followed by 6 hours in N sulphuric acid at 100°. The procedure was as follows :

The sample usually 20-40 mg., was weighed into a long-necked glass bulb, 2 ml. of 96% formic acid was added and the neck sealed. It was secured upright in a boiling water bath and left for 24 hours, after which it was cut open and a weighed quantity of reference sugar (known not to occur in the polysaccharide) was added and the formic acid solution then transferred to a distilling flask where the formic acid was distilled off in vacuo at 45-50°. Usually several additions of a few mls. of water were found necessary before all the acid was removed. Then 2 ml. of N sulphuric acid was added to the syrup and the solution transferred to another glass bulb which was sealed and placed in a boiling water bath for a further 6 hours. After

cooling the tube was opened and the contents transferred to a centrifuge tube; the sulphuric acid was neutralised with barium carbonate and the precipitate removed by centrifugation. The next step, the removal of the bulk of the uronic acid, was found desirable because of the difficulty introduced by the trails occurring between the fast moving lactone spots and the slow moving uronic acid spots. At first the use of Amberlite anion exchange resins (IR-4B and IRA-400) were tried, but not proceeded with because of the possibility that the resins would remove some of the free sugars also (Roseman, Abeles and Dorfman 1952). It was found that the uronic acids could be removed from the neutral hydrolysate without disturbing the ratio of the free sugars by precipitation as the barium salts with 2 volumes of ethanol. The precipitate was removed by centrifugation; sometimes it was found desirable to let the precipitate and solution stand overnight in the refrigerator before centrifuging. The supernatant from this was treated with Amberlite IR-120 cation exchange resin, and then transferred to another tube in which it was evaporated to a syrup by a gentle steam of air blowing into the tube, standing in a beaker of warm water.

(ii) Chromatography. The syrup from the hydrolysate was applied with a micropipette (volume ca. 0.03 ml. made from capillary tubing)

to chromatogram sheets cut from Whatman no. 1 paper. The sheets were 13x58 cm., with a serrated bottom edge. Two lines were drawn lengthwise 2.5 cm. from each edge, and spots marked along the starting line, one each in the middle of the side-strips and 4 equally distributed in the middle section. One pipetteful was applied evenly to the 4 middle spots, the paper being dried after each application with a hair-drier; a half-pipetteful was distributed between the 2 side-spots. From every hydrolysate 4 - 6 chromatograms were prepared.

The solvent used for earlier separations was benzene-butanol-pyridine-water (10:50:30:30) (Albon and Gross 1950) which gave good separation of the sugars but which often interfered in the estimations because of erratic high blank values. This was replaced by butanol-ethanol-water (5:1:4) (Hough, Jones and Wadman 1949) which was found to give equally good separation while having reproducible small blank values.

After being run in the tank for a suitable period (often 6 days) the chromatograms were hung in a fume cupboard for about 2 days to permit the solvent to evaporate away. The side-strips were cut and sprayed with aniline oxalate and the colours developed by heating.

~~developed by heating~~. Every side strip was examined in ultra-violet light for the clearer demarcation of slight spots, and sometimes the detection of otherwise invisible spots. The areas from the central portion of the chromatograms were cut so that every sugar was obtained on a separate piece. At this stage, when the pyridine-containing solvent was used, the paper sections were kept in a vacuum desiccator over water for several hours to remove the last traces of pyridine; with the butanol-ethanol solvent this was unnecessary. The sugars were eluted from the sections using 10-15 ml. of cold water, after the method described by Laidlaw and Reid (1950). It was found convenient to cut the paper sections across diagonally and to superimpose the two triangular halves thus obtained, and to elute them together, point downwards. The shape and double paper surface permitted only a straight water front to develop, and the drops from the paper never fell outside the collecting tube. This arrangement did not appear to interfere with complete elution.

(iii). Periodate oxidation. The eluates from the chromatogram sections were collected in tubes fitted with ground joints and stoppers. 1 ml. of 0.3 M sodium metaperiodate was added to each tube and the stopper tightly applied. The tubes were then heated in boiling water from 30 minutes

in an apparatus which kept the top part of the tubes water-jacketed. After the heating the tubes were cooled in water and about 10 drops of ethylene glycol added to each. Because of the fairly large volume of solution in the tubes it was found that the glycol required about a minute to destroy the excess periodate; it was found convenient therefore, during serial determinations, to add the ethylene glycol to all the tubes and replace the stoppers before commencing the titrations with dilute standard sodium hydroxide. Methyl red or bromthymol blue were used as indicators; a control solution containing ethylene glycol and indicator was always used for comparison. With each set of chromatograms, blanks corresponding to a range of areas were determined, and found to depend on the area. For convenience curves relating area to blank titre were drawn and used for calculating the blanks of sections of intermediate areas.

The weight of sugar present in each fraction was calculated directly from the value of the corrected titre. Corrections were not made for the deviations from the theoretical yield of formic acid, because the total errors involved in the procure are probably much greater than these deviations. The following formic acid-carbohydrate equivalences were used :

Hexose	1 mole sugar = 5 moles formic acid
	1 ml. 0.01 N NaOH = 0.3600 mg.hexose
Pentose	1 mole sugar = 4 moles formic acid
	1 ml. 0.01 N NaOH = 0.3753 mg.pentose
6-Deoxyhexose	1 mole sugar = 4 molesformic acid
	1 ml. 0.01 N NaOH = 0.4103 mg.6-deoxyhexose

After calculating the weights of the sugars present in each chromatogram, the percentage of each component in the polysaccharide was derived by multiplying the weight of the component by the factor $\frac{R \times 100}{r \times P}$ where R = wt. of reference sugar added to the hydrolysate
r = wt. of reference sugar found in the chromatogram
P = wt. of polysaccharide hydrolysed.

(iv) Uronic acid estimation.

Because of the difficulties introduced by lactone trails on chromatograms and the anomalous yield of formic acid from periodate oxidation, the uronic acid content of the polysaccharide was determined by other, specific methods. After preliminary experiments with several colorimetric methods for uronic acids, including those of Hanson Mills and Williams (1944) and Jarrige (1947) it was decided that colorimetric methods were not sufficiently reliable for a study of polysaccharide composition when so many other components were present. Good results were obtained with the

decarboxylation method of McCready, Swenson and McLay (1946). The apparatus was slightly different from that described by the authors, and is shown below (Fig. 1). The procedure was as follows : The sample to be analysed, usually 20-40 mg., was put in the dry reaction-flask and some small glass "ballotini" added to prevent sudden boiling. 30 ml. of 19% hydrochloric acid were added and the ground neck of the flask lubricated with a drop of syrupy phosphoric acid, and the flask attached to the condenser and the air inlet tube. The whole apparatus was flushed out with a fairly rapid current of air to remove traces of carbon dioxide before addition of the sodium hydroxide solution. 5 minutes was found to be sufficient for this purpose. Then the air current was stopped and the top of the absorption tower opened by removing the adaptor, and 25 ml of approx. 0.02 N sodium hydroxide and 6-8 drops of butanol added. The absorption tower and flask were disconnected from the inlet tube for 15-30 seconds to allow the butanol to drain past the glass bulb. The apparatus was reconnected and the oil bath rapidly heated, in situ, to 145° while a slow air current was passed. After the temperature was stabilised and the expanded air was out of the system, the air flow was adjusted to a suitable rate (60-80 bubbles/min. at the outlet tube). At the end of 90 minutes the absorption

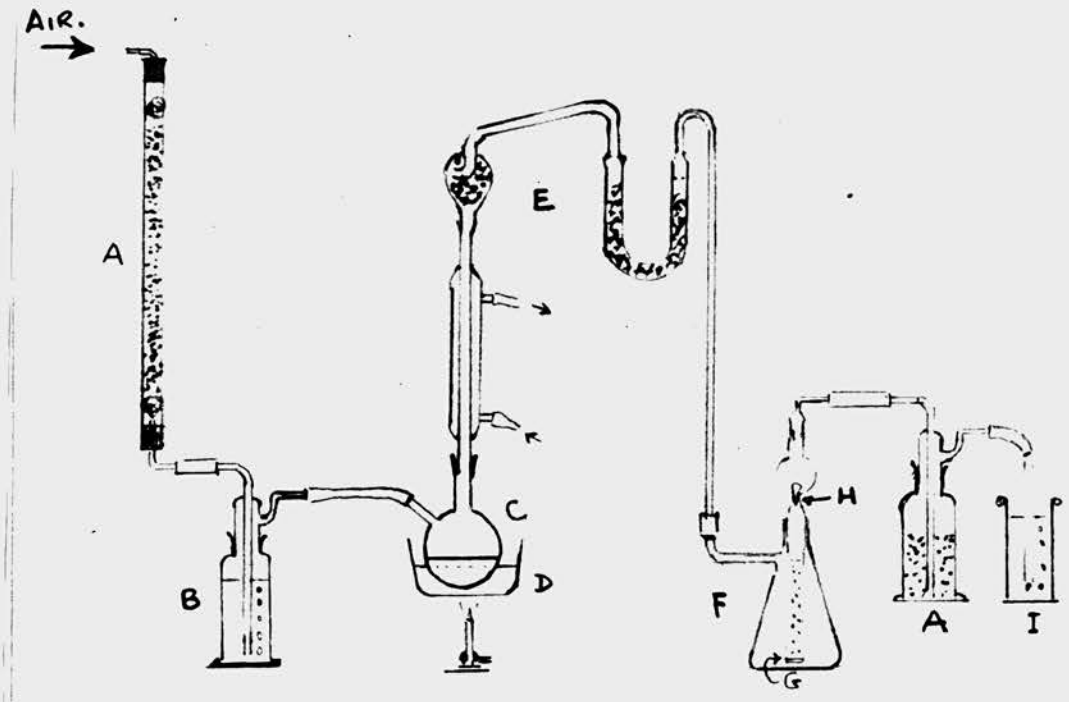


Fig. 1. Decarboxylation Apparatus

A. Soda-lime
B. Saturated $Ba(OH)_2$
C. Reaction flask (150 ml.)
D. Oil bath
E. Splash head and U-tube, charged with Zn turnings
F. Absorption tower (25 ml. NaOH)
G. Sintered glass disc
H. Bulb
I. Water (to permit flow rate to be estimated)

flask and tower were disconnected from the apparatus and the sodium hydroxide sucked down into the flask and the tower washed thoroughly with water(4 x 10ml), and 10 ml. of 10% barium chloride solution added. Two drops of phenolphthalein were added and the solution back-titrated with accurately standarised (approx. 0.02 N) hydrochloric acid. The difference between the titration and a control blank was equivalent to the carbon dioxide released from the uronic acid.

1 ml. N HCl = 0.194 mg. uronic acid

The results were found to be reproducible to within 1%

Notes on the decarboxylation apparatus and method.

1. It was found easier to control the air flow by pressure than by suction. If stop-cock grease, containing a little rubber was used in place of vaseline there were few leaks.

2. It was found necessary to introduce a small glass bubble to act as a spray trap at the constriction of the absorption tower, because droplet spray sometimes carried away significant amounts of alkali.

3. Olive oil was found to be the best oil-bath liquid.

4. The main difficulty found with the apparatus was to obtain a suitable type of zinc for the hydrochloric acid traps. Commercial granulated zinc appeared to be coated with zinc oxide which possibly contained some carbonate as well; it did not withstand many determinations with the apparatus before hydrochloric acid was able to pass through into the absorption tower. The problem was very satisfactorily solved by turning zinc rods (Leclanche electrodes) on a lathe, and cutting the turnings into smaller pieces. By charging the U-tube and splash-head with this no further difficulty was encountered.

5. Glass wool was used to hold the zinc in place in the splash-head. If not previously washed with acid the glass wool was sufficiently alkaline to contain

significant amounts of absorbed carbon dioxide which gave high results. After a few times in use the zinc granules in the splash-head became cemented together and the glass wool was dispensed with.
6. After each determination the reaction flask was found to have a purple coloured ring, where the surface of the liquid inside had been. This was easily removed with strong alkali.

(v). 6-Deoxyhexose estimations. Fucose was found in many of the polysaccharides examined in the present study ; rhamnose was also found in one of them. These sugars were estimated by periodate oxidation from chromatograms of hydrolysates, as described above, but as a means of confirmation the Dische and Shettles (1948) colorimetric method was also applied. The method was claimed by the authors to be specific for 6-deoxyhexoses but in trial experiments it was found that all the other components of the polysaccharides would interfere, but their colour producing power relative to fucose and rhamnose was slight, and by obtaining correction factors the fucose content of the polysaccharides estimated by the colorimetric method paralled the results obtained by periodate oxidation.

The method was as follows : 4.5 ml. of sulphuric acid (1 volume water: 6 volumes conc. sulphuric acid) was added to 1 ml. of polysaccharide solution while cooling in ice. The mixture was

then warmed for a few minutes, at 20-22° and then heated for exactly 10 minutes in a boiling water bath, and cooled rapidly. To the cold solution 0.1 ml. of 3% aqueous cysteine hydrochloride was added with shaking. The colour was measured after an interval of 1½ - 2 hours, by measuring the difference in absorption at 3960 Å and 4300 Å in a spectrophotometer. In the present study a Unicam SP 500 Photoelectric Quartz spectrophotometer was used.

Dische and Shettles considered the method to be suitable for 2 - 10 µg. amounts but in the present study a straight line relationship was obtained over the range 0-50 µg fucose, both when alone or in the presence of large amounts of glucose and glucuronic acid when corrections had been made for the latter sugars.

Serological methods.

Precipitation tests were carried out with some of the A.aerogenes polysaccharides to demonstrate the reaction of the isolated polysaccharides with antiserum, and to establish the serological identity of some of them. The antiserum was kindly provided by Dr. P.N. Edmunds, who prepared it by inoculating rabbits with A.aerogenes (A3) cells, and was the same as that used by Wilkinson, Duguid and Edmunds (1954).

Doubling dilutions of the antiserum in saline from 1/2 to 1/32, were placed in narrow tubes and a

0.1% solution of the polysaccharide in saline was superimposed. The tubes were incubated for 15 minutes at 37° and examined, when a positive reaction was indicated by the appearance of a white ring at the interface. For precipitin-absorption tests the absorbing reagent (0.1% polysaccharide solution) was added dropwise with constant agitation to an equal volume of antiserum and the mixture incubated at 37° for 30 minutes. After removal of the precipitate by centrifugation the supernatant was tested with the polysaccharide solution to find whether precipitating activity had been lost as a result of the absorption. If activity remained, more of the polysaccharide solution was added until absorption was complete.

EXPERIMENTAL AND RESULTS.

A. Experiments relating to the extraction of capsular polysaccharides.

The influence of formaldehyde on lysis during the soda method of extraction.

It was found that the capsules of A.aerogenes strain A3 were firm enough to remain on prolonged stirring and shaking with water, and with saline solutions containing up to 20% sodium chloride. The empirical observation was made that the capsules of strain A3 could be dissolved by either boiling a saline suspension at neutral pH for 15 minutes or by stirring a suspension in 1% sodium hydroxide

in the cold for 15-30 minutes. The capsular polysaccharides thus obtained were found to have higher nitrogen values than the slime polysaccharide which, after the absence of hexosamines was ascertained suggested that boiling or soda treatment of the capsular cells caused lysis and contamination of the polysaccharide with the cytoplasmic contents. This was also indicated by the sharp decrease in turbidity of the suspensions during soda treatment.

The following experiment showed that lysis during the soda method of extraction was appreciably reduced if the suspension was first allowed to stand in 1% formaldehyde solution for 15 minutes.

A culture of strain A3 was grown for 48 hrs at 37° on glucose synthetic liquid medium in Roux bottles. The cells were harvested by centrifugation and were washed several times with saline, and a suspension of Spekker reading 1.0 prpared. Two 20 ml. portions were transferred to 4 cm. Spekker cells, in which the soda extraction of the capsules were to be carried out; one portion with, and the other without, formalin pre-treatment. To one cell 0.59 ml. of 36% formaldehyde,sclution was added to give a final concentration of 1% formaldehyde, while to the other cell 0.59 ml. of saline was added, to cause an equal volume change. At intervals during the 15 minutes standing the opacity of the suspensions were measured and found to remainconstant

The formalin suspension was very slightly less turbid than the other. Then 6.5 ml. of N sodium hydroxide was added to each cell, and the opacity of the suspensions measured frequently during the following 30 minutes, stirring with glass rods before every reading was made. The opacity readings were made in the Spekker ; the results are presented graphically in fig. 2. The experiment was repeated with a less dense suspension (initial opacity 0.45) and similar results were obtained. In both experiments the final opacity difference was 31% of the initial opacity. If the final opacity of the soda treated suspensions is taken as a measure of the number of intact cells remaining, these results indicate that formaldehyde treatment leads to a significant decrease in lysis.

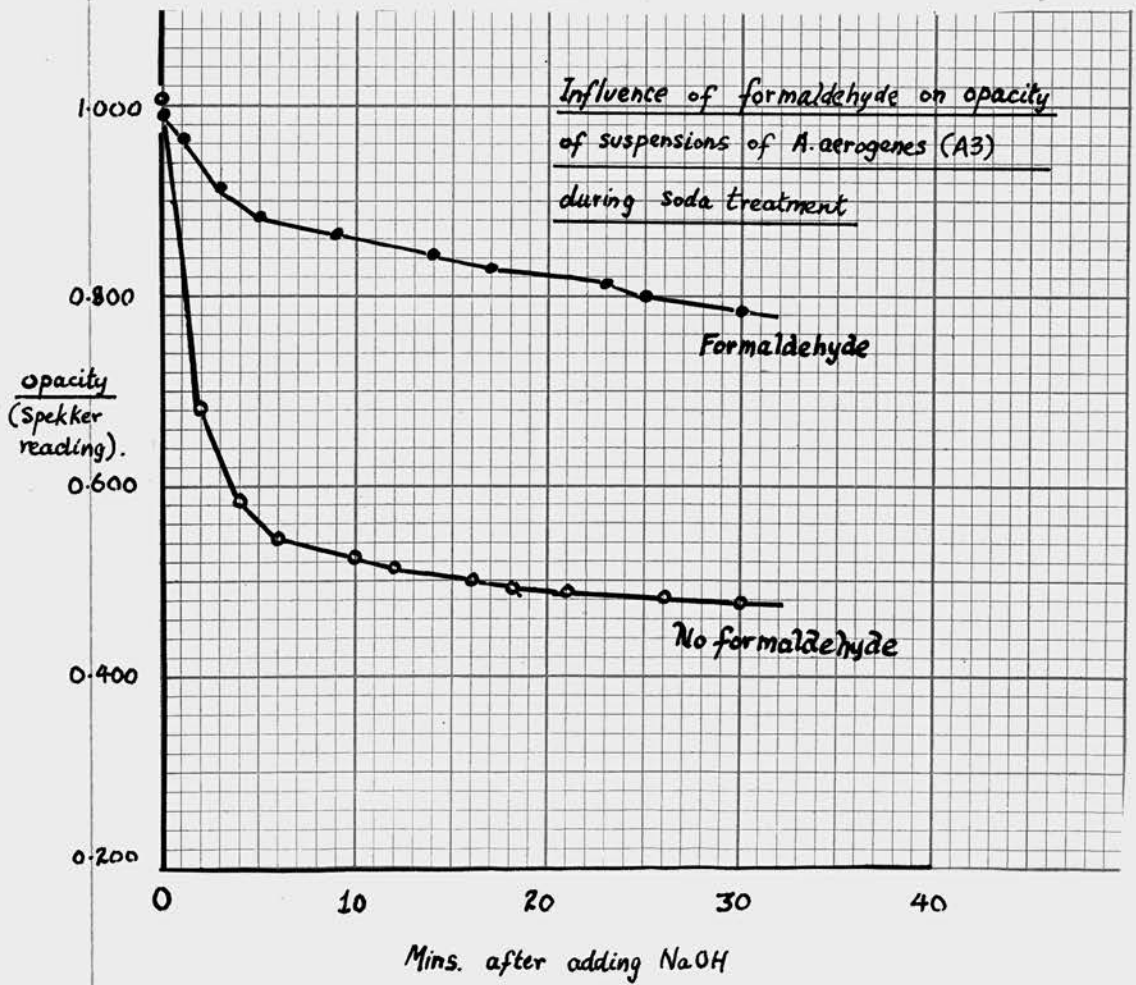
To confirm the protective influence of formaldehyde treatment the distribution of nitrogen between the cells and solution was determined in the case of the second suspension referred to above. After the soda treatment the contents of the Spekker cells were centrifuged and the supernatants withdrawn. The sediments were washed twice with saline and then suspended in 5 ml. Kjeldahl determinations were carried out on aliquot portions of the supernatants and the suspensions, and the following results were obtained :

Cells treated	N in total supernatant	0.062 mg.	(25%)
with formaldehyde	N intotal cells	0.190	(75%)
		0.252 mg.	

Cells not treated	N in total supernatant	0.140 mg.	(52%)
with formaldehyde	N in total cells	0.130	(48%)
		0.270 mg.	

From the results it will be seen that pre-treatment with formaldehyde reduced the nitrogen content of the extracted polysaccharide solution by half.

Fig. 2



B. Experiments relating to the methods of estimating the component sugars of the polysaccharides.

Trial experiments were carried out on various general and specific methods for sugar estimations, in order to select those most suitable for the present investigations. Of the general methods which could be used in conjunction with paper chromatography, the periodate oxidation method (Hirst and Jones 1949) and the colorimetric Somogyi method (Nelson 1944, Somogyi 1945) were tried, and the periodate method chosen because of its greater convenience in serial determinations. It is less sensitive than the Somogyi method but has the important advantage of being an absolute method. In the present study it would have been cumbersome to have had to prepare calibration curves with every determination of each component.

Experiments with periodate oxidation.

In the course of eluting the sugar fractions from the chromatograms by the method of Laidlaw and Reid (1950) the volume of eluate was not always the same. The following result indicated that differences in volume over the range 6 - 21 ml. do not influence the oxidation of glucose.

Effect of volume on periodate oxidation of glucose.

1 ml. of 0.3 M sodium metaperiodate added to each tube containing 1.24 mg. glucose. Heated for 30 minutes in boiling water bath.

Total volume	Titre*	Corrected Titre	Glucose + estimated	%
6 ml.	3.29ml.	3.24 ml.	1.180 mg.	95.1
	3.28	3.23	1.177	94.9
11 ml.	3.28	3.23	1.177	94.9
	3.27	3.22	1.174	94.5
16 ml.	3.30	3.25	1.184	95.4
	3.27	3.20	1.166	94.0
21 ml.	3.28	3.23	1.177	94.9
blank	0.05			
	0.05			

* 0.01012 N sodium hydroxide

\+ Calculated by using the relationship on p.

Control experiments were carried out with rhamnose, ribose and arabinose, the sugars whichwere used as reference sugars in the quantitative analysis of the polysaccharide hydrolysates, and the yields of formic acid were found to be 96%, 95% and 96% respectively.

Control experiments were also carried out with glucurone and galacturonic acid. With glucurone the yield of formic acid was found to be unaffected by the duration of oxidation (from 10 - 60 minutes);

and was constant at 4.1 molecules/molecule. In the case of galacturonic acid the yields of formic acid were found to decrease with increasing duration of oxidation, but the results were lower than those reported by Hirst and Jones (1949) ; the yields of formic acid (expressed as mols./mol.) were found to vary as follows : 10 min. 3.91; 2o min. 3.86; 30 min. 3.63; 60 min. 3.10. The highest yield of formic acid was obtained when oxidation was carried out for 100 minutes at room temperature (yield 4.08 mols./mol).- These experiments were not pursued when it was found that the decarboxylation method for estimating uronic acids was more suitable.

Experiments with the anthrone method (Fairbairn 1953).

The anthrone method, which is finding increasing application as a rapid and sensitive means of estimating polysaccharides, especially in biological studies, is of little direct value in composition studies, but it was found to be useful as an independent check on the composition data derived by the normal methods of hydrolysis and quantitative paper chromatography. This was done by comparing the experimental anthrone value of the polysaccharides with the anthrone values calculated from the composition data. In order to calculate anthrone values it was necessary to know the relative anthrone-colour producing powers of all the component sugars.

The rate of colour development with glucose was determined and 8 minutes heating found to give

maximum colour (fig.3) ; the sharpness of the maximum showed the necessity of including glucose standards with every batch of tubes, and that any variation in heating among the tubes could lead to appreciable errors. The 95% sulphuric acid method of Seifter, Dayton, Novic and Muntwyler (1950) was found to have a similar sharp maximum (fig.3). It was observed during these experiments that the colour of the reaction mixture became progressively less green and more orange as the duration of heating increased. The spectrum of the anthrone-glucose colour was measured at intervals using the Spekker and the eight colour filters. (Table 3, fig.4). The green component was seen to be destroyed as the orange-brown component was formed.

Table 3. Spectrum of glucose-anthrone complex

Filter no.	601	602	603	604	605	606	607	608
" Max absorption (A)	4300	4700	4930	5200	5500	5830	6250	6750
Blank	0.471	0.076	0.046	0.039	0.038	0.036	0.039	0.030
5 min.	0.509	0.142	0.116	0.130	0.191	0.293	0.370	0.235
10	0.548	0.190	0.178	0.180	0.230	0.324	0.380	0.246
12	0.590	0.211	0.202	0.202	0.238	0.321	0.369	0.255
15	0.602	0.229	0.221	0.218	0.239	0.300	0.336	0.238
20	0,595	0.256	0.243	0.232	0.240	0.288	0.312	0.223
30	0.639	0.300	0.305	0.286	0.270	0.275	0.272	0.218
60	0.755	0.390	0.399	0.385	0.342	0.309	0.271	0.228

Fig. 3.

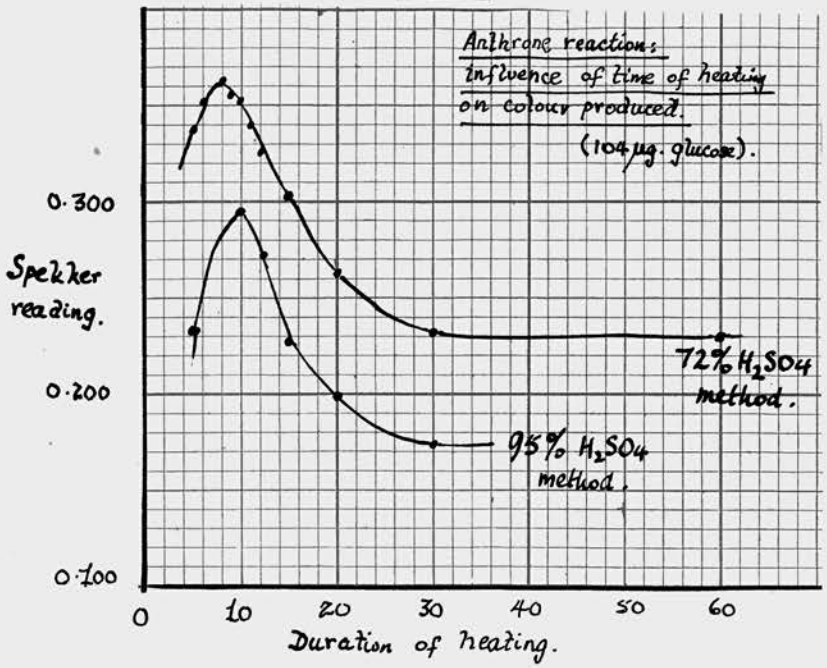
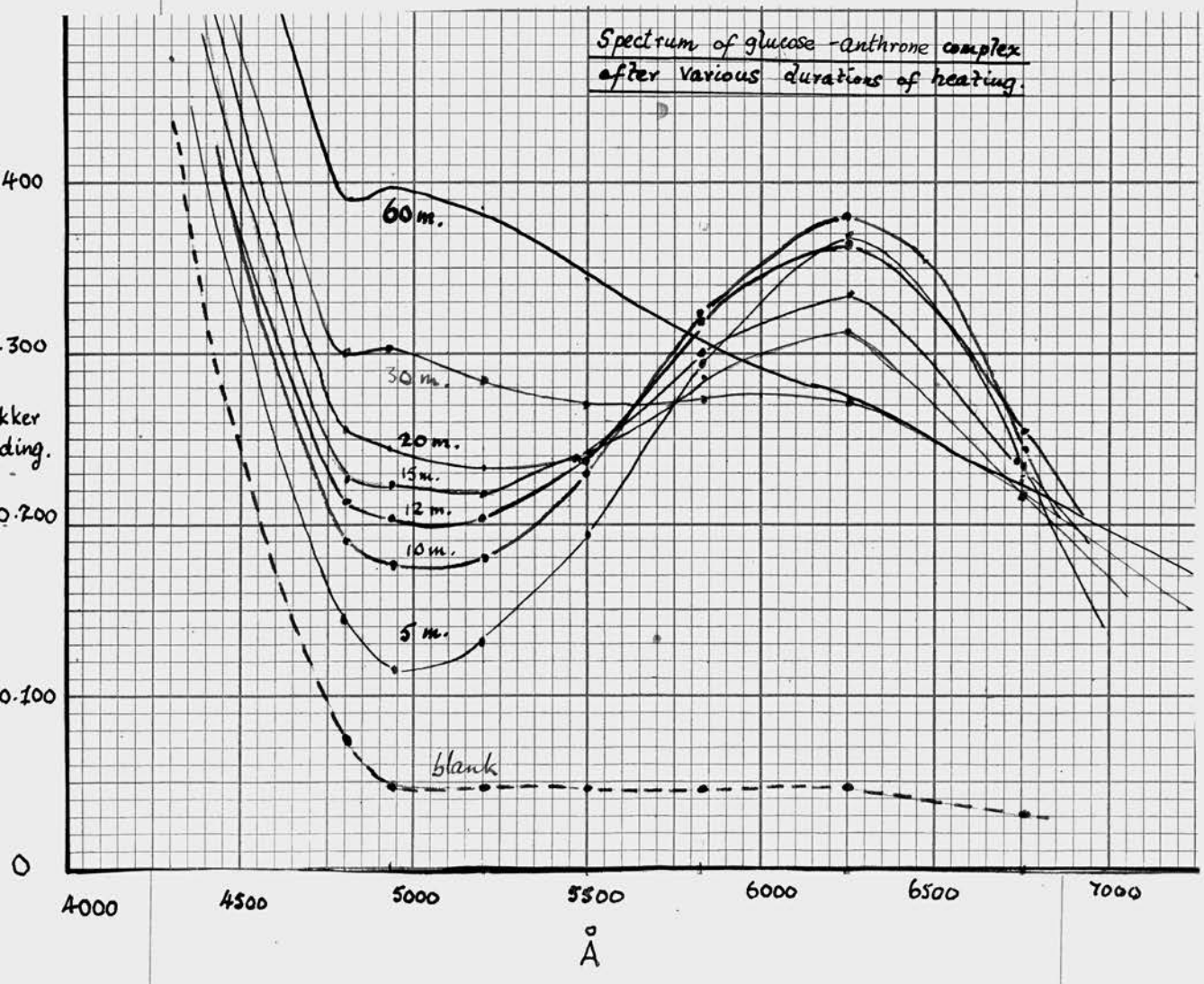

Anthrone reaction:
influence of time of heating
on colour produced.
(104 µg. glucose).
0·300
Spekker reading.
0·200
0·100
72% H_2SO_4 method.
95% H_2SO_4 method.
0
10
20
30
40
50
60
Duration of heating.

Fig. 4


Spectrum of glucose-anthrone complex
after various durations of heating.
60 m.
30 m.
20 m.
15 m.
12 m.
10 m.
5 m.
blank
4000
4500
5000
5500
6000
6500
7000
Å

The colours produced by other sugars in the anthrone reaction were measured. As well as the sugars known to be present in the polysaccharides other monosaccharides were included, so that the behaviour of most types of monosaccharides was compared. It was not possible to carry out the reaction with all the sugars simultaneously and small differences were observed in the value of the glucose standard included with each batch. To make the results strictly comparable they have been corrected to accord with a common glucose value. 104 µg. glucose = 0.350 (Table 4, fig. 5).

Table 4. Anthrone Values.

2 ml. of sugar solution and 10 ml. of 0.1% anthrone in 72% sulphuric acid solution, heated in boiling water for 8 minutes, cooled rapidly, and colour measured with Spekker using Ilford orange-red (607) filters.

Table 4. See over.

From these results "anthrone equivalents" were calculated, i.e. the anthrone-colour producing power of 100 µg. of sugar relative to 100 µg. glucose. The equivalents were used to calculate the anthrone value from the composition data, for comparison with the experimental value. The anthrone equivalents found here are close to those found by Morris (1948).

Table 4. Anthrone Values.

Glucose		Arabinose		Dulcitol	
Wt.	Reading	Wt.	Reading	Wt.	Reading.
52µg	0.187	43µg	0.008	42µg	0
104	0.350	86	0.020	84	0.004
208	0.660	129	0.025	126	0.005
312	0.949				
Fructose		**Fucose**		**Galactose.**	
Wt.	Reading	Wt.	Reading	Wt.	Reading
51µg	0.196	65µg	0.216	47µg	0.102
102	0.370	130	0.410	94	0.185
153	0.521	195	0.570	141	0.264
Glucurone		**Glucosamine**		**Mannose**	
Wt.	Reading	Wt.	Reading	Wt.	Reading
56µg	0.012	45µg	0	13µg	0.031
112	0.019	90	0.002	66	0.125
168	0.036	180	0.006	132	0.228

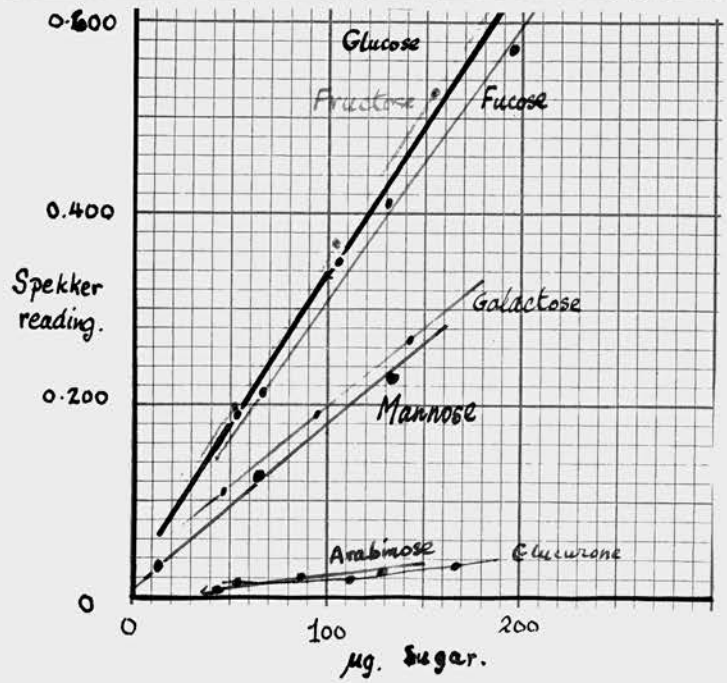


Fig. 5.

Anthrone values.

Anthrone equivalents.

Glucose	1.00
Arabinose	0.07
Dulcitol	0
Fructose	1.06
Fucose	0.92
Galactose	0.60
Glucosamine	0
Glucurone	0.05
Mannose	0.52

Experiments with the Dische and Shettles method for 6-deoxyhexoses.

The colorimetric method for estimating 6-deoxyhexoses described by Dische and Shettles (1948) was used to check the accuracy of the fucose and rhamnose results obtained by chromatographic separation of the polysaccharide hydrolysates and periodate oxidation. Fucose was used in all the control experiments because it was found in 16 of the polysaccharides examined, while rhamnose was found in one.

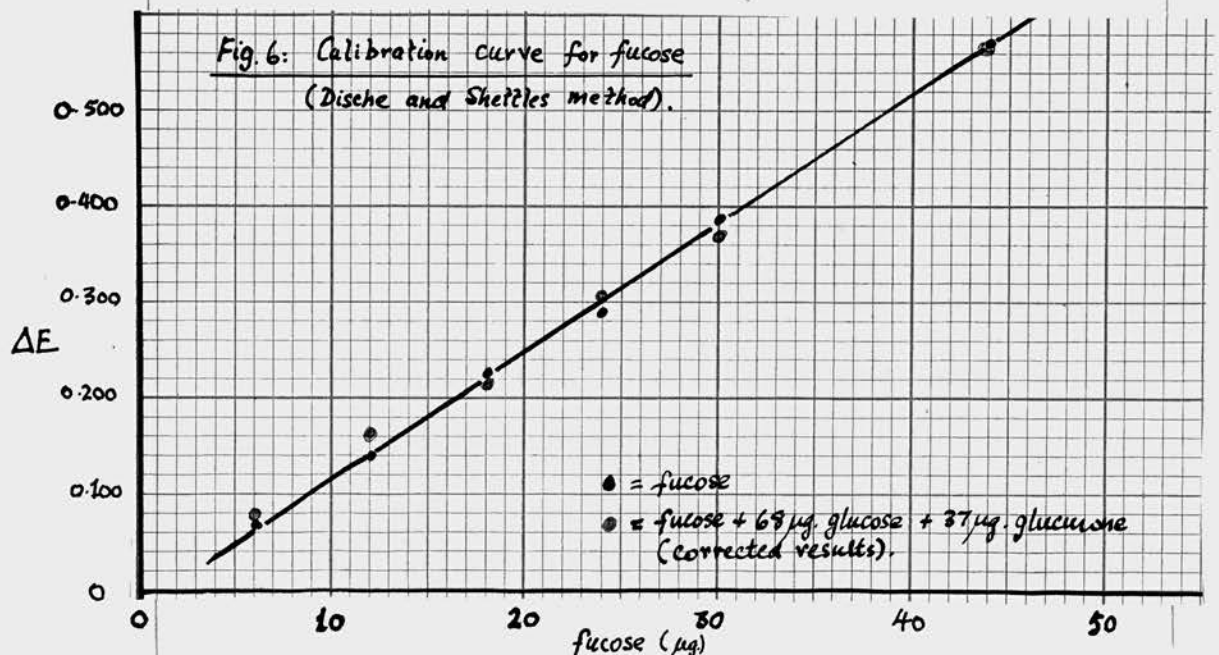
Control experiments were carried out to determine the reproducibility and reliability of the method, to prepare calibration curves and to ascertain the extent of interference by other sugars. It was found that determinations carried out in the same batch was concordant, but the variation from batch to batch made it necessary to include fucose standards with every batch of determinations.

Calibration curves were prepared with fucose alone, and with fucose in the presence of glucose and glucurone. (Table 5 ; fig 6).

Table 5. Calibration curve for fucose.

1 ml. of solution per tube, treated as described above in method.

Fucose	Glucose	Glucurone	E.3960	E.4300	ΔE	ΔE. corrected.
6 µg	—	—	0.064	0	0.064	
12	—	—	0.159	0.019	0.140	
18	—	—	0.238	0.015	0.223	
24	—	—	0.299	0.011	0.288	
30	—	—	0.399	0.016	0.383	
44	—	—	0.610	0.038	0.572	
—	68 µg	37 µg	0.211	0.122	0.089	
6	"	"	0.296	0.127	0.169	0.080
12	"	"	0.396	0.144	0.252	0.163
18	"	"	0.446	0.145	0.301	0.212
24	"	"	0.560	0.168	0.392	0.303
30	"	"	0.600	0.155	0.455	0.366
44	"	"	0.875	0.210	0.665	0.576



The corrected values were close to those for fucose alone, showing that fucose can be determined by this method in amounts ranging from 6 - 50 µg., and in the presence of 100 µg. of glucose and glucurone. It was also found that the colours produced by glucose and glucurone were proportional to the amounts of these sugars (Table 6, fig.6d). In the fucose determinations carried out on the polysaccharides, controls for all the components were included, to allow the corrections to be made by direct proportion.

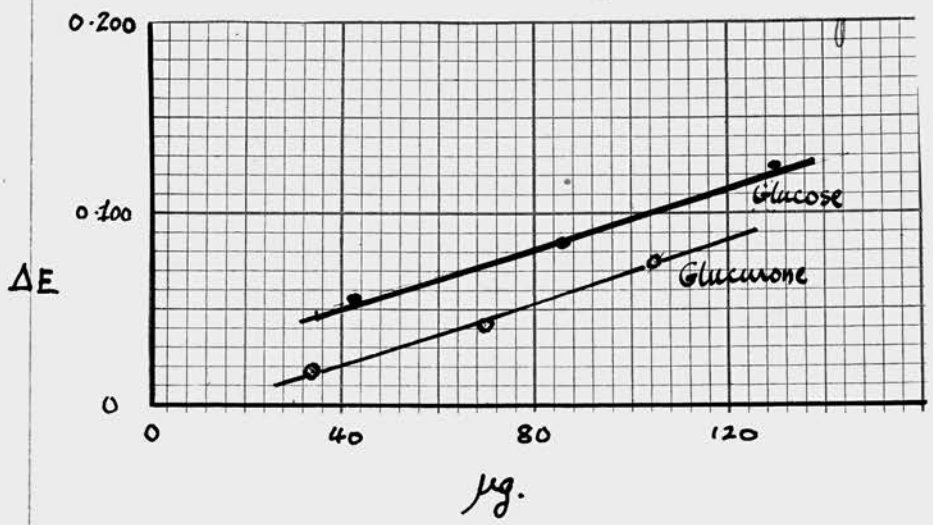
Table 6. Dische and Shettles reaction with glucose and glucurone.

1 ml. per tube, treated as described above in methods.

Amount of sugar taken.		E 3960	E 4300	ΔE
Glucose	43 µg.	0.128	0.074	0.054
	86	0.233	0.150	0.083
	129	0.372	0.246	0.126
Glucurone	34 µg.	0.040	0.021	0.019
	69	0.081	0.040	0.041
	105	0.137	0.062	0.075

The colour producing power of all the component sugars present in the fucose-containg polysaccharides were determined, and the values relative to fucose (as 1.0) found to be: rhamnose 1.0, glucose 0.058, glucurone 0.021 and galactose 0.009. Dische and

Fig. 6a: Calibration curves for glucose and glucurone (Dische and Shettles method).



Shettles recommended the use of internal standards, but this was thought to be unnecessary when the above mentioned corrections were applied. The values of an internal and an external blank were found to agree within the limits of the method(0.197 and 0.178 respectively). The observation made by the authors that unhydrolysed polysaccharides gave higher results than hydrolysed samples was confirmed when it was found that A.aerogenes strain A3(S1) polysaccharide was found to contain 14% fucose when estimated unhydrolysed, and 13% when hydrolysed, (both values uncorrected).

Experiments with colorimetric methods of estimating uronic acids.

The problem to be met in the estimation of the uronic acid components of the polysaccharides under investigation, was to find a method which would measure the amount of uronic acid accurately in the presence of large excesses of other sugars. It was thought that one of the many colorimetric methods based on 'Tollens' naphthoresorcinol reaction would be suitable. A method which included extraction of the colour-complex from the reaction mixture was thought to be more likely to yield accurate results, because of the apparently specific solubility of uronic acid-naphthoresorcinol complex in ether (Tollens 1908) and other organic solvents (reviewed by Jarrige 1947)

The procedure described by Hanson, Mills and Williams (1944), in which the colour-complex is extracted with amyl alcohol was tried. The authors claimed that glucose did not interfere, even when present in amounts equal to that of the uronic acid, but control experiments showed that glucose interfered seriously and diminished the recovery of uronic acid. When increasing amounts of glucose (20. 50. 70 and 100 µg.) were

added to glucurone (74 µg.), the uronic acid, as estimated by the method, decreased in the following order : (expressed as percentage of colour when no glucose was present) : 97, 92, 84 and 77%. As it was known that the polysaccharides contained at least 60% of other components this method was obviously unsuitable. In the face of often conflicting evidence advanced by many authors (Maughan, Evelyn and Browne 1938; Kapp 1940; Meyer, Bloch and Chaffee 1942; Ratish and Bullowa 1943; Diechmann and Dierker 1946) as to the conditions required for optimum extraction of the uronic acid-naphthoresorcinol complex, extraction modifications of the colorimetric method were not investigated further.

The Jarrige (1947) method was used in control experiments and found to be more satisfactory. Although glucose interfered it was to a much lesser extent than above. A calibration curve was prepared with glucurone and a straight-line relationship found over the range 0 - 50 µg. (Table 7, fig. 7.)

Table 7. Relationship between glucurone amount and colour produced by Jarrige method of uronic acid Estimation.

Glucurone.	Spekker reading.
0 µg.	0
12	0.060
24	0.118
36	0.187
48	0.248

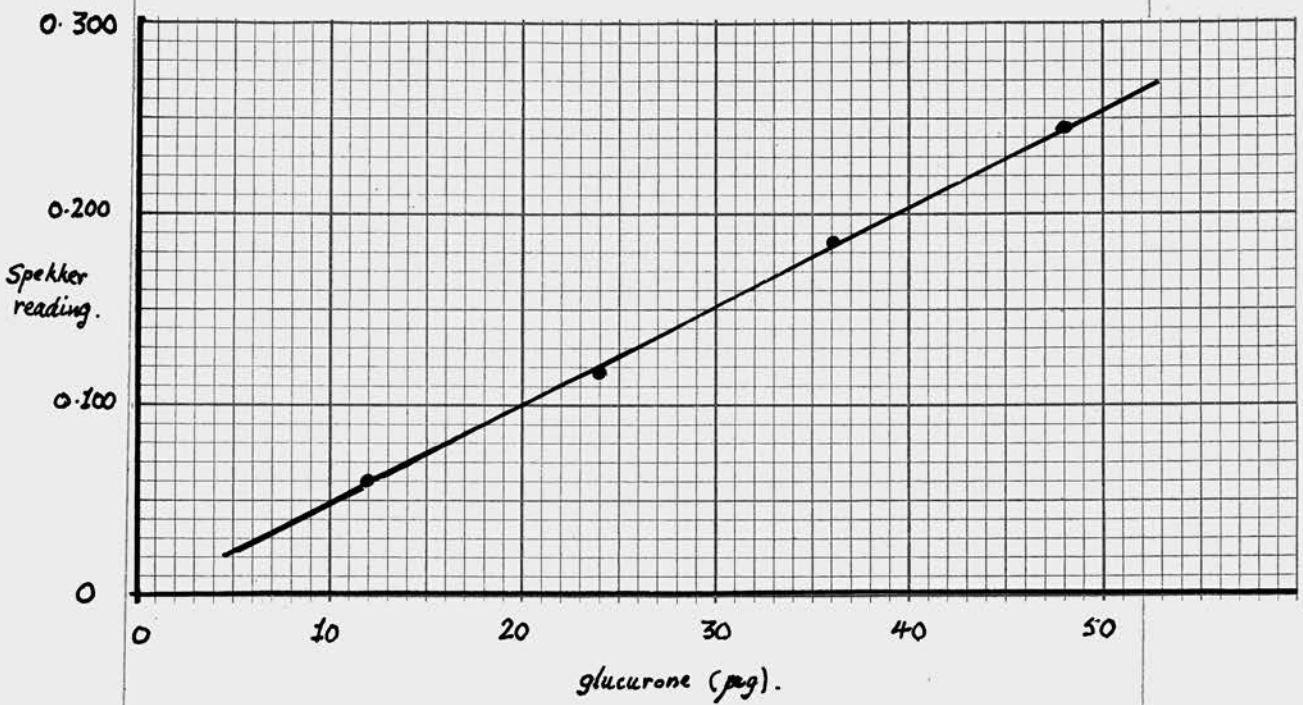


Fig: 7: Glucurone calibration curve (Jarrige method).

The extent of interference from glucose was determined by adding increasing amounts of glucose to glucurone samples and carrying out the estimations in the usual way. (Table 8). The results showed that the interference from glucose was constant (mean value of uronic acid recovery 112%) up to a glucose : uronic acid ratio of 5:1 and that the Jarrige method was sufficiently reliable for the estimation of uronic acid in these polysaccharides.

Table 8. Interference of glucose in glucurone estimation.

Glucose added.	Glucurone added	estimated	Recovery
0 µg	19.8 µg	19.8 µg	100
10	19.8	22.2	112
25	19.8	21.8	110
50	19.8	22.6	114
100	19.8	22.0	111
200	19.8	26.5	134
50	0	0	

Since the estimations were carried out in conditions not likely to cause complete hydrolysis of the polysaccharide, a sample of polysaccharide (A3 capsule) was hydrolysed in N sulphuric acid at 100° and at intervals, samples were withdrawn and the Jarrige method applied. (table 9).

It is not surprising that the estimated uronic acid did not rise above 20%, because it is now known that N sulphuric acid would not cause complete hydrolysis to the free uronic acid. Until the Jarrige method was abandoned in favour of the decarboxylation method the standard condition of hydrolysis for uronic acid estimations was 15 hours with N sulphuric acid at 100°. Although glucurone standards were used in conjunction with these determinations it was found that the results would be applicable to galacuronic acid, because equal amounts glucuronic and galacturonic acid produced the same amount of colour.

Table 9. Uronic acid estimations on a sample of hydrolysed A3 capsular polysaccharide (hydrolysed in N sulphuric acid at 100°.

Duration of hydrolysis.	Uncorrected uronic acid.	Corrected* uronic acid.
0	10.5%	9.3%
1 hr.	11.2	10.0
2	12.3	11.0
6	16.8	15.0
15	20.3	18.1

* multiplying by factor $\frac{100}{112}$

Experiments with alcohol-precipitation of barium glucuronate.

The lactone trails found in the chromatograms of the hydrolysed polysaccharides reduced the

accuracy of the results obtained by quantitative paper chromatography. Since the uronic acid components were to be estimated by a non-chromatographic method (decarboxylation) it was thought desirable to remove the uronic acids from the hydrolysates before the chromatographic separation of the other components. This could be done satisfactorily by precipitation of the barium salts of the uronic acids with alcohol, but it was necessary first to show that this would not alter the ratio of the remaining component sugars. The following experiment was carried out with this aim.

A mixture of glucose, arabinose and glucurone was prepared by taking 22.3, 22.1 and 22.0 mg. of each sugar, respectively. The mixture was dissolved in 3.6 ml. of water and acidified with 0.4 ml. of 10 N sulphuric acid, to make 4 ml. of N sulphuric acid. The solution was neutralised with barium carbonate and the precipitate removed by centri- fugation. The supernatant was withdrawn and 2 volumes of ethanol added to it. A finely divided precipitate formed which was allowed to settle out overnight at 4°. After centrifugation the supernatant was examined on a chromatogram and found to contain some uronic acid but there was no lactone trail. The glucose and arabinose were estimated at the stages indicated below by withdrawing samples, treating with Amberlite IR-120 resin, and chromatographic aration, followed by periodate oxidation. The results dicated that withing the limits of experimental error,

there was no change in the relative amounts of glucose and arabinose.

Sample taken from	Glucose and arabinose in mixture Glucose	arabinose	RATIO
1. Initial mixture by weighing.	22.3 mg	22.1 mg	1.01 : 1
by estimation,	0.746	0.705	1.06 : 1
	0.912	0.892	1.02 : 1
2. Supernatant after neutralisation with barium carbonate.	0.573	0.534	1.07 : 1
	0.609	0.576	1.06 : 1
3. Supernatant after precipitation with 2 volumes of ethanol.	1.031	0.980	1.05 : 1
	1.204	1.153	1.04 : 1

Experiments with moisture uptake.

Measurements were made to determine the absorption of moisture by the polysaccharides. The amount of moisture, and the rate with which it was found to be absorbed, indicated the necessity of drying them in vacuo over phosphorus pentoxide, and the need for speed in weighing out samples for analysis.

A sample of polysaccharide (A.aerogenes, strain A3(S1) was dried over phosphorus pentoxide in vacuo and then allowed to stand in contact with the atmosphere. The sample was weighed at frequent intervals until constant weight was attained. The results are shown in fig. 8.

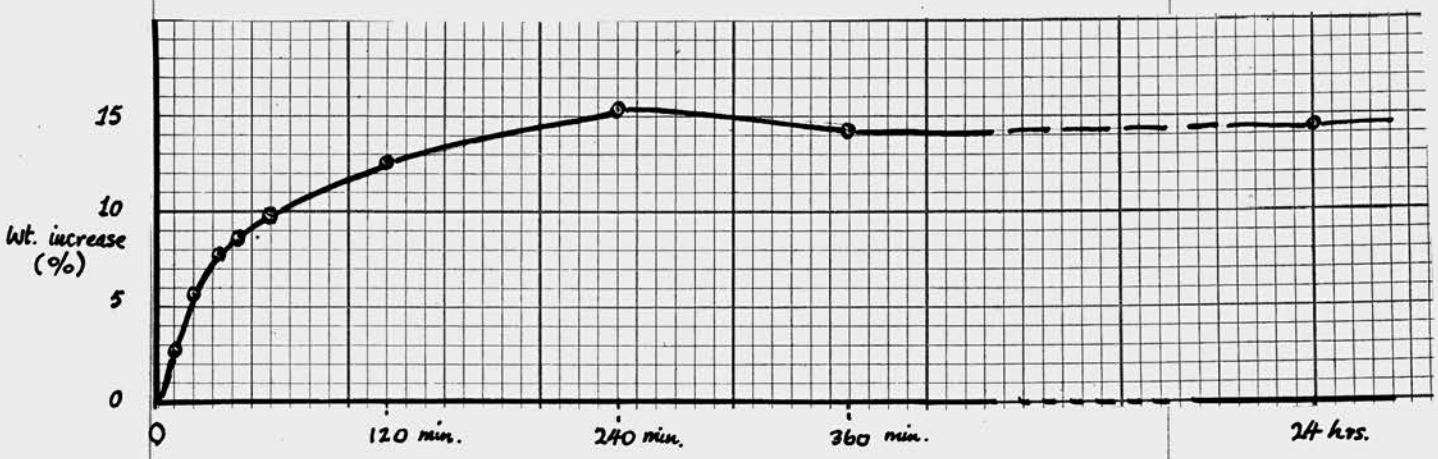


Fig. 8: Moisture taken up by A. aerogenes A3(Sl) polysaccharide.

C. RESULTS OF EXOPOLYSACCHARIDE ANALYSIS.

The polysaccharide of *A.aerogenes* strain A3(S1) was examined more thoroughly than any other and the results are given in more detail, because this strain was to be used in a study of the influence of carbon substrate on polysaccharide composition (described in Part II of this thesis). The other polysaccharides are dealt with more briefly. The results for the neutral component sugars (estimated by paper chromatographic separation and periodate oxidation) are given in some detail separately for each polysaccharide, but the mean values for these and all the other estimations are given in table 11. The molecular ratios of the component sugars present in each polysaccharide, and the results of the serological experiments are shown in tables 12 and 14 respectively.

1. Aerobacter aerogenes exopolysaccharides.

(a) Polysaccharide of strain A3 (S1) (non-capsulate, slime-producing strain).

The procedure used for culturing the cells and isolating the polysaccharide followed the general lines described above in Materials and Methods. The yield was generally about 1 g./ 1.2 litres of 48 hour culture. Three different samples of the polysaccharide were prepared, each purified in a slightly different way. The first sample was not boiled during the purification procedure, it was not de-proteinised and was finally isolated by freeze-drying. The second sample was not boiled but was de-proteinised and was isolated by precipitation with 2 - 3 volumes of acetone from 1% sodium acetate solution. The third sample differed from the second only in that it was boiled. These three A3(S1) polysaccharide preparations are referred to hereafter as the freeze-dried, unboiled sodium salt and boiled sodium salt preparations respectively.

The homogeneity of the polysaccharide (A3S1 freeze-dried preparation) was shown by ultracentrifugation. (Kindly carried out by Dr. C.T. Greenwood, Chemistry Dept., Edinburgh University). The sedimentation constant (s_{20}) was determined in 0.1 N sodium hydroxide and found to have the value 1.8×10^{-13}. The sedimentation diagram is shown in fig. 9.

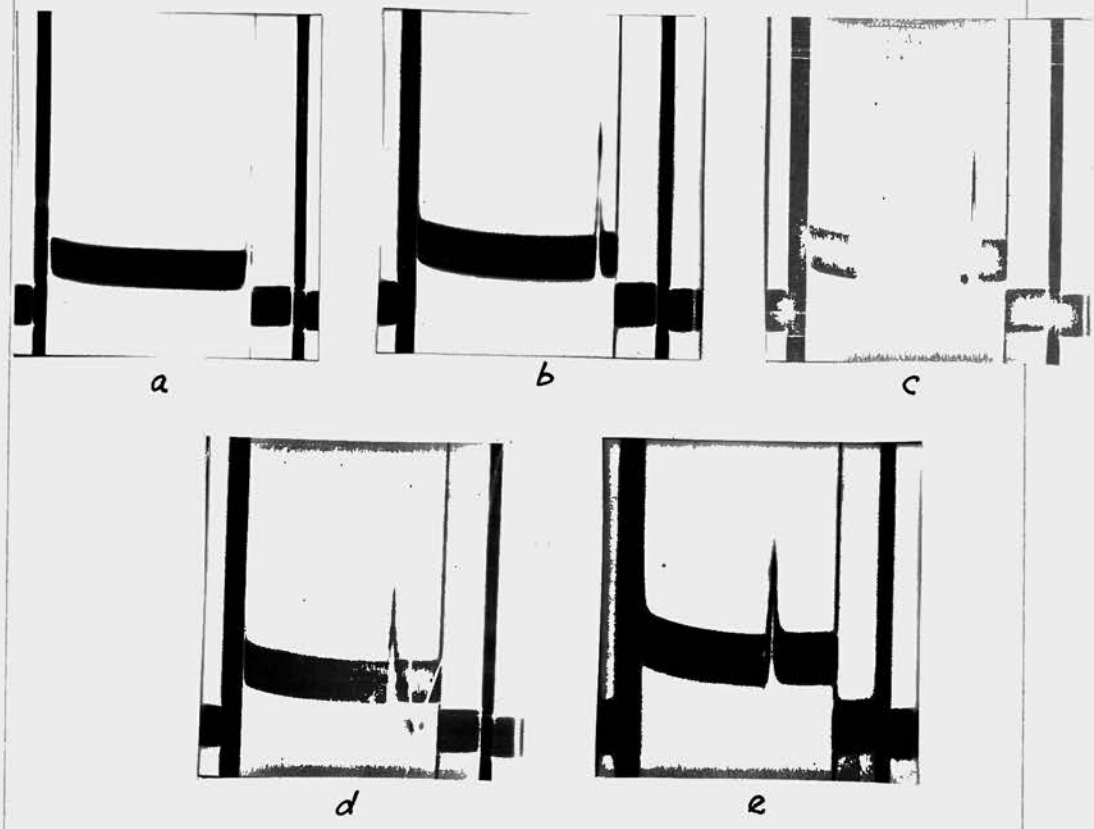


Fig. 9. Sedimentation diagrams for A. aerogenes strain A3(S1) freeze-dried exopolysaccharide. Speed 59, 700 rpm. Solvent 0.1 N NaOH. Concentration 2.5%. Times of exposure: (a) 5 min., (b) 37 min., (c) 71 min., (d) 103 min., (e) 133 min., from starting the centrifuge.

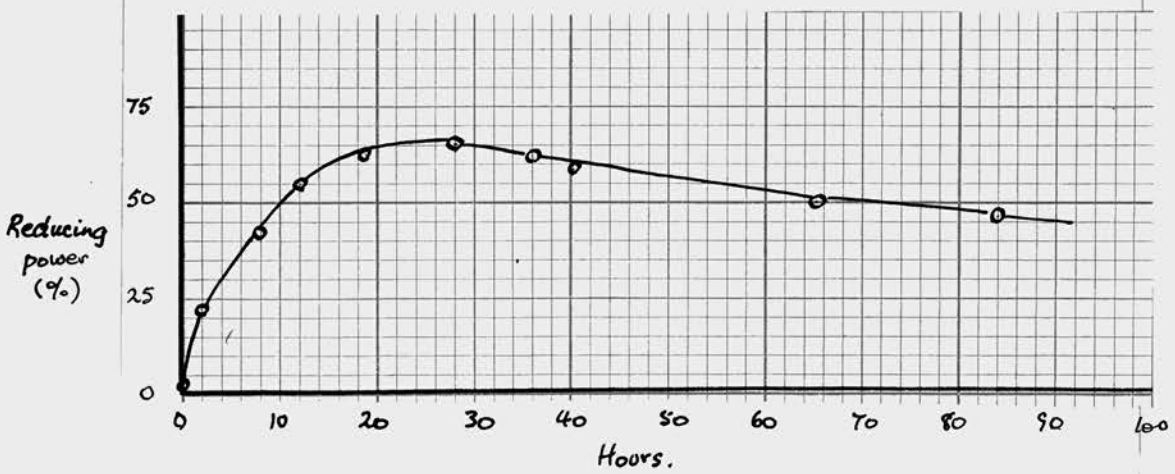


Fig. 10: Hydrolysis of A. aerogenes [A3(S2)] polysaccharide ($N\ H_2SO_4$)

Identification and estimation of component sugars.

(1) Hydrolysis.

Drastic conditions were found to be necessary for the optimum hydrolysis of the polysaccharides. A hydrolysis curve was obtained for A3(S1) freeze-dried polysaccharide by following the reducing power of a solution in N sulphuric acid at 100°. A sample of polysaccharide (20 mg.) was dissolved in 100 ml. of N sulphuric acid and heated under reflux in a boiling water-bath. At intervals 2 ml. samples were removed and neutralised with N sodium hydroxide and the reducing power measured by the Somogyi method. At the end of the experiment the volume of the remaining solution was found to be only 2 ml. less than expected, showing that there was no loss by evaporation and therefore no change in carbohydrate concentration. The results (table 10 fig.10) show that under these conditions the maximum reducing power (65%) was reached after about 24 hours hydrolysis, and that prolonged hydrolysis led to a decline in reducing power.

Table 10. Hydrolysis of A3(S1) freeze-dried polysaccharide.

Duration of hydrolysis	Reducing power (relative to glucose)
0 hrs	2.5%
2	22
8	42
12	55
19	63
28	65
36	62
40	59
65	50
84	41

Chromatographic evidence showed that N sulphuric acid hydrolysis was not sufficient for complete hydrolysis. Chromatograms were run with samples taken after 12, 28 and 65 hours hydrolysis, and all appeared to be similar. They all showed the presence of glucose, fucose and a small amount of galactose. No uronic acid or lactone spots were observed but some oligosaccharide spots were detected; the chromatograms of the 12 and 28 hour samples also showed some material at the starting line.

It was evident that stronger hydrolysis conditions were necessary: a sample (19 mg) of the polysaccharide was hydrolised with 95% formic acid (1 ml.) in a sealed tube for 7 hours at 100°. After the acid had been removed by distillation in vacuo at 45 - 50° the syrup was heated under reflux with water (1 ml.) for 5 hours, to hydrolyse formyl esters. A chromatogram showed the presence of two oligosaccharides as well as glucose, fucose and galactose, but free uronic acid was not observed. The ogilosaccharides were isolated from other paper chromatograms and hydrolysed with N sulphuric acid for 6 hours at 100°, and after neutralisation with barium carbonate and treatment with Amberlite resin IR-120 the solutions were examined on paper chromatograms. The faster moving oligosaccharide contained glucose, fucose and galactose, while the slower

oligosaccharide contained a uronic acid in addition to these sugars.

To examine the uronic acid fraction and determine whether it was hydrolysed to the free acid or was still bound to other sugars after the above hydrolysis, a larger scale hydrolysis using 100 mg. of polysaccharide was carried out with the above conditions of hydrolysis (7 hours with 95% formic acid (5 ml.) followed by 6 hours with N sulphuric acid (5 ml.)). The solution was neutralised with barium carbonate and treated with Amberlite resin IR-120, and then evaporated to low volume by distillation in vacuo at 45-50°. The whole syrup was transferred to paper chromatogram sheets which were run in the benzene-butanol-pyridine-water solvent for 44 hours. The uronic acid fraction was located by examining side-strips, and were eluted from the chromatograms with cold water in the normal way. The eluates were combined and treated with Amberlite resin IR-120, and then evaporated to a syrup by distillation in vacuo; the syrup was dried over phosphorus pentoxide and weighed 23 mg. It was dissolved in water (15 ml.) and titrated with dilute standard (0.0107N) sodium hydroxide using bromothymol blue as indicator ; the titre (6.09 ml.) was equivalent to 12.6 mg. of uronic acid. Aliquots of the solution were used for determinations by a variety of methods. The Jarrige colorimetric method gave a

result equivalent to a total of 5.5 mg. of uronic acid in the solution. The colorimetric Somogyi method indicated a reducing power equivalent to 4.4 mg. of glucurone or 6.0 mg. of galacturonic acid. Periodate oxidation indicated 11.5 mg. of glucurone. These results suggested that the uronic acid fraction of the hydrolysate did not consist of free acid but combined in an aldobiuronic acid or larger oligosaccharide. A sample of the solution (3 ml.) was hydrolysed in N sulphuric acid at 120° for 24 hours (sealed ampoule in an air oven). The contents were neutralised and prepared for chromatographic separation as described above. A chromatogram showed spots with the R_G value of an uronic acid as well as glucose and fucose. When it was seen that the presumed uronic acid was in fact an oligosaccharide, the remainder of the solution was hydrolysed under the same conditions and the relative amounts of the component sugars estimated by paper chromatographic separation and periodate oxidation. The proportions of uronic acid, glucose and fucose was found to be of the order 1:1:5.

The above experiment showed that more drastic conditions were required to obtain complete hydrolysis. Samples of the polysaccharide were hydrolysed with 95% formic acid under more severe

conditions. One sample was hydrolysed for 7 hours at 120° while another was hydrolysed for 24 hours at 100° ; in both samples further hydrolysis was carried out with N sulphuric acid for 6 hours at 100° after the formic acid had been removed by distillation in vacuo. Both hydrolysates appeared to be the same on chromatograms; both showed the presence of a lactone spot and a lactone trail running from the lactone to the uronic acid spot near the starting line, in addition to the other sugars referred to above. The presence of lactones and trails was taken as indicating complete hydrolysis of the polysaccharide. Of these two hydrolytic conditions, the former in which hydrolysis was carried out with formic acid at 100° for 24 hours was chosen because it appeared to cause less destruction, as judged by the degree of charring ; this was confirmed by comparing the results of analyses carried out under both conditions. It would have been desirable to have obtained hydrolysis curves for these conditions but this was not done because of the difficulty introduced by the need for two succesive hydrolyses. A sample of polysaccharide was hydrolysed using the 24 hour formic acid / 6 hour sulphuric acid method and the reducing power found to be equivalent to 78% glucose, a higher value than was obtained in the hydrolysis with N sulphuric acid alone.

The 24 hour formic acid/ 6 hour N sulphuric acid treatment was chosen as the standard method for hydrolysing all the polysaccharides for qualitative and quantitative analysis. It was thought possible that these hydrolyses conditions might cause some decarboxylation of the uronic acid component which would lead to false results, but a control experiment in which glucurone and galacturonic acid were subjected to the hydrolysis treatment showed that no decarboxylation products could be detected.

In conjunction with the experiments on hydrolysis conditions described above, the component sugars of the freeze-dried preparation of A3(S1) polysaccharide were estimated after hydrolysis under a variety of conditions. The estimation were carried out by paper chromatography, elution and periodate oxidation, as described in Methods.

Component sugars of A3(S1) freeze-dried polysaccharide.

See over ➡

The reference sugars were found to contain other sugars as impurities. Arabinose (0.5%) and galactose (0.5%) were found in the rhamnose, and galactose (1%) in the arabinose. The above results were corrected for galactose.

In analyses 1 - 6 the uronic acid was not

COMPONENT SUGARS OF A3 (S1) FREEZE-DRIED POLYSACCHARIDE.

	1	2	3	4	5	6	7	8	9
Wt. of polysaccharide taken for analysis (mg)	35.0	45.8	31.1	32.8	33.3	30.8	18.8	21.5	18.8
Reference sugar (mg)	13.11 Rh	23.3 Rh	21.3 A	26.9 A	21.2 A	25.4 A	19.1 A	22.0 A	23.6 A
Hydrolysis conditions									
(i) formic acid Vol	1 ml	1 ml	1 ml	2 ml	1 ml	2 ml	2 ml	2 ml	2 ml
Time	7 hr.	7 hr.	24 hr.	24 hr.	7 hr.	7 hr.	24 hr.	24 hr.	24 hr.
Temp.	100°	100°	100°	100°	120°	120°	100°	100°	100°
(ii) N sulphuric acid (6hr. at 100°) Vol	1 ml	1 ml	1 ml	2 ml	1 ml	2 ml	2 ml	2 ml	2 ml
Component sugars :-									
Uronic acid %	19	23	37	39	32	34	-	-	-
Galactose %	2	2	1	1	1	1	1	1	1
Glucose %	36	41	36	37	31	33	36	38	46
Fucose %	12	8	10	11	6	10	9	9	12

(Rh = rhamnose A = arabinose).

Each set of results in the table is the average obtained from 2-4 determinations carried out on every hydrolysate. The results are expressed as the nearest whole number.

precipitated from the hydrolysate. In analyses 1 and 2 no lactone or trail was observed and the uronic acid was estimated by periodate oxidation assuming it to be glucuronic acid and to give rise to 5 molecules of acid per molecule. In analyses 3 - 6 lactones and trails were present in addition to uronic acid. The lactone estimations were made by assuming that it was glucurone and that it would give rise to 4.15 molecules of titrable acid per molecule. The same assumption was made for the lactone present in the trails, which was estimated in small sections in which no other sugars were present, and then calculating the lactone present in the whole trail. It was necessary to correct the glucose, galactose and fucose results for the presence of the lactone trail in their respective sections of the chromatograms. The total uronic acid found in analyses 3 - 6 was obtained by adding the results found for the free uronic acid, lactone and trail.

The hydrolyses for analyses 3 - 6 were carried out simultaneously and the hydrolysate of 4 found to contain less charred material than those of 3,5 and 6, indicating that less destruction had taken place.

The chromatograms of analyses 1 - 8 were developed with the benzene-butanol-pyridine-water solvent, and were found to have erratic and large blanks.

The chromatograms of analysis 9 were developed in the butanol-ethanol-water solvent which gave blanks that were smaller and more reproducible. Since the composition of all the other polysaccharides were determined using chromatograms run in the latter solvent, the results of analysis 9 were used for comparison with other polysaccharides.

(ii). Identification of component sugars

The component sugars of the A3(S1) polysaccharides were identified by paper chromatography. The hydrolysates, after neutralisation and treatment as described above, were run with sugar standards on chromatograms irrigated with three different solvents. The identity of a component was accepted when it was found to travel alongside a standard in all the solvents, and to give the same colours as the standard with the various spray reagents. The solvents used were: ethyl acetate-pyridine-water (5:2:5) (Jermyn and Isherwood 1949), benzene-butanol-pyridine-water (1:5:3:3) and butanol-ethanol-water (5:1:4). The spray reagent that was most used was saturated aqueous aniline oxalate, but p-anisidine hydrochloride and naphthoresorcinol were also used. By this means, the A3(S1) polysaccharides were shown to contain glucose, galactose, fucose and an unidentified uronic acid which formed a lactone. The absence of hexosamine was confirmed by spraying chromatograms with ninhydrin and with the Morgan and Elson reagents (Partridge 1948b).

Because none of the above-mentioned solvents gave good separations of uronic acids, some chromatograms were irrigated several times in the ethyl acetate-pyridine-water solvent, according to the multiple development technique advocated by Jeanes, Wise and Dimler (1949). It was found that multiple

development up to 12 times did not improve the relative separation of the spots; they merely became more diffuse and thus did not aid the identification of the uronic acid. Chromatograms of the hydrolysates together with glucurone, galacturonic and mannuronic acid standards were run in acidic solvents which have been claimed to give good separation of uronic acids, but without success. The A3(S1) uronic acid on these chromatograms did not behave exactly like any of the standards; sometimes it would resemble one standard and sometimes another so that the results were ambiguous. The solvents which were tried in this connection were butanol-acetic acid-water (4:1:5) (Partridge, 1946) and ethanol-acetic acid-water (3:1:3) (Rao, Beri and Rao 1951). Attempts were made to identify the uronic acid by using the non-chromatographic specific colorimetric tests devised by Dische (1947, 1948) but the results were again ambiguous. The only reliable fact that emerged from the attempts to identify the uronic acid was that it formed a lactone, and therefore was unlikely to be galacturonic acid.

The identification of glucose and fucose was confirmed by large-scale isolation from a cellulose column and the preparation of derivatives. Trial separations on cellulose columns were carried out with a synthetic mixture of approximately the same composition as the A3(S1) polysaccharides, and

water-saturated butanol found to give better results than butanol-ethanol-water (5:1:4). The polysaccharide sample used for the large scale isolation consisted of 2 g. of impure material. This was hydrolysed for 24 hours in 200 ml. of 95% formic acid by heating under reflux at 100°. This large volume of acid was used to maintain a low polysaccharide concentration in order to minimise charring. The solution was distilled in vacuo until all the formic acid was removed and then heated under reflux for 6 hours at 100° with 100 ml. N sulphuric acid. The solution was then transferred to 50 ml. centrifuge tubes and neutralised with barium carbonate. After centrifugation the supernatant was withdrawn and the precipitate washed twice with a total of 60 ml. of water each time. The supernatant and washings were combined and distilled in vacuo to a smaller volume (30 ml.), and then poured into methanol (60 ml.) and left overnight at 4°. After centrifugation the supernatant was withdrawn and the buff-coloured precipitate washed with 65% aqueous methanol; the washings were combined with supernatant which was found still to contain some uronic acid in addition to glucose, galactose and fucose. The supernatant was kept at 4° for 2 days when a slight amount of precipitate settled out. The clear supernatant was decanted and the precipitate, after washing with 65% aqueous methanol, was combined with the main precipitate. The

supernatant was examined chromatographically and found to contain uronic acid, glucose and fucose in the proportion 1:5:1. Since the proportions in the polysaccharide are of the order 3:5:1, this showed that the supernatant now contained only a third of the total uronic acid and that therefore two-thirds of the uronic acid was in the precipitate. A hitherto unobserved spot was seen on these chromatograms; its rate of movement relative to that of fucose in the butanol-ethanol-water solvent was 1.2. An attempt was made to estimate it but it was present in amounts that were too small for this purpose. It was only visible in ultraviolet light. It was possible that the unknown was fucoketose, formed in slight amounts by epimerisation of fucose during contact with barium carbonate. To test this and to obtain a sample of fucoketose to use as a chromatographic standard, fucose solutions were treated overnight with alkali at room temperature (one solution was left in contact with barium carbonate and another with 0.1 N sodium hydroxide). Both solutions were applied to chromatograms with some of the supernatant and very faint spots found in all three, alongside each other, identifying the unknown as a product of alkali action on fucose, most probably fucoketose.

Because the uronic acid in the supernatant might interfere with separation by forming a lactone trail in the column, it was removed by passing the solution through a column of anion-exchange resin.

Amberlite IRA-400 resin was used, in the carbonate form which has been shown by many authors not to cause carbohydrate transformations or to retain neutral sugars like the more strongly basic hydroxide form. (Roseman, Abeles and Dorfman 1952, Hulme 1953, Rebenfeld and Pacsu 1953, Woolf 1953). The carbonate form of the resin was prepared by washing 100 g. successively with 5 litres of water, 750 ml. of 2N hydrochloric acid, 5 litres of water, 500 ml. of 2% sodium carbonate and finally with 10 litres of water, by which time the effluent was neutral. The supernatant solution was passed through the resin column and emerged distinctly alkaline. It was collected in a flask in which some Amberlite IR-120 cation-exchange resin had been put to counter-act the alkalinity. The contents of the flask were found to be at pH 3. The column was washed with water until the effluent was non-reducing (colorimetric Somogyi test). The solution was filtered off from the resin and a small volume evaporated to a syrup for chromatographic examination, during which the smell of formic acid became noticed. Glucose and fucose were found to be the main constituents of the solution, in the presence of small amounts of uronic acid and the presumed fucoketose. It was probably the formic acid that accounted for the acidity of the solution. The rest of the solution was distilled in vacuo and the syrup dried over phosphorus pentoxide to constant

weight (1.5 g.). When 10 - 15 ml. of water-saturated butanol was added to the syrup in order to dissolve it and add it to the cellulose column, it was found that the syrup would not dissolve. Water and ethanol were added in small amounts until the syrup dissolved while maintaining one liquid phase. The final volume was approximately 30 ml.

The cellulose column (21 x 1½ in) was set up in a 37° incubator to maintain it at a constant temperature, because the laboratory was subjected to large temperature fluctuations. The dissolved syrup was added dropwise to the top of the cellulose, the top inch of which had been allowed to drain free of solvent. The first 300-400 ml. of solvent that was used was only half saturated with water, to prevent water from separating out in the column. Then the fully saturated butanol was applied. The effluent was collected in 20 ml. tubes changed by hand at 10 ml. intervals. To locate the sugar fractions, 1 ml. was taken from every 10th tube and evaporated to dryness in an air stream and the anthrone test carried out. This gave a rapid indication of the presence of sugar; to identify the sugar another sample was taken from the tube, evaporated to a syrup and put on a paper chromatogram. In connection with the anthrone tests, satisfactory results were obtained only when all the butanol had been removed. The distribution of sugars in the collecting tubes was found to be as follows

Tube	Sugars present
0 - 54	--
55 - 95	fucose
96 - 124	fucose and increasing amounts of glucose
125 - 140	glucose
141 - 300	glucose and v. faint trace of uronic acid
301 - 330	--

The fucose fraction was isolated by combining tubes 55 - 95 and distilling in vacuo at 50°. Water was added and the distillation continued to dryness; this was done to try to remove butanol and butanol products but even after 12 additions it was not completely successful. The syrup was dissolved in water (10 ml.), and filtered through sintered glass to remove traces of cellulose fibres. The sintered glass was washed with water (5 ml.) which was added to the filtrate. The solution was distilled in vacuo and the syrup dried over phosphorus pentoxide and paraffin wax, the latter to remove the remaining traces of butanol. Weight of fucose syrup 93 mg. For the measurement of optical rotation the syrup was dissolved in 6 ml. of water.

$[\alpha]_D^{15}$ - 69° (c = 1.6, d = 0.5)

L-fucose mutarotation value - 76°, Rodd (1952)

The sign of the rotation showed that the fucose present in A3(S1) polysaccharide is the L-isomer. The identification was confirmed by the preparation of fucose phenylhydrazone, following the details described by Charalambous (1953) on a reduced scale. The fucose syrup was dissolved in 1 ml. of ethanol and half of this taken for the preparation of the derivative. The alcoholic solution was evaporated to a thick syrup

and a drop of glacial acetic acid and 0.01 ml. of phenyl-hydrazine added. The reaction mixture was cooled in ice and within a short time yellow crystals were formed. More solid was obtained by gently evaporating the liquid in an air current while it was being cooled. The solid was filtered and carefully washed with cold ethanol and ether. After drying, the melting point was found to be 156 - 158°. An authentic sample of fucose phenylhydrazone was obtained and found to have a melting point 159 - 160°. A mixed melting point was determined and found to show no depression. (Mixed m.p. 156 - 159°). (All m.p. determinations were carried out with a Köfler micromelting apparatus). The optical rotation and derivative confirmed that the sugar isolated as fucose from the A3(S1) polysaccharide was L-fucose.

The glucose fraction was collected by combining tubes 125 - 245; the remainder of the glucose-containing tubes were not used because they contained only small amounts of sugar. The glucose was obtained as a syrup by the same procedure as the fucose, described above. The weight of the glucose dried syrup was 300 mg. For the measurement of the optical rotation the syrup was dissolved in 10 ml. of water. (d = 1) $[\alpha]_D^{15}$ + 50°.

The identification was confirmed by the preparation of β-penta-acetylglucose, following the method described by Mann and Saunders (p. 113) suitably modified for the much smaller quantity of glucose. Half of the glucose syrup was taken by dissolving it in

a small volume of water, removing half and evaporating to vacuo. Anhydrous sodium acetate (100 mg.) and acetic andydride (1 ml.) were added, and the solution heated under reflux for an hour in boiling water. The solution was poured into cold water (25 ml.) when a dark viscous oil separated out. The water was decanted and shaken twice with chloroform (5 ml.) to extract dissolved pentaacetylglucose. The chloroform was then added to the oil and was extracted 3-4 times with water (10 ml.) to remove any acetic acid that was present. The chloroform solution was then evaporated to dryness at the water-bath, several drops of ethanol were added and the solution was left at room temperature overnight. The small amount of solid that appeared was washed several times with cold ethanol and ether, and dried. The melting point was found to be 124 - 126°. The melting point of an authentic specimen of β-pentaacetyl-glucose was determined and found to be 127 - 128°. A mixed melting point was determined and no depression observed. (Mixed m.p. 124 - 127°). This confirmed that the sugar isolated from A3(S1) polysaccharide was D-glucose.

The uronic acid in the Amberlite IRA-400 column was eluted with 0.2N hydrochloric acid. The elution was continued until the effluent was no longer reducing (ca. 100 ml. was required). A chromatogram showed that the effluent contained only uronic acid. The precipitated uronic acid, obtained when the polysaccharide

hydrolysate was precipitated with methanol, was redissolved and reprecipitated several times and was finally combined with the uronic acid from the resin column. Traces of IRA-400 resin which were present in the solution were removed by shaking with Amberlite IR-120 resin. The solution was filtered from the resin and neutralised with N sodium hydroxide. It was distilled in vacuo and dried over phosphorus pentoxide. (Wt. of syrup 600 mg.). The syrup contained only slight traces of other sugars but it was feared that some of the uronic acid might still be combined with other sugars in aldobiuronic acids. It was thought that the following procedure would be more reliable than direct oxidation of the syrup to the corresponding saccharic acid. The uronic acid was to be oxidised with bromine, distilled in a molecular still and then identified as the diamide, prepared via the dimethyl ester. The advantage of this procedure was that the bromine oxidation would convert only the uronic acid to the saccharic acid, while the other neutral sugars would be oxidised to the corresponding aldonic acids; the saccharic and aldonic acids would be separated in the molecular still and the identification would then be finally made on a pure derivative.

The uronic acid syrup was dissolved in water (15 ml.) and liquid bromine (2 ml.) added. The flask was stoppered and left with occasional shaking at 40° for 7 days. The bromine was removed by blowing a rapid

current of air through the solution. The solution was filtered and distilled in vacuo to form a syrup which was transferred to a molecular still where it was heated to 200° at very high vacuum. It was maintained at this temperature for 5 minutes during which time a small amount of dark coloured oil collected in the receiver. The distillate was dissolved in methanol (12 ml.) and acidified with 18% hydrochloric acid (2 ml.) and was heated for 2 hours under reflux in boiling water after which it was left overnight at 0°. The solution of the dimethyl ester was neutralised with silver carbonate, filtered through sintered glass and charcoal, and was distilled in vacuo to a syrup. Several drops of methanol saturated at 0° with gaseous ammonia was added to the syrup, and the stoppered flask left at 0° for 2 days. A precipitate of small crystals was filtered, washed with cold ethanol and dried. The melting point was found to be 166 - 168° (decomp.). A sample of glucosaccharic diamide was prepared and found to have a melting point at 163 - 164° (decomp.). When a mixed melting point was carried out a large depression was observed (mixed m.p. 140 - 145°). Both specimens were dried thoroughly and the mixed melting point repeated and found to be the same. This suggested that the uronic acid from A3(S1) polysaccharide was not glucuronic acid. Unfortunately there was not enough material with which to carry out further mixed melting points, and the attempts to identify the uronic acid

terminated.

(iii) Estimations

Using the standard hydrolysis conditions, the component sugars of the A3(S1) polysaccharide samples were estimated. (The results for the freeze-dried preparation are given above, p.131).

Component sugars of A3(S1) unboiled and boiled Na salts.

	Boiled Na salt			Unboiled Na salt		
	1	2	mean	1	2	mean
Wt. of sample (mg.)	22.8	20.0		17.1	26.3	
Reference sugar (mg.) (arabinose)	16.9	17.2		14.4	19.8	
galactose %	1	1	1	1	1	1
glucose %	49	51	50	46	45	46
fucose %	10	10	10	9	6	8

The acid equivalent of the freeze-dried polysaccharide was determined by titrating a sample (80 mg.) with dilute standard sodium hydroxide, using phenolphthalein as indicator. The acid equivalent was found to be 760, corresponding with a uronic acid content of 23.2%. The uronic acid estimations carried out on the freeze-dried polysaccharide by the various methods were all of the same order, but the decarboxylation results were taken as correct. The fucose results obtained by the method of Dische and Shettles were in agreement with the chromatographic results, and were probably more reliable, because in the latter method the fucose determinations were open to large errors, due to the fact that the fucose sections of the chromatograms had

very large blanks in relation to the fucose titres. The results of these estimations are given in table 11.

The over-all accuracy of the results was checked by calculating the anthrone ratios. The anthrone values were calculated from the composition data, using the anthrone equivalents on p.110, and these calculated anthrone values were divided by the experimentally determined anthrone results. The ratios of the three A3(S1) samples were all close to unity, showing that within the limits of error all the anthrone-reactive components in the polysaccharides had been accounted for.

Anthrone Ratios of A3(S1) Polysaccharides

	Freeze-dried		Unboiled Sodium Salt		Boiled Sodium Salt	
	%	Anthrone colour (glucose equivalent)	%	Anthrone colour (glucose equivalent)	%	Anthrone colour (glucose equivalent)
Uronic acid	26.8	1.3	29.1	1.4	28.2	1.4
Galactose	1	0.6	1	0.6	1	0.6
Glucose	46	46	46	46	50	50
Fucose	9	8.3	9	8.3	10	9.2
		56.2		56.3		61.2
Calculated anthrone value (nearest whole number)		56		56		61
Experimental anthrone value		54		55		64
Anthrone value		1.04		1.02		0.95

There is reason to believe that the small amount of galactose found in all the A3(S1) exopolysaccharides is not a component, but arises from contamination of the exopolysaccharides with small amounts of somatic or intracellular polysaccharide, which has been found to be mainly a galactan (See Part III). Disregarding the galactose, the proportions of glucose, uronic acid and fucose was found to be almost the same for the three preparations, showing that the different purification treatment did not affect the composition.

A3(S1) polysaccharide	glucose : uronic acid : fucose
freeze-dried	100 : 59 : 20
unboiled Na salt	100 : 61 : 20
boiled Na salt	100 : 56 : 20

(b) Polysaccharides of A.aerogenes strain A3.

This strain produced abundant exopolysaccharide in the form of firm capsules and loose slime, which were isolated separately.

Neutral component sugars of A3 slime polysaccharide.

Polysaccharide sample (mg.)	1	2	3	4	mean results
	20.1	18.3	8.0	12.6	
Reference sugar (arabinose, mg.)	17.0	17.3	7.1	10.4	
Galactose%	1	1	1	1	1
Glucose%	48	46	46	46	47
Fucose%	8	8	8	7	8

(All samples were analysed in duplicate. Results are expressed as the nearest whole number).

The capsules of A3 were removed from the cells by two procedures, by boiling and by treatment with 1% sodium hydroxide after formalin pre-treatment; the

products are referred to as A3 capsular polysaccharide A and B respectively.

Neutral component sugars of A3 capsular polysaccharide A.

	1	2	mean result
Polysaccharide sample (mg.)	13.1	12.9	
Reference sugar (arabinose, mg.)	12.5	9.9	
galactose%	2	2	2
glucose%	43	42	43
fucose%	9	9	9

(Both samples were analysed in duplicate. Results are expressed as the nearest whole number.)

Neutral component sugars of A3 capsular polysaccharide B.

	1	2	3	4	5	6	7	mean
Polysaccharide sample (mg.)	24.6	19.5	16.4	12.6	20.0	19.4	25.7	
Reference sugar (arabinose, mg.)	17.9	15.6	14.0	9.9	19.4	21.0	21.9	
galactose%	3	3	3	3	2	4	4	4
glucose%	37	36	37	36	37	39	39	37
fucose%	4	4	6	7	4	8	7	6

(All samples were analysed in duplicate. Results are expressed as the nearest whole number.)

(c) Polysaccharide of A.aerogenes strain A1.

This strain produced very large capsules which were removed from the cells by soda-treatment. The relatively small amount of slime was isolated together with the capsular polysaccharide.

Neutral component sugars of A1 slime and capsular polysaccharide.

	1	2	mean
Polysaccharide sample (mg.)	18.7	30.2	
Reference sugar (arabinose, mg.)	15.7	20.0	

Neutral component sugars of A1 slime and capsular polysaccharide (Contd.)

	1	2	mean
galactose%	1	1	1
glucose%	47	49	48
fucose%	10	10	10

(Both samples were analysed in triplicate. Results are expressed as the nearest whole number.)

(d) Polysaccharide of A.aerogenes strain A4.

This strain produced abundant exopolysaccharide in the form of capsules and slime both of which were isolated together. The capsules were removed by soda-treatment.

Neutral component sugars of A4 slime and capsular polysaccharide

	1	2	mean
Polysaccharide sample (mg.)	22.8	28.3	
Reference sugar (ribose, mg.)	16.7	23.4	
galactose%	51	51	51
glucose%	22	22	22
mannose%	3	2	3

(Both samples were analysed in triplicate. Results are expressed as the nearest whole number.)

(e) Polysaccharides of A.aerogenes strain A29.

This strain produced abundant exopolysaccharide in the form of capsules and slime, both of which were isolated together. The capsules were removed from the cells by soda-treatment.

Neutral component sugars of A29 slime and capsular polysaccharides

	1	2	mean
Polysaccharide sample (mg.)	22.3	22.8	

Neutral component sugars of A29 slime and capsular polysaccharides (Contd.)

	1	2	mean
Reference sugar (ribose, mg.)	20.0	19.9	
galactose%	21	21	21
mannose%	43	44	44

(Both samples were analysed in triplicate. Results are expressed as the nearest whole number.)

2. Aerobacter cloacae exopolysaccharides

(a) Polysaccharide of A.cloacae strain NCTC 5920.

This non-capsular strain produced moderate amounts of slime polysaccharide. The homogeneity of the polysaccharide was shown by ultracentrifugation (carried out by Dr. C.T. Greenwood). The sedimentation constant (S_{20}) in 0.1 N sodium hydroxide was calculated to be 0.8×10^{-13}; the sedimentation diagram is shown in fig. 11.

Neutral component sugars of NCTC 5920 polysaccharide

	1	2	mean
Polysaccharide sample (mg.)	24.6	23.7	
Reference sugar (arabinose, mg.)	24.4	22.3	
galactose%	26	26	26
glucose%	17	16	17
fucose%	25	24	25

(Both samples were analysed in triplicate. The results are expressed as the nearest whole number.)

The acid equivalent of the NCTC 5920 slime polysaccharide was determined on a specially purified freeze-dried sample (146 mg, 0.92% ash). The acid equivalent was found to be 680, equivalent to a uronic

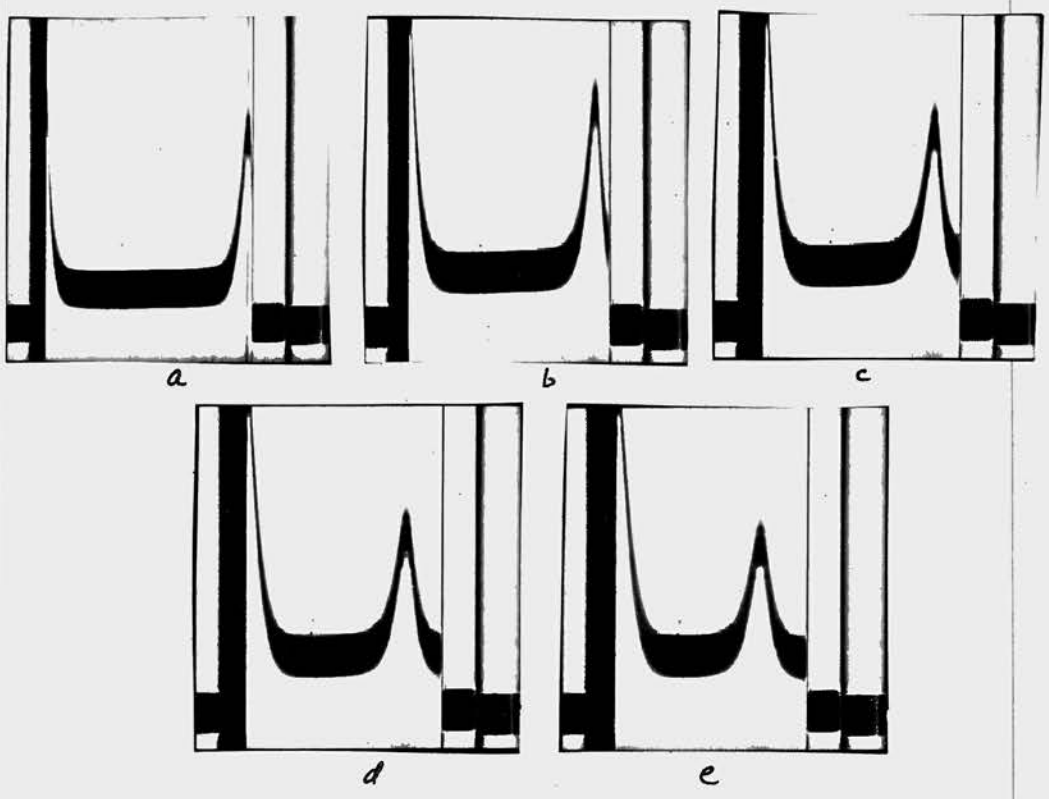


Fig. 11. Sedimentation diagrams for A. cloacae NCTC 5920 exopolysaccharide. Speed 59, 700 rpm. Solvent, 0.1 N NaOH. Concentration 2%. Times of exposure: (a) 30 min., (b) 79 min., (c) 135 min., (d) 171 min., (e) 213 min., from starting the centrifuge.

acid content of 25.9%.

(b) Polysaccharide of A.cloacae NCTC 5936.

This non-capsulate strain produced small amounts of slime polysaccharide. Quantitative determinations were not carried out, but the component sugars were identified to be glucose, galactose, fucose and a uronic acid (estimated to be 21% by the Jarrige method).

3. Klebsiella exopolysaccharides

(a) Polysaccharide of Klebsiella type 2 (Kl. pneumoniae type b)

This organism produces large amounts of slime and capsular polysaccharides. The sample of polysaccharide used in the present study was one which had been prepared in this Department in connection with viral haemagglutination work, by boiling washed cells, the details of which treatment are unknown. The product was probably mainly capsular polysaccharide contaminated with some intracellular polysaccharide. The sample was of low purity, as evidenced by the high ash, nitrogen and phosphorus figures. It was not purified before analysis because of the small quantity available, but it was investigated to provide a comparison with the results reported by Heidelberger, Goebel and Avery (1925).

Neutral component sugars of Klebsiella type 2.

	1	2	mean
Polysaccharide sample (mg.)	17.2	18.3	
Reference sugar (ribose, mg.)	18.3	16.7	

Neutral component sugars of Klebsiella type 2 (Contd.)

	1	2	mean
galactose%	17	17	17
glucose%	26	28	27
mannose%	6	7	7

(Both samples were analysed in triplicate. Results are expressed as the nearest whole numbers.)

(b) Polysaccharide of Klebsiella type 26(S1).

This non-capsulate strain produced abundant slime polysaccharide.

Neutral component sugars of K1. type 26(S1) polysaccharide

	1	2	mean
Polysaccharide sample (mg.)	20.5	24.3	
Reference sugar (ribose, mg.)	17.8	13.1	
Galactose%	25	26	26
glucose%	35	35	35
mannose%	23	23	23

(Both samples were analysed in triplicate. Results are expressed as the nearest whole numbers).

(c) Polysaccharide of Klebsiella type 29(S1).

This non-capsulate strain produced moderate amounts of slime polysaccharide.

Neutral component sugars of K1 type 29(S1) polysaccharide

	1	2	mean
Polysaccharide sample (mg.)	26.1	37.3	
Reference sugar (ribose, mg.)	18.7	22.1	
galactose%	31	30	31
mannose%	13	12	13

(Both samples were analysed in triplicate. Results are expressed as the nearest whole numbers).

4. Escherichia coli exopolysaccharides

Polysaccharide of E.coli strain A 102.

This non-capsulate strain produced small amounts of slime polysaccharide.

Neutral component sugars of A102 polysaccharide.

	1	2	mean
polysaccharide sample (mg.)	20.6	14.5	
Reference sugar (arabinose, mg.)	13.0	--*	
galactose%	17	17	17
glucose%	17	17	17
fucose%	16	16	16
rhamnose%	2	1	2

(Both samples were analysed in triplicate. Results are expressed as the nearest whole numbers.)

* These results were calculated by taking the fucose content to be 16% (found in the first analysis). No reference sugar was added to this hydrolysate because a slight trace of mannose was seen in a chromatogram of this polysaccharide and it was hoped to estimate it. Arabinose was the only reference that could be used with this polysaccharide, and it would have overlapped with the mannose. The mannose was present in amounts too small to estimate.

Hexosamine was proved absent by spraying chromatograms with the Morgan and Elsen reagents (Partridge 1948).

Note on the identity of the uronic acids.

All the polysaccharides were found to contain uronic acid components but none were identified despite

repeated attempts with chromatographic methods. They were all found to form lactones, thus indicating that probably none of them was galacturonic acid.

Serological results

The serological activity of A3(S1) freeze-dried polysaccharide was demonstrated by titrating A3 antiserum (diluted 1:4) against increasing dilutions of the polysaccharide in saline. A positive precipitation was observed in the ring test with dilutions up to 1:1,000,000, showing that the polysaccharide retained its serological activity to a high degree, and was unaffected by the isolation and purification treatment.

The immunological relationships of the polysaccharides of A.aerogenes strains A3(S1), A3 and A1 were studied. The results (table 14) show that A3(S1), A3 slime, A3 capsular and A1 slime and capsular polysaccharides are immunologically identical, confirming the results found by Wilkinson, Duguid and Edmunds (1954).

TABLE 11. Results of analyses carried out on the exopolysaccharides of A.aerogenes (A3(S1), A3, A1, A4, A29), A.cloacae (NCTC 5920) Klebsiella (K2, K26(S1), K29(S1)) and E.coli (A102).

	A3(S1)* 1	A3(S1)* 2	A3(S1)* 3	A3 Slime	A3 Capsule A	A3 Capsule B	A1	A4	A29	NCTC 5920	K2 ‡	K26(S1)	K29(s1)	A102
Ash %	1.3	4.0	7.6	7.8	6.2	9.2	5.5	2.3	3.2	4.3	18.3	5.0	8.9	3.5
N %	1.05¶	0.18	0.17	0.08	0.53	0.88	0.46	0.65	0.59	0.30	1.45¶	0.12	0.30	3.1¶
P %	0.10	0.12	0.04	0.46	0.11	0.38	0.02	0.25	0.02	0.19	2.87	0.59	1.08	0.25
$[\alpha]_D^{20}$	-50°	-57°	-40°	-59°	§	-82°	-77°	+95°	+220°	+110°	+56°	+138°	+128°	§
Reducing power of unhydrolysed polysaccharides (% glucose)	1.8	1.6	0.7	1.2	0.9	0.6	0.7	0.7	0.7	1.6	1.0	0.8	1.0	1.8
Glucose %	46	50	46	47	43	37	48	22	-	17	27	35	-	17
Galactose %	1	1	1	1	2	4	1	51	21	26	17	26	31	17
Fucose (chromatographic) %	12	10	8	8	9	6	10	-	-	25	-	-	-	16
Fucose (colorimetric) %	9	9	10	9	9	7	11	-	-	28	-	-	-	21†
Mannose %	-	-	-	-	-	-	-	3	44	-	7	23	13	Trace
Rhamnose %	-	-	-	-	-	-	-	-	-	-	-	-	-	2
Uronic acid (decarboxylation)%	27	28	29	30	27	26	29	25	28	22	21	17	25	23
Uronic acid (colorimetric) %	20	-	-	17	—	22	18	—	—	24	—	—	—	—
Anthrone value (calculated) %	56	56	61	57	54	47	60	55	37	57	42	60	44	44
Anthrone value (experimental) %	54	55	64	59	59	54	60	52	40	58	41	55	40	50
Anthrone ratio	1.04	1.02	0.95	0.97	0.92	0.87	1.00	1.06	0.93	0.98	1.02	1.09	1.10	0.88
Total carbonydrate components %	83	83	86	87	81	74	89	101	93	93	72	101	69	78
Total of all components ≠ %	90	93	95	96	90	89	98	107	100	99	95	106	81	100

≠ 6 x N% added. Ash and excess P added directly.

* A3(S1)1= freezed dried
2= unboiled. No salt.
3= boiled. No salt.

§ Solution too turbid to read.

† Fucose and rhamnose.

≠ Trace of xylose found.

¶ Polysaccharide not deproteinised.

Table 12.

Molecular ratios of component sugars in the exopolysaccharides of A.aerogenes (A3(S1), A3, A1, A4, A29), A.cloacae (NCTC 5920), Klebsiella (types 2, 26(S1), 29(S1)) and E.coli (A102).

Polysaccharide	Component sugars (Parts present)
A3(S1) freeze-dried	glucose (9), uronic acid (5), fucose (2)
" unboiled Na salt	glucose (10), uronic acid (5), fucose (2)
" boiled Na salt	glucose (8), uronic acid (5), fucose (2)
A3 slime	glucose (9), uronic acid (6), fucose (2)
A3 capsular A	glucose (9), uronic acid (5), fucose (2)
" B	glucose (10), uronic acid (6), fucose (2)
A1	glucose (8), uronic acid (4), fucose (2)
A4	galactose (17), uronic acid (8), glucose (7), mannose (1)
A29	mannose (2), galactose (1), uronic acid (1)
NCTC 5920	galactose (3), fucose (3), glucose (2) uronic acid (2)
K1 type 2	glucose (8), uronic acid (6), galactose (5) mannose (2)
K1 type 26(S1)	galactose (4), glucose (3), mannose (3), uronic acid (2)
K1 type 29(S1)	galactose (5), uronic acid (4), mannose (2).
A102	uronic acid (10), glucose (8), galactose (8), fucose (8), rhamnose (1)

Table 13.

Molecular ratios of component sugars in the exopolysaccharides of A.aerogenes strains A3(S1), A3 and A1, relative to glucose = 100.

Polysaccharide	Component sugars		
	glucose	uronic acid	fucose
A3(S1) freeze-dried	100	54	22
unboiled Na salt	100	52	20
boiled Na salt	100	58	24
A3 slime	100	59	21
A3 capsule A	100	58	23
" B	100	65	21
A1	100	56	25

Table 14.

Highest dilutions of antiserum giving precipitation reactions with various polysaccharides of A.aerogenes.

Antigen (Polysaccharide)	A3 antiserum absorbed with the following polysaccharides				
	Nil	A3(S1)*	A3 slime	A3 capsule B	A1
A3(S1) *	16	0	0	2	0
A3 slime	16	0	0	0	0
A3 capsular B	16	0	0	0	0
A1	16	0	0	0	0

* A3(S1) freeze-dried preparation.

DISCUSSION

All bacterial hetero-exopolysaccharides that have been examined with the aid of modern techniques have been found to contain uronic acid or hexosamine components. The exopolysaccharides of the strains of A. aerogenes, A. cloacae, Klebsiella and E. coli examined in the present study were all found to be poly-uronides, and therefore to conform with the general pattern. They all contained small amounts of nitrogen known not to come from hexosamine, but to be due most probably to the presence of protein and nucleic acid contaminants; the latter material probably also accounts for much or all of the phosphorus found present. With the exception of the results for the K29(S1) polysaccharide, the totals of the components found in each polysaccharide (given in table 11) were all of the same order (90 - 107%) and indicate that all the components were accounted for in the analyses; this was supported by the anthrone ratios. The low value of the total for the K29(S1) polysaccharide arises most probably from the resistance of the polysaccharide to hydrolysis; unfortunately pressure of time prevented investigation into suitable hydrolysis conditions for this polysaccharide.

An unusual feature that was found was that six of the strains (A. aerogenes strains A3(S1), A3, A1; A. cloacae strains NCTC 5920, NCTC 5936; E. coli A102) produced polysaccharides containing fucose, which in the case of strain A3(S1) was identified as L-fucose.

Until the recent report by Norris, de Sipin, Zilliken, Harvey and György (1954) that fucose was a component of the polysaccharide of a mucoid mutant of Lactobacillus bifidus, fucose had never been found in a bacterial polysaccharide, although rhamnose has been identified in the polysaccharides of a variety of micro-organisms (see table 2). It is believed that the combinations of sugars found in the fucose-containing polysaccharides have not been met before in polysaccharides from any source. It is interesting to note that all the polysaccharides contained either mannose or fucose, and that they were not found to occur together.

The galactose found in the polysaccharides of strains A3(S1), A3 and A1, is believed not to be a component of the exopolysaccharides, but to arise from contamination with a somatic polysaccharide, which has been found to contain a large proportion of galactose (see Part III of this thesis). This view is supported by the discovery of larger amounts of galactose in the capsular polysaccharide of strain A3 than in the slime because the former was isolated by treatment which was likely to lead to greater contamination by the somatic polysaccharide. In the discussion that follows, the galactose found in the A3(S1), A3 and A1 polysaccharides will therefore be disregarded.

Since no previous report has been made of the composition of the polysaccharides of A. aerogenes and A. cloacae, with the exception of the few inconclusive

attempts described in the Introduction, the results found for these polysaccharides cannot be discussed in the light of previous results. In the present study the most closely examined polysaccharide was that produced by A. aerogenes strain A3(S1) which was found to contain D-glucose (9 parts), uronic acid (5 parts) and L-fucose (2 parts). It was shown by ultracentrifugation to be homogeneous, but this method would not detect contaminating polysaccharides if present in amounts less than 5%, and thus does not rule out the possibility that the 1% galactose found in the polysaccharide could arise from a contaminating polysaccharide. The sedimentation constant (S_{20}) of a 2.5% solution of the polysaccharide in 0.1N sodium hydroxide was 1.8×10^{-13}. At present no information is available regarding the shape of the molecule, but by using the expression

$$M = 2.45 \times 10^{22} \, (f/f_o)^{\frac{3}{2}} \, \bar{V}^{\frac{1}{2}} \, (S_{20} / (1 - \bar{V}\rho))^{\frac{3}{2}}$$

where M = molecular weight

f/f_o = frictional ratio

$\bar{V}$ = partial specific volume

ρ = density of the solution

and assuming various values for the frictional ratio, possible values for the molecular weight of the polysaccharide were calculated.

M = 6,600	where f/f_o = 1.1 (i.e., spherical)
16,000	2.0
30,000	3.0

M = 85,000 where f/f_o = 6.0 (frictional ratio found for pneumococcus type II polysaccharide (Record and Stacey 1948) This is the highest value for f/f_o found in a bacterial polysaccharide.

(results calculated assuming $\bar{v}$ = 0.60, p = 1)

The molecular proportions of the components present in the polysaccharide indicate a repeating unit of glucose (9 residues), uronic acid (5 residues) and fucose (2 residues), with a molecular weight of 2630. The minimum value for M (6,600) suggests that the polysaccharide molecule contains at least 3 repeating units (M 7890) with a total of 48 residues. Other data is required before it is possible to be more specific.

The polysaccharide of A. cloacae strain NCTC 5920 was found to contain galactose (3 parts), fucose (3 parts), glucose (2 parts) and uronic acid (2 parts). Ultracentrifugal measurements were also made with this polysaccharide: its sedimentation constant (S_{20}) in 0.1N sodium hydroxide (2% polysaccharide solution) was 0.8×10^{-13}. The absence of knowledge of the molecular shape poses the same problems as with the polysaccharide of strain A3(S1), but by making the same assumptions as above, and using the same equation, it is found that

M = 2,000	where f/f_o = 1.1
4,800	2.0
8,800	3.0
25,000	6.0

The repeating unit of the polysaccharide appears to be: galactose (3 residues), fucose (3 residues), glucose (2 residues) and uronic acid (2 residues), with a molecular weight of 1,610, suggesting that if the polysaccharide molecule is spherical, it will contain one repeating unit.

The results found for A. aerogenes strain A4 polysaccharide indicate that it contains glucose (7 parts), galactose (17 parts), uronic acid (8 parts) and mannose (1 part). However, if the small amount of mannose found is regarded as being derived from a contaminating somatic or intracellular polysaccharide, the composition of the polysaccharide would be glucose (1 part), galactose (2 parts) and uronic acid (1 part).

The presence of glucose and a uronic acid in the polysaccharide of Klebsiella type 2 (Friedländer's bacillus type B) reported by Heidelberger, Goebel and Avery (1925) was confirmed, but galactose and mannose were also found, together with traces of xylose which may be from a contaminating polysaccharide. The polysaccharide of E. coli strain A102 differed in an important respect from the polysaccharides of other E. coli strains that have been examined (see table 2) in that it contained a uronic acid in place of the glucosamine found in the other polysaccharides. The trace of mannose found in the chromatograms of the polysaccharides of strain A102 was probably from a contaminating somatic or intracellular polysaccharide.

If the small amount of rhamnose found in the polysaccharide is regarded as a true component, the composition is found to be uronic acid (10 parts), glucose (8 parts), galactose (8 parts), fucose (8 parts) and rhamnose (1 part). But because it is present in such small amounts it is possible that the rhamnose is derived from a contaminating somatic or intracellular polysaccharide, in which case the polysaccharide would contain uronic acid, glucose, galactose and fucose in equimolecular amounts. If the rhamnose is a true component, the exopolysaccharide of strain A102 would be noteworthy as probably the first polysaccharide found to contain both the naturally occurring methylpentoses - fucose and rhamnose.

No previous attempt has been made to show by chemical means that the slime and capsular polysaccharides of an organism are the same. Edwards and Fife (1952) and Wilkinson, Duguid and Edmunds (1954) demonstrated the immunological identity of the slime and capsular polysaccharides of Klebsiella type 14 and A. aerogenes strain A3 respectively, but serological methods of proving identity are open to the doubt that the apparent identity may be the result of cross-reactions between antigens possessing similar patterns of immunologically dominant groups while the other parts of the molecules may be different. By separately isolating and analysing the slime and capsular polysaccharide of A. aerogenes

strain A3 it was shown that they were identical in composition; this is readily seen from the molecular ratios in tables 12 and 13. Complete proof must await structural studies and molecular weight determinations on the slime and capsular material. The result is in keeping with the hypothesis that capsules derive their shape and rigidity from skeletal frame-works or networks, such as have been found by Tomcsik (1951) and Ivanovics and Horvath (1953). According to this hypothesis capsules are formed by the filling of the interseptal spaces of the skeleton with exopolysaccharide; as the latter diffuses outwards from the framework it becomes loose slime. The capsular and slime polysaccharides would thus be identical.

It was shown for the first time, in the present study, that the exopolysaccharide of a non-capsulate slime-forming variant (A3(S1)) retains the composition of the exopolysaccharide of the parent strain, (A3). The report by Wilkinson, Duguid and Edmunds (1954) that strains A3 and A3(S1) were identical immunologically was confirmed by showing that the isolated exopolysaccharides of these strains were identical immunologically. This result is also in keeping with the skeletal hypothesis of capsule formation, according to which the slime-forming variants of capsular species could be postulated to arise through loss of the ability to produce the skeletal framework.

Edmunds (1954) showed that A. aerogenes strains A1 and A3 were immunologically identical. In the present study it was shown that the exopolysaccharides of these strains were identical in composition and immunological specificity.

The high degree of immunological specificity found among the Klebsiella-Aerobacter types (see Edwards and Fife, 1952; Edmunds, 1954) is remarkable in view of the present observation that the exopolysaccharides of the 6 different types that were examined (using the Edwards and Fife notation these were: K2, K8, K26, K29, K54 and K57) all contained similar amounts of uronic acid (20 - 30%), which would lead one to expect frequent cross-reactions because uronic acids dominate the immunological patterns of the polysaccharides in which they occur. The low incidence of cross reaction suggests that the spatial distribution of the uronic acid residues must be different in each polysaccharide, at least sufficiently different to prevent immunological cross reactions, which in turn indicates that all the polysaccharides of the Klebsiella-Aerobacter group must have different complex structures.

SUMMARY Part I

1. The exopolysaccharides of five strains of A. aerogenes, two of A. cloacae, three of Klebsiella and one of E. coli were isolated and examined.

2. They were found to be unusual polyuronides with the following compositions (expressed in molecular ratios.)

<u>A. aerogenes</u>

strain	A1	glucose (8), uronic acid (4), fucose (2)
"	A3	glucose (9), uronic acid (5), fucose (2)
"	A3(S1)	glucose (9), uronic acid (5), fucose (2)
"	A4	galactose (17), uronic acid (8), glucose (7), mannose (1)
"	A29	mannose (2), galactose (1), uronic acid (1)

<u>A. cloacae</u>

NCTC 5920	galactose (3), fucose (3), glucose (2), uronic acid (2)
NCTC 5936	glucose, galactose, fucose, uronic acid

<u>Klebsiella</u>

type	K2	glucose (8), uronic acid (6), galactose (5), mannose (2)
"	K26(S1)	galactose (4), glucose (3), mannose (3), uronic acid (2)
"	K29(S1)	galactose (5), uronic acid (4), mannose (2).

<u>E. coli</u>

strain A102	uronic acid (10), glucose (8), galactose (8), fucose (8), rhamnose (1).

D-glucose and L-fucose were confirmed in the polysaccharide of <u>A. aerogenes</u> strain A3(S1) by isolation and preparation of derivatives. None of the uronic acid components were identified.

3. The slime and capsular polysaccharides of <u>A. aerogenes</u> strain A3 were isolated separately and shown to be identical.

4. The exopolysaccharide of a slime-forming variant

(*A. aerogenes* strain A3(S1)) was shown to retain the composition of the exopolysaccharide of its parent capsulate strain (A3).

5. The immunological relationship of the exopolysaccharides of *A. aerogenes* strains A1, A3 and A3(S1) were examined and shown to be identical. The exopolysaccharide of strain A3(S1) in dilutions of 1,000,000 was found to react with homologous immune serum.

PART II

THE INFLUENCE OF CARBON SUBSTRATE ON THE COMPOSITION OF THE EXOPOLYSACCHARIDE OF AEROBACTER AEROGENES (STRAIN A3(S1))

THE INFLUENCE OF CARBON SUBSTRATE ON THE COMPOSITION OF THE EXOPOLYSACCHARIDE OF AEROBACTER AEROGENES (STRAIN A3(S1))

No attempt has been made to investigate quantitatively the influence of carbon substrate on the composition of bacterial heteroexopolysaccharides. It is a commonly observed fact that, apart from type transformations and antigenic variations of the smooth-rough type, the immunological specificity of an organism remains constant regardless of the medium on which it is cultured. For example, Morgan and Beckwith (1939) showed that the immunological specificity of a strain of E.coli was unaffected when it was cultured on a synthetic medium containing sucrose, glucose, rhamnose or xylose as sole carbon source. But immunological methods of proving identity are open to the objection that the apparent identity is the result of cross-reactions between antigens possessing similar patterns of immunologically dominant groups while the remainder of the molecules are different. Forsyth and Webley (1949) made a qualitative examination of the exopolysaccharides of a number of species of genus Bacillus grown on synthetic media in which sucrose, fructose, glucose, galactose and arabinose were used singly as sole sources of carbon and energy, and found that the component sugars of the polysaccharides remained unchanged.

In the present study an examination was made of the exopolysaccharide of A.aerogenes isolated from cells grown in liquid synthetic medium containing different sugars as sole source of carbon and energy. Strain A3(S1) was chosen for several reasons. It produced abundant slime, and being non-capsulate the cells could be more easily centrifuged from the viscous cultures and thus facilitated the isolation of the polysaccharide. Its neutral component sugars (glucose and fucose) were easily separable on paper chromatograms, and the fucose results could be checked by the independent colorimetric method.

MATERIALS AND METHODS

Organism: A.aerogenes strain A3(S1) was used. (See part I)

Cultural conditions: The organism was grown on synthetic liquid media containing 1% carbohydrate and the same inorganic salts in the same concentrations as in the medium described in Part I. The sugars on which the organism was grown were glucose, galactose, mannitol, xylose, rhamnose, fucose, glucurone and sucrose. When the glucurone medium was prepared it was adjusted to pH 7.3 by adding N sodium hydroxide. With each sugar 3.6 l. of medium was prepared, except in the case of fucose, which because of its scarcity was used to prepare 2.4 l. of medium containing 0.5% fucose.

Growth was carried out in 1 l. screw-capped bottles (200 ml. of medium per bottle) in the rotor, as described in Part I. The bottles were inoculated with loopfuls of the organism which had previously been adapted to the sugar in the medium by being cultured on solid synthetic medium containing the sugar as the sole source of carbon and energy. The cultures were grown for 2 - 3 days at 37°, by which time they were highly viscous. With the glucurone cultures the pH was found to be high (8 - 9) after 24 hours growth, and N hydrochloric acid was added under aseptic conditions to each bottle to adjust the pH to about 7.

Isolation of the exopolysaccharides: The method used for isolating the exopolysaccharide from each culture was the same as that described in Part I for the isolation of slime polysaccharide. The yields of polysaccharide were all of the same order - 1.0 - 1.2 g. of purified polysaccharide from 3.6 l. of culture. The fucose culture produced 0.6 g. of polysaccharide. All the polysaccharides were isolated as boiled sodium salts.

Analytical methods: The methods described above in Part I were used.

RESULTS

The exopolysaccharides produced by strain A3(S1) when grown on the different sugars as sole carbon and energy source were all analysed in the same way as the

polysaccharides in Part I, to elucidate their composition. Two samples of each polysaccharide were hydrolysed under standard conditions (24 hours with 95% formic acid at 100°, followed by 6 hours with N sulphuric acid at 100°) and the neutral component sugars present in each hydrolysate were estimated in duplicate or triplicate. Arabinose was used as reference sugar with all the polysaccharides. All the other analyses were also carried out in duplicate. The results have been summarised in table 15, where the data for the glucose-grown polysaccharide are also included (taken from Part I). The results show that there is no detectable qualitative or quantitative difference between any of polysaccharides produced by strain A3(S1) when grown with any of the sugars. The anthrone values were all around unity, indicating the estimations were complete. The slight amounts of galactose found in the polysaccharides were regarded as coming from a contaminating polysaccharide (see Part III). It is unfortunate that the uronic acid components could not be identified from their chromatograms, for the same reasons that prevented the identification of the uronic acids of the polysaccharides studied in Part I.

The polysaccharide produced by the organism when grown on sucrose as sole carbon and energy source was not analysed quantitatively; it was found to contain the same sugars as the other polysaccharides, in the

same relative amounts in as far as could be judged from visual examination of the chromatograms. Fructose was not found even when the polysaccharide was hydrolysed by gentle conditions, e.g.

30 minutes with 0.1 N sulphuric acid or

4½ hours with N sulphuric acid, both at 100°.

The sucrose-grown polysaccharide also gave a negative Seliwanoff test.

Table 15. Results of analyses carried out on the exopolysaccharide of strain A3(S1) when grown on different sugars as sole source of carbon and energy.

	Glucose *	Galactose	Mannitol	Xylose	Fucose	Rhamnose	Glucurone
Ash %	7.6	7.1	6.3	4.5	3.7	4.0	2.9
N %	0.17	0.15	0.15	0.39	0.65	0.79	0.88
P %	0.04	0.04	0.06	0.19	0.45	0.49	0.43
Reducing power of unhydrolysed polysaccharide (% glucose)	0.7	0.8	1.4	1.3	1.2	0.84	1.3
Glucose %	46	43	43	49	44	47	46
Galactose %	1	1	1	1	1	1	1
Fucose (chromatographic) %	8	7	5	8	7	8	8
Fucose (colorimetric) %	10	9	9	10	9	9	9
Uronic acid (decarboxylation) %	29	28	25	28	27	26	29
Anthrone value (calculated) %	61	53	53	61	54	57	56
Anthrone value (experimental) %	64	59	53	60	55	52	57
Anthrone ratio	0.95	0.90	1.00	1.02	0.98	1.10	0.98
Total carbohydrate components %	86	81	78	89	81	83	85
Total of all components %	95	89	85	96	89	92	93

* Data for boiled sodium salt taken from Part I.

Table 16.

Molecular proportions of the component sugars in the exopolysaccharide of strain A3(S1) when grown in the presence of the sugars shown in the table as sole carbon and energy source.

Substrate	Proportions of glucose-uronic acid-fucose	
	Relative to fucose (fucose = 2)	Relative to glucose (glucose = 100)
glucose	8 : 5 : 2	100 : 58 : 24
galactose	9 : 5 : 2	100 : 60 : 23
mannitol	9 : 5 : 2	100 : 54 : 23
xylose	9 : 5 : 2	100 : 53 : 23
fucose	9 : 5 : 2	100 : 57 : 23
rhamnose	9 : 5 : 2	100 : 51 : 21
glucurone	9 : 5 : 2	100 : 58 : 22

DISCUSSION

The composition of the exopolysaccharide of A.aerogenes strain A3(S1) was shown to remain unchanged when the organism was grown on media containing glucose, galactose, arabinose, mannitol, rhamnose, fucose and glucurone as the sole source of carbon and energy. The identical composition of the polysaccharides is readily seen from the table of molecular proportions of the components found in each preparation (table 16). The proportions calculated in the ordinary way, relative to the smallest component (in this case fucose) showed that all the polysaccharides contained the component sugars in the same proportions: glucose (9 parts), uronic acid (5 parts) and fucose (2 parts). The only exception to this was the glucose-grown polysaccharide which had 8 parts of glucose instead of 9, but this difference is probably within the limits of error involved in the determinations of glucose and fucose. It should be noted that for the calculation of these results the colorimetric fucose values were used, because they seemed to be more consistent and reliable than the results found by chromatography and periodate oxidation. When the molecular proportions were calculated relative to glucose (taken arbitrarily to be 100, so that the proportion of each sugar could be expressed as two significant figures, thus permitting the appearance of smaller variations) some small

variations were found, but these were small and within the limits of error of the method. The mean molecular proportions relative to glucose were found to be 100:56:23 (glucose:uronic acid:fucose)

The organism was grown in sucrose-containing medium to find whether it possessed the ability to synthesise a levan in addition to its polyuronide, as was found to be the case with B.polymyxa and B. megatherium (Hestrin, Avineri-Schapiro and Aschner 1943; Forsyth and Webley 1949). The absence of fructose in the hydrolysate showed that no levan was produced by strain A3(S1).

The results of the present experiments show for the first time that the composition of a heteropolysaccharide remains unaltered when the organism producing the polysaccharide is grown on a variety of different sugars as the sole sources of carbon and energy. The sugars used included representatives of different types of monosaccharides - hexoses, methylpentoses, a pentose, a uronic acid (lactone) and a hexitol, and included the sugars which occur as components of the polysaccharide. These results show that the composition of bacterial heteroexopolysaccharides remain constant, and therefore that heteropolysaccharides must be synthesised by some means that are capable of reproducing the specific molecules.

Nothing is yet known about the mechanism of

heteropolysaccharide synthesis, but it has been shown that homopolysaccharides are synthesised by unit steps which may be expressed by the general equation

$$\text{D-glycoside} + \text{R} \rightleftharpoons \text{R-glycoside} + \text{D}$$

where D-glycoside is the donor of high energy glycosyl units, and R is the carbohydrate receptor. Two main types of glycosyl donors have been found to participate in homopolysaccharide synthesis: sugar phosphates, and di- and higher saccharides. In heteropolysaccharide synthesis it may be assumed that glycosyl donors function in a similar way. Unless all the carbon substrates are metabolised by the organism to a common intermediate from which all glycosyl donors are formed, and there is evidence from the incorporation of free glucosamine into hyaluronic acid by Strep. haemolyticus (Topper and Lipton, 1952) to show that this is not so, it follows that by varying the nature of the carbon substrate during growth and polysaccharide synthesis it can be assumed that the equilibrium concentrations of the glycosyl donors are varied so that their relative proportions are different. If heteropolysaccharide synthesis is carried out by a comparatively non-selective enzyme, or enzymes which join together glycosyl units according to the availability of glycosyl donors, then it would be expected that the nature of the heteropolysaccharides should vary according to the concentration of the glycosyl donors,

and thus according to the nature of the carbon substrate. The results of the present study show that this does not happen, and therefore some other synthetic mechanism is involved. There appear to be two possible alternative mechanisms: synthesis by a series of completely specific enzymes, or synthesis by a template mechanism as visualised for protein synthesis. The former mechanism would involve a large number of specific enzymes to synthesise the smallest repeating unit of the heteropolysaccharide molecule, which in the case of A3(S1) polysaccharide contains 9 parts of glucose, 5 of uronic acid and 2 of fucose; in addition to which further enzymes would be required to link the repeating units together to form the heteropolysaccharide molecule. This large number of enzymes is unlikely, in particular since the ability to synthesise exopolysaccharide, and therefore to form mucoid colonies, is apparently controlled by a single mutable step, and this according to current views, by one enzyme.

A template mechanism is thus favoured. Such a mechanism is supported by the narrow molecular weight distributions found in many bacterial heteropoly-saccharides. Close control of molecular size can be more easily visualised to occur with a template mechanism than with one involving the action of polymerising enzymes alone. In view of its importance in type transformations it is likely that

deoxyribonucleic acid is involved in the template. Thus the evidence suggests that heteropolysaccharides and homopolysaccharides differ in their mode of synthesis.

SUMMARY Part II

A non-capsulate slime-forming strain (A3(S1)) of A.aerogenes was cultured in simple synthetic medium containing glucose, galactose, mannitol, xylose, fucose, rhamnose, glucurone and sucrose (used singly) as sole carbon and energy source. The exopolysaccharide produced by the organism from each sugar was examined and its composition found to be the same in each case: glucose (9 parts), uronic acid (5 parts) and fucose (2 parts). No levan was produced by the organism when grown on sucrose. The constant composition of the polysaccharide is interpreted to indicate that heteropolysaccharides are synthesised by some form of template mechanism.

PART III

THE SOMATIC AND INTRACELLULAR POLYSACCHARIDES OF AEROBACTER AEROGENES STRAIN A3(O).

THE SOMATIC AND INTRACELLULAR POLYSACCHARIDES OF AEROBACTER AEROGENES STRAIN A3(O).

An examination was made of the polysaccharides of A. aerogenes strain A3(O), a non-capsulate and non-slime-producing smooth variant of strain A3, in order to gain some knowledge of the somatic and intracellular polysaccharides of A. aerogenes about which very little is known, and to complement the results found for the exopolysaccharides. A non-exopolysaccharide-producing strain was chosen because it would produce only the somatic and intracellular polysaccharides and thus prevent any possibility of contamination with exopolysaccharide. This strain in particular was chosen because it was known to have the same somatic antigen (and therefore the same somatic polysaccharide) as strains A3(S1), A3 and A1 (Wilkinson, Duguid and Edmunds 1954), and therefore the results found for strain A3(O) could be applied to strains A3(S1), A3 and A1. It was hoped that by elucidating the composition of the somatic polysaccharide of these strains to show that the galactose found in small amounts in their exopolysaccharides was likely to have arisen through contamination with small amounts of somatic polysaccharide.

The only previous attempt to investigate a somatic or intracellular polysaccharide of A. aerogenes was made recently by Levine, Stevenson, Tabor, Bordner

and Chambers (1953). They isolated a glycogen-like polysaccharide from an unspecified strain by two methods: extraction with 30% potassium hydroxide for 3 hours at 100°, and ultrasonic lysis. They were unable to extract the polysaccharide with boiling water. After purification the polysaccharide products were found to have low nitrogen and phosphorus values and to have the general properties of glycogen. The optical rotation was found to be of the order of $[\alpha]_D$ + 200°. The polysaccharide dissolved in water to form opalescent solutions which reacted with iodine to form the characteristic reddish-brown colour; both these properties were lost rapidly when the solutions were treated with saliva. Paper chromatographic analysis of the hydrolysate showed the presence of a single component which was identified by its R_f value as glucose. The reducing value of the hydrolysed polysaccharide was equivalent to 91 - 93% glucose. The infra-red spectrum of the polysaccharide was similar to that of glycogen.

MATERIALS AND METHODS

Organism: The strain of A. aerogenes used (A3(O)) was a variant isolated by Dr. P.N. Edmunds from strain A3 by culturing in broth containing homologous antiserum. It differed from the parent strain in being non-capsulate and non-slime-forming, and in giving smooth non-mucoid growths.

Cultural conditions: The synthetic liquid medium described in Part I was used, with slightly increased nitrogen (ammonium sulphate 0.05%). Growth was carried out for 24 hours at 35° in 5 l. flasks aerated by bubbling sterile air through the medium. Each flask was inoculated with 10 ml of 24 hour broth culture of the organism.

Extraction and purification of the polysaccharides: The polysaccharides were isolated in fractions from the washed cells by using a series of increasingly severe extraction procedures, described in detail in the experimental section below.

Analytical methods: The analytical methods described above in Part I were used. Hydrolysis of the polysaccharides was carried out under different conditions, described below in the text.

EXPERIMENTAL AND RESULTS

Extraction and isolation of the polysaccharides.

The cells from 10 l. of a 24-hour culture of strain A3(0) were harvested by centrifugation in a Sharples "supercentrifuge", and were washed three times with saline (ca. 200 ml). The black sediment found on top of the deposit of cells was removed, and the whole crop suspended in water (500 ml). The total bacterial dry weight, estimated by withdrawing 1 ml. aliquots and drying them at 120° to constant weight, was found to be about 5 g. After the pH of the suspension was checked and found to be 7, it was

heated in boiling water under reflux. At intervals (0, 1, 3 and 4 hours) 10 ml. samples were withdrawn for the purpose of following the course of the extraction with the anthrone method. The samples were centrifuged and the supernatants withdrawn; all the supernatants except the first (0 hour) were opalescent. The residues were washed with water (10 ml.) and were made up to 10 ml. in volumetric flasks. Anthrone determinations were carried out on 0.1 ml. samples of the supernatants and on 0.2 ml. samples of the suspensions. The results were as follows:

Extraction of polysaccharide with boiling water from cells of strain A3(O) (results expressed as glucose)

	Polysaccharide distribution in 10 ml. sample		
Duration of heating	supernatant	deposit	total
0 hr	0.055 mg	0.665 mg	0.720 mg
1	0.499	0.245	0.744
3	0.548	0.172	0.720
4	0.554	0.164	0.718

The extraction was stopped after 4 hours by which time it was seen that no further polysaccharide was being extracted; the polysaccharide distribution between the supernatant and residue was 77% and 23% respectively. The cold suspension was centrifuged at high speed and the pale yellow opalescent supernatant withdrawn. The residue was washed with water (6 x 100 ml.), and the first washings added to the supernatant. An attempt was made to clear the latter with "hyflo-supercel" but with little success. A

A small scale experiment showed that in the absence of added salts 2 volumes of acetone caused no precipitation of the polysaccharide from the supernatant, but in the presence of sodium acetate the addition of 2 volumes of acetone caused rapid precipitation. Sodium acetate (10 g) was added to the supernatant (600 ml.) and 2 volumes of acetone were added; within a few minutes a white flocculent precipitate formed and was left to develop overnight at 4°. The precipitate was separated by centrifugation and washed with 75% aqueous acetone (2 x 40 ml.). The pale white powder was dissolved in water (40 ml.) giving a buff-coloured solution which frothed on shaking. The solution was de-proteinised by the Sevag procedure; the de-proteinisation process was repeated 16 times by which time only a slight protein gel formed at the interface between the two liquids. The solution was dialysed against running tap-water for 42 hours, after which it was found to be an opalescent non-viscous solution. It was centrifuged at high speed but no deposit was obtained. The polysaccharide was isolated from the solution by freeze-drying: 0.65 g of soft white flakes was obtained. This fraction was designated "polysaccharide I".

The extraction of polysaccharide from the cell debris was taken a stage further. The washed boiled residue was suspended in 10% potassium hydroxide

(100 ml.) and heated in a boiling water-bath under reflux in an atmosphere of nitrogen. Samples (2.5 ml.) were removed at intervals (0, 1 and 2 hours) to follow the course of the extraction by the same method as described above for the water extraction; the samples were neutralised with 2 N sulphuric acid before centrifuging etc. The anthrone determinations gave the results shown below, indicating that the extraction was complete after 2 hours.

Extraction of polysaccharide with 10% KOH from boiling-water-extracted A3(O) cells.
(results expressed as glucose)

Duration of heating	polysaccharide distribution in 2.5 ml. sample		
	supernatant	residue	total
0 hr	15 µg	126 µg	141 µg
1	103	23	126
2	109	21	130
2*	115	18	133

* neutralised with glacial acetic acid.

The extraction was stopped after 2 hours; the polysaccharide distribution was now 85% (20%) in the supernatant and 15% (3%) in the residue, the figures in parenthesis being the results in terms of the initial total polysaccharide in the A3(O) cells. The alkaline suspension was neutralised with glacial acetic acid and centrifuged. The supernatant was withdrawn and the residue washed with water (3 x 30 ml.) the first two washings were added to the supernatant. Acetone (2 vols) was added to the supernatant (120 ml.) and the beaker was cooled in ice for 30

minutes, after which the precipitate was separated by centrifugation and washed with acetone (2 x 50 ml.). The powder dissolved easily in water (30 ml.) to give a pale yellow solution which was de-proteinised by the Sevag procedure. The process was repeated only 8 times with this extract, and the solution was then dialysed for 44 hours against running tap-water. Finally the solution was centrifuged at high speed to remove solid matter, and the solution was freeze-dried, yielding 0.10 g. of dried polysaccharide. This was designated "polysaccharide II".

The anthrone determinations indicated the presence of only 3% of the initial polysaccharide in the alkali-extracted residue. However, it was decided to continue with further extractions in case the anthrone results were under-estimating the remaining polysaccharide; which could have been the case if the polysaccharide contained sugars which produce little or no colour in the anthrone reaction. Furthermore, the results for this extraction, obtained by the anthrone method, were rendered less reliable owing to interference in the reaction by an unknown cause which gave rise to a purple colour in addition to the usual green.

The washed neutralised alkali-extracted residue was dried with acetone and suspended in 2 N sulphuric acid (25 ml.) and heated in boiling water for 30 minutes. The residue was centrifuged and washed

with 2N sulphuric acid (3 x 5 ml.) and the washings added to the supernatant (35 ml.) which was treated with 4 volumes of acetone and left overnight at 4°. As no precipitate was obtained from the supernatant even after the addition of a further 4 volumes of acetone, it was not investigated further. The residue from the 2 N acid extraction was dissolved in 60% sulphuric acid (10 ml.) at room temperature and a yellow-brown solution obtained which was left for 10 hours at 4°, during which time no darkening of the solution was observed. The acid was diluted with water (6 volumes) when immediate precipitation was caused; the precipitate was removed by centrifugation and washed with 2 N sulphuric acid after which it was dried with acetone. This final residue which was obtained in the form of dark brown granules (35 mg.) was designated "residue".

The acid supernatant was tested for the presence of polysaccharide by adding sodium acetate to a small sample and precipitating with 2 volumes of acetone; a flocculent white precipitate was obtained, indicating that the acid had extracted something from the cell residues. Before precipitating the main supernatant solution it was examined on a paper chromatogram to find whether any hydrolysis had been caused by the cold 60% acid; free glucose and oligosaccharides were found in addition to unhydrolysed material which remained at the starting

line. The acid supernatant was neutralised with barium carbonate and the precipitate removed by centrifugation. The precipitate was washed with water (3 x 100 ml.) and the washings added to the supernatant, which was then distilled in vacuo at 45-50° to low volume (20 ml.). Sodium acetate (0.1 g) was added to the concentrated solution and the polysaccharide precipitated with acetone (2 volumes) and left overnight at 4°. The precipitate was centrifuged and washed with 75% aqueous acetone and then dried with pure acetone and ether. This fraction was designated "polysaccharide III". (18 mg.)

Purification

Only polysaccharides I and II were purified, because the other fractions were not available in large enough amounts. Polysaccharides I and II were found to have high nitrogen and phosphorus values (N 7.5%, 8.5%; P 5.4%, 6.6% respectively) equivalent to 40% - 50% of nucleic acid in each preparation. Nucleic acid contamination was confirmed by the presence of large amounts of ribose on the chromatograms of hydrolysed polysaccharide I. The method for removing nucleic acid described by Aubert (1951) was used on both polysaccharides.

Polysaccharide I (0.6 g) was dissolved in water (50 ml.) and 10% lead acetate solution (5 ml.) was added; a white flocculent precipitate was produced immediately. The precipitate was increased when the

solution was adjusted to pH 3.9 by dropwise addition of glacial acetic acid (approx. 3 ml.). The suspension was centrifuged and the precipitate was washed with 10% acetic acid (30 ml.) which was added to the supernatant; the precipitate was discarded. Acetone (3 volumes) was added to the supernatant and a white flocculent precipitate was obtained which was separated by centrifugation and redissolved in 10% acetic acid (30 ml.). The solution was neutralised with solid sodium carbonate. When acetone was added to precipitate the polysaccharide it was found that the solution was immiscible with acetone and two layers were formed; prolonged stirring had no effect. It was thought that this phenomenon was caused by the high salt concentration of the solution. The acetone was decanted and the aqueous layer dialysed for 40 hours against running tap-water, during which a slight precipitate settled out of the solution. After centrifugation, the opalescent dialysate (55 ml.) was tested for the presence of further-precipitable nucleic acid, by adding a few drops to acidified lead acetate solution (pH 3.9): as no precipitate was obtained, further purification was not attempted. A small sample of the solution was acidified with sulphuric acid to give a N solution, and was hydrolysed for 24 hours at 100°. The paper chromatogram showed the presence of ribose but in much smaller amounts than was found in the initially isolated

polysaccharide, showing that a detectable purification had been achieved. The polysaccharide was isolated by adding acetone (3 volumes) to the solution and leaving the precipitate to settle out overnight at 4°. The supernatant was decanted and after being tested with more acetone to find that it contained no unprecipitated polysaccharide, it was discarded. The precipitate was washed several times with acetone and ether, and was dried over phosphorus pentoxide. The purified polysaccharide (I) weighed 130 mg.

Polysaccharide II was purified in a similar manner. The whole sample was dissolved in water (15 ml.) and 10% lead acetate solution (1 ml.) was added. Glacial acetic acid was added dropwise to the solution to adjust the pH to 3.9, and the precipitate thus formed was centrifuged and washed with 10% acetic acid (10 ml.); the washings were added to the supernatant. Acetone was added to the supernatant which was left overnight at 4°. The supernatant was poured off from the flocculent white precipitate which had settled out. The precipitate was washed with acetone and ether, and was finally dried over phosphorus pentoxide. The sample of purified polysaccharide II weighed 10 mg.

Properties and Composition of the polysaccharides.

Polysaccharides I, II and III dissolved easily in water, forming non-viscous solutions, in marked contrast with the exopolysaccharides. None of the

solutions gave colour reactions when tested with iodine. The specific rotation of polysaccharide I was $[\alpha]_D^{15}$ + 105° (c 0.19, water; d = 1).

The nitrogen and phosphorus results (4% and 2% respectively) for the purified specimen of polysaccharide I indicated that purification had halved the nucleic acid present in the polysaccharide, but that the material was still heavily contaminated, with about 25% nucleic acid. This was confirmed by the low anthrone values for polysaccharides I and II, found to be 39% and 22% respectively.

The component sugars of the polysaccharides were identified on paper chromatograms run in butanol-ethanol-water solvent (5:1:4 v/v). Trial experiments showed that 5 hours hydrolysis with N sulphuric acid at 100° was insufficient for complete hydrolysis. The conditions described for the hydrolysis of each polysaccharide was chosen empirically and found to give complete hydrolysis in 24 hours. The absence of hexosamine was ascertained in polysaccharide I and II by spraying the chromatograms with the Morgan and Elson reagent.

Component sugars of A3(O) polysaccharides

Fraction	Hydrolysed for 24 hr at 100° with:	Component sugars
Polysaccharide I	N sulphuric acid	galactose, little glucose, ribose (from nucleic acid)
" II	"	Galactose, little glucose
" III	"	Glucose, little galactose

Component sugars of A3(O) polysaccharides (Contd.)

Residue	95% formic acid followed by 3 hr with N sulphuric acid	glucose

The components were identified by comparison with standards run alongside on the same chromatogram sheets. The galactose and glucose in polysaccharides I and II were confirmed by adding galactose to part of the hydrolysates and comparing them on chromatograms with the hydrolysates to which no galactose was added. The chromatographic patterns of the hydrolysates with and without added galactose were identical, confirming the identification of the spots. The presence of galactose in polysaccharide I was confirmed by the isolation of mucic acid. The polysaccharide (1 g. of crude boiling-water-extracted material) was dissolved in water (10 ml.) and an equal volume of concentrated nitric acid added. The solution, in a boiling tube, was heated for an hour in a boiling water bath. A small amount of white crystals separated on cooling; the solution was left for several days at 4°, and then concentrated to a smaller volume (6 ml.) by the action of a gentle stream of air. More crystals separated when the tube was cooled in ice. The solution was filtered at the pump and the crystals were washed thoroughly with cold water and dried with acetone. Without further purification the crystals were found to have a sharp melting point. (Yield 40 mg.)

M.p. of crystals	209 - 210° decomp.
M.p. of authentic mucic acid	210 - 211° decomp.
Mixed m.p.	210° decomp.

The isolation of mucic acid from oxidised polysaccharide I confirmed the presence of galactose in the polysaccharide.

It would have been desirable to have examined the homogeneity of the polysaccharides by carrying out fractionations of the polysaccharide preparations but they were present in amounts that were too small for chemical fractionation. It was found by examining two different preparations of polysaccharide I that the galactose:glucose ratio varied significantly, suggesting the possibility that polysaccharide I was a mixture of a galactan and a glucan. The polysaccharides were analysed by hydrolysing 20 mg. samples in N sulphuric acid (2 ml.) at 100° for 24 hours. The solutions were neutralised with barium carbonate, treated with Amberlite resin IR-120, and applied as syrups to the chromatograms. No reference sugars were used in these determinations because only the relative amounts of the two components were to be found. The chromatography and the estimations by periodate oxidation were carried out in the manner described for the exopolysaccharides, in Part I above. The galactose:glucose ratio in the two preparations was found to be 15:3 and 15:1 respectively.

Serological results

The immunological reactions of polysaccharide I were examined to find whether it was the somatic antigenic polysaccharide of the A3 group of A. aerogenes strains. Immune serum obtained by injecting rabbits with cells of strain A3(0) was kindly provided by Dr. P.N. Edmunds. Precipitation tests, carried out as described in Methods in Part I of this thesis, were carried out using polysaccharide I solution to titrate the A3(0) antiserum. The serum was absorbed with A3(0) cells and with polysaccharide I, and the absorbed sera tested in precipitation tests with polysaccharide I, and in agglutination reactions with washed A3(0) cells. The results are shown in table

Highest dilutions of antiserum giving precipitation and agglutination reactions with various antigen preparations of strain A3(0).

Antigen	Test	A3(0) antiserum absorbed with:- Nil	Polysaccharide I	A3(0) cells
polysaccharide I	P	32	0	0
A3(0) cells	A	10,240	2560	0

P = precipitation A = agglutination

The reactivity of polysaccharide I with A3 antiserum was also tested, in precipitation reactions. A3 antiserum absorbed with various exopolysaccharides were titrated with polysaccharide I, with the results shown in the table.

Highest dilution of antiserum giving precipitation reaction with polysaccharide I antigen.

A3 antiserum absorbed with	Antigen: Polysaccharide I Highest dilution.
Nil	8
A3S1 freeze-dried	8
A3 slime	8
A3 capsule B	4
A1	8
Polysaccharide I	0

When the various exopolysaccharide antigens were titrated with A3 antiserum absorbed with A3(O) polysaccharide I their titres were found to be the same as for the unabsorbed serum, i.e., the highest dilutions were 16 (see p.158)

DISCUSSION

The results found for the polysaccharides of A. aerogenes strain A3(0) must be regarded as being preliminary, but they provide some information regarding the general nature of these polysaccharides. The polysaccharide fractions isolated by the different extractions were most probably mixtures of monohexosan polysaccharides, the different fractions containing the hexosans in different proportions. Thus polysaccharide I appeared to be mainly a galactan in the presence of smaller amounts of a glucan. Polysaccharide II contained the same components in much the same proportions, while polysaccharide III appeared to contain less galactan and more glucan. The residue contained very little polysaccharide, most of it glucan.

The glucan found in the polysaccharides was probably glycogen, but when the polysaccharides were tested with iodine none gave the characteristic reddish-brown colour. Polysaccharide II however, was observed to possess iodine-binding properties, for when the polysaccharides in dilute solution, to which were added small amounts of iodine, were left to stand for several hours it was found that the iodine had volatilised from all the solutions except that of polysaccharide II, suggesting the possibility of the presence of a degraded glycogen. The present results contrast with those reported by Levine et al., who

found large amounts of glycogen in an unspecified strain of A. aerogenes. They also reported that boiling water did not extract glycogen from the cells, but did not mention whether the cells contained any other polysaccharide or whether these were extractable by boiling water. The discovery of large amounts of galactose in the somatic and intracellular polysaccharides supports the view that contamination with these polysaccharides is responsible for the small amounts of galactose found in the exopolysaccharides of strains A3(S1), A3 and A1.

There is little doubt that the ribose found in the hydrolysates of polysaccharide I was from the contaminating ribosenucleic acid (RNA). This was shown by the marked decrease in the relative intensity of the ribose spot on the chromatograms after the polysaccharide had been purified. It is interesting that polysaccharide II showed no trace of ribose although its nitrogen and phosphorus content indicated heavy contamination with nucleic acid. This may have been because all the RNA had been extracted by boiling water together with polysaccharide I, leaving the deoxyribosenucleic acid to be extracted by the alkali together with polysaccharide II. No deoxyribose was found on the chromatograms of polysaccharide II, but this is not surprising in view of the instability of the sugar; it may have been destroyed by the alkaline extraction, and by the acid

hydrolysis prior to chromatography.

The serological tests with A3(0) antiserum indicated that polysaccharide I contained one of the antigens responsible for the immunological specificity of strain A3(0) cells, i.e. that there are at least two antigens responsible for the antigenicity of A3(0) cells and only one is present in polysaccharide I. This was shown by the fact that absorption of A3(0) antiserum with polysaccharide I reduced fourfold the titre of the agglutination reaction with A3(0) cells but could not eliminate it completely, despite the repeated addition of solid polysaccharide I to the absorbed serum which was sufficient to eliminate the reaction between A3(0) antiserum and the absorbing polysaccharide.

The serological tests with A3 antiserum indicated that the component polysaccharides of polysaccharide I contribute to the antigenicity of the capsulate strain, confirming the results found by Wilkinson, Duguid and Edmunds (1954). The decrease in titre of the reaction between polysaccharide I and the A3 antiserum absorbed with A3 capsular polysaccharide B indicated that the latter polysaccharide contained a small but immunologically significant amount of polysaccharide I, confirming the view that the galactose found in the exopolysaccharides of strains A381, A3 and A1 arise from contamination with the somatic and intracellular polysaccharides. This is not incompatible with the observation that absorption of A3 antiserum with the

other exopolysaccharides did not reduce the titre of the precipitation reaction with polysaccharide I, because their lower galactose figures indicated that they contained much less of the contaminating polysaccharide than the A3 capsular polysaccharide.

The polysaccharides of strain A3(0), and therefore the somatic and intracellular polysaccharides of strains A3(S1), A3 and A1, differed from the exopolysaccharides of the latter strains in two respects. The former polysaccharides appear to be simple hexosans and are therefore much less complex than the triheteroglycan polyuronides which constitute the exopolysaccharides, and also they contain galactose as the main component while the exopolysaccharides contain none. There is no obvious relationship between the exopolysaccharides and the somatic and intracellular polysaccharides of A. aerogenes strains A3S1, A3 and A1.

SUMMARY Part III

The somatic and intracellular polysaccharides of a smooth strain (A3(0)) of A. aerogenes were extracted by a series of increasingly drastic conditions: boiling water, boiling 10% potassium hydroxide and cold 60% sulphuric acid. The first and second stages remove 77% and 20% of the total polysaccharide respectively. The fractions appear to be mixtures of a galactan and a glucan in various

proportions. The galactan comprises the bulk of the total polysaccharide of this strain, and is believed to account for the small amounts of galactose found in the exopolysaccharides of A. aerogenes strains A1, A3 and A3(S1). The identity of the galactose was confirmed by oxidation of some of the main fraction and isolation of mucic acid.

The main fraction of isolated polysaccharide reacted with A3(O) antiserum but could not completely absorb all the antibody against A3(O) cells.

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