1 Introduction and definition

1.1. Introduction

The surface of the microbial cell is a rich source of carbohydrate-containing molecules. Some of these are unique types, confined to a limited range of microorganisms. These are the components of the microbial cell walls such as yeast mannans, bacterial teichoic and teichuronic acids, lipopolysaccharides and peptidoglycan. However, in addition to these wall components, polysaccharides may be found either associated with other surface macromolecules or totally dissociated from the microbial cell. These are **exopolysaccharides**, extracellular polysaccharides showing considerable diversity in their composition and structure. Some of these polymers may bear a strong chemical similarity to cell-wall components, but the majority are distinct chemical structures totally unrelated to cellular constituents.

Exopolysaccharides occur widely, especially among prokaryotic species, both among those that are free-living saprophytes and among those that are pathogenic to humans, animals and plants. Most microalgae yield some type of exopolysaccharide but they are less common among yeasts and fungi. However, some of those isolated from fungi do possess interesting physical and pharmacological properties.

Definition of exopolysaccharides is more difficult than definition of the carbohydrate-containing polymers found in microbial walls. The term exopolysaccharide has been widely used to describe polysaccharides found external to the structural outer surface of the microbial cell and it can be applied to polymers of very diverse composition and of different physical types. The term *glycocalyx*, introduced by Costerton, fails to differentiate between the different chemical entities found at the microbial surface. It has been used to represent a complex array of macromolecular species inlcuding components which are truly extracellular, together with wall polysaccharides and many other non-carbohydrate-containing chemical species. Although it may describe structures seen under the microscope or electron microscope, it is inadequate in chemical terms.

The exopolysaccharides do not in themselves normally contribute to microbial structure; the other components of the cell surface are unaltered if exopolysaccharides are absent. They do, however, form structures which can be recognised *per se* by either light or electron microscopy. In a few microbial genera, they form components of more complex structures which may be involved in different morphogenetic cycles such as those found in
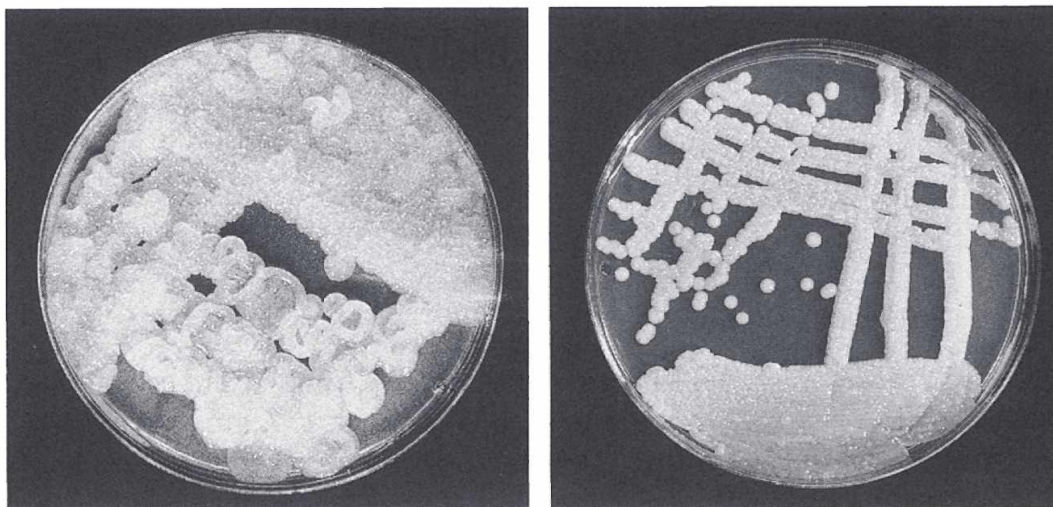
1

Fig. 1.1. The exotic colonial appearance of two polysaccharide-producing pseudomonads.

the families Azotobacteriaceae and the Myxobacteriaceae. In each of these bacterial groups, exopolysaccharides are associated with normal vegetative cells and with resting cells in the form of microcysts. In the Azotobacteriaceae, microcysts and vegetative cells differ little in shape and both are surrounded by exopolysaccharide of similar composition. In the Myxobacteriaceae, the microcysts and polysaccharide are contained within complex fruiting bodies; the vegetative cells also excrete considerable quantities of extracellular polysaccharide and resemble the Myxomycetes in leaving 'slime trails' on the surface of solid media.

1.2.   Definition

The presence of exopolysaccharides associated with microbial cells grown on solid surfaces is frequently recognisable from the mucoid colony morphology. This may be linked, in some bacterial isolates, to unusual appearance (Fig. 1.1). In liquid medium, exopolysaccharide-producing cultures may become very viscous or, exceptionally, may solidify as a gel. The exopolysaccharide may form part of a *capsule* firmly attached to the bacterial cell surface. Alternatively, it may be observed as loose *slime* secreted by the microorganisms but not directly attached to the cell. On solid surfaces exposed to aqueous environments, whether within the human or animal body, in fresh water or in the oceans, bacterial growth is seen as *biofilms*, in which the microbial cells are associated with large amounts of exopolysaccharide. Unfortunately, many of the descriptive reports of extracellular polysaccharides in the laboratory and in natural environments have failed to recognise the relationship between the physical forms of these macromolecules and the physiological conditions present. Changes in the growth conditions can drastically alter the composition, physical

properties and organisation of the polysaccharides at the bacterial surface.

Visualisation of exopolysaccharides under the light microscope is possible either through negative staining or through the use of specialised stains, which frequently utilise the polyanionic function of many of these polymers. Negative staining has the advantage of distinguishing between capsules and extracellular slime. Techniques such as scanning electron microscopy (SEM) frequently provide strong evidence for the presence of exopolysaccharide, but are not capable of providing confirmatory chemical evidence. Equally, the modern techniques of transmission electron microscopy (TEM) can reveal considerable information about the surface structures, including the polysaccharides involved, but are unable to distinguish between different chemotypes; neither do they have sufficient resolution to provide much information on the microstructure of the bacterial capsule. Unfortunately, the very high water content of the polysaccharides, in excess of 99%, makes preparation and resolution difficult. However, improved electron microscopic preparation methods, including pretreatment with anticapsular IgG and freeze-etching, can reveal the exopolysaccharide in an uncollapsed state. Capsules can be visualised as being fibrous in composition. Thin sections of gelatine-enrobed bacteria show fibrous strands extending radially from the bacterial surface (Fig. 1.2). Apparent association between the fibres may represent the ordered form of the polymer molecules. Improved electron microscopic methods can also be used to study the structure of pure polysaccharide when prepared in aqueous solution. This *may* provide supporting evidence for their conformation in solution and for intramolecular interaction.

1.3. Composition

Exopolysaccharides are primarily composed of carbohydrates, but in addition to the various sugars, there may be organic and inorganic substituents. The carbohydrates found in microbial exopolysaccharides are extremely diverse. Most of the sugars are those commonly found in animal and plant polysaccharides. D-glucose, D-galactose and D-mannose in the pyranose forms are present in many exopolysaccharides. The 6-deoxyhexoses, L-fucose and L-rhamnose, are also frequently present. A distinction between eukaryotes and prokaryotes can be seen in the presence of pentoses. Eukaryotic polysaccharides may contain pentoses such as D-ribose or D-xylose, but they are of less common occurrence in extracellular polymers derived from prokaryotes. The Cyanobacteria provide an exception. In this group of bacteria, pentoses may be found in the sheath polysaccharides.

In addition to the more common monosaccharides, some polysaccharides may contain one or more rare sugars. These may include L-hexoses or furanose forms of the hexoses, glucose and galactose. There are also
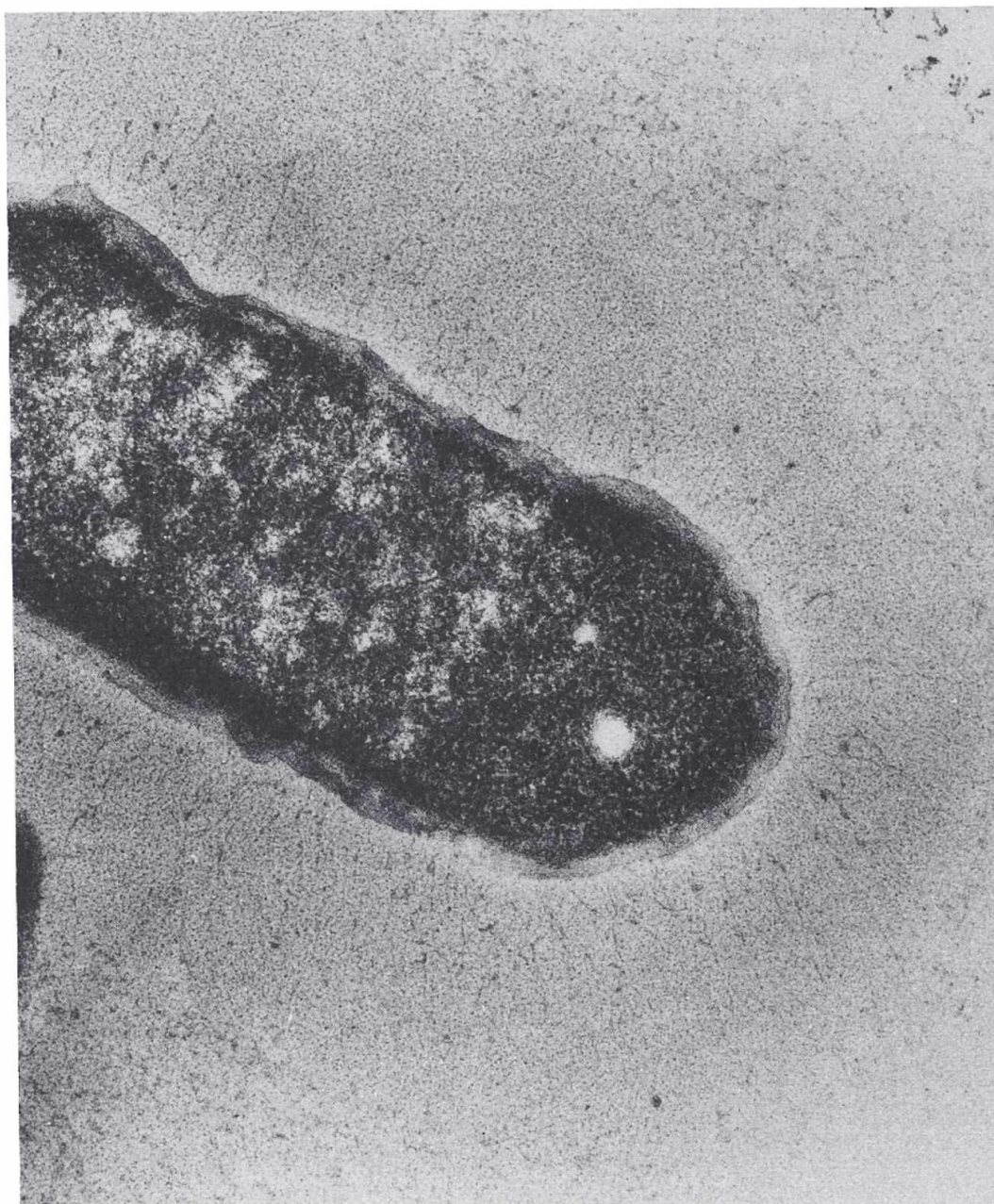
Fig. 1.2. Electron micrograph of a capsulate *Escherichia coli* strain K29. The bacteria are fixed with glutaraldehyde, enrobed with gelatine and embedded. The capsular material forms a layer about 600 nm thick around the bacterium. (Reproduced from Bayer *et al.* (1985) with permission.)

various *N*-acetylamino sugars, although as yet these have not been found in any of the microbial polysaccharides of industrial importance. Amino sugars are present in polysaccharides from species such as *Escherichia coli* but absent from the products of genera such as *Xanthomonas* or *Klebsiella*. The commonest amino sugars are *N*-acetyl-D-glucosamine and *N*-acetyl-D-galactosamine. *N*-acetyl-D-mannosamine is occasionally found and there are also several rarer amino sugars such as fucosamine and talosamine.

Inevitably, as a wider range of microbial sources of exopolysaccharides is examined, new monosaccharides will be discovered.

Many or perhaps even most microbial exopolysaccharides are polyanionic in nature. This results from the presence in many of them of uronic acids, with D-glucuronic acid being easily the commonest. D-galacturonic acid is less common and only a small number of polysaccharides containing D-mannuronic acid have been identified. Although most polysaccharides contain only a single uronic acid, D-mannuronic acid is usually present in bacterial alginates together with L-guluronic acid. Some polysaccharides from *Rhizobium* species are known to contain both D-glucuronic acid and D-galacturonic acid. An exopolysaccharide with L-iduronic acid has recently been reported from the anaerobic rumen bacterium *Butyrivibrio fibrisolvens*. This sugar is found in the sulphated proteoglycans present in higher eukaryotes but had not until now been reported in prokaryotic material. Hexosaminuronic acids have been identified in a wide range of bacterial polysaccharides. Indeed, the exopolysaccharide from *Staphylococcus aureus* strain M contains both 2-acetamido-2-deoxy-D-fucose and 2-acetamido-2-deoxy-D-galacturonic acid.

Another unusual sugar found in exopolysaccharides is ketodeoxyoctonic acid (KDO), 3-keto-deoxy-D-*manno*octulosonic acid. This sugar is of almost universal occurrence in the core portion of the lipopolysaccharides from the walls of Gram-negative bacteria. It was originally thought to be limited to these polymers but has recently been identified in eukaryotic material and as a component of several bacterial exopolysaccharides from strains of *Escherichia coli*.

1.4. Organic substituents

In addition to carbohydrates, the microbial exopolysaccharides contain various ester-linked substituents and pyruvate ketals. These are very widely found in bacterial exopolysaccharides, although they have as yet seldom been reported from eukaryotic polymers. Acetate, as an ester-linked substituent, does not contribute to the overall charge on the polysaccharide molecule, whereas the pyruvate ketals add to the anionic nature of the polymers. Pyruvate is normally present in stoichiometric ratios with the sugar components of the exopolysaccharide and is usually attached to a neutral hexose. It is, however, occasionally attached to uronic acid, and one exopolysaccharide from *Klebsiella aerogenes* contains a pyruvylated methylpentose. The pyruvate is commonly present along with uronic acid residues, both contributing to the overall anionic charge. Indeed, in an exopolysaccharide secreted by *Rhizobium leguminosarum*, *R. trifolii* and *R. phaseoli*, the octasaccharide repeat unit is unusual as it contains two moles of D-glucuronic acid, one mole of pyruvate linked as a ketal to D-galactose, and another mole similarly linked to D-glucose (Zevenhuizen, 1984). The

two pyruvylated hexoses are the terminal and subterminal residues, respectively, of a tetrasaccharide side-chain. In some exopolysaccharides, pyruvate alone is responsible for the anionic nature of the polymer. This is true of a polysaccharide from E. coli (Anderson et al., 1987) and of material from Zoogloea ramigera. Both of these polymers contain D-glucose and D-galactose as the only monosaccharides, the pyruvate being linked to galactose. Another similar polymer, obtained from a number of isolates of Pseudomonas marginalis, contains the same sugars along with pyruvate and succinate (Osman and Fett, 1989). The commonest ester-linked component is acetate, but propionyl groups have also been found in E. coli K14 exopolysaccharide and ester-linked glyceryl residues form part of the structure of the polymer from Pseudomonas elodea before it is converted to the deacylated form which is sold as gellan or gelrite. In each case, these unusual esters are present as well as acetate and will contribute to the lipophilic nature of part of the polysaccharide molecule. Succinyl groups, as the half ester, are also found along with acetyl groups and may represent up to 7% of the exopolysaccharide weight from certain Rhizobium species and from Agrobacterium species. Also present in some bacterial polysaccharides, as ester-linked residues attached to D-galactose, is 3-hydroxybutanoate. This substituent is present in material from R. trifolii and R. leguminosarum. Lactyl groups are found in some Klebsiella aerogenes exopolysaccharides. Although formate was at one time thought to be another acyl group present in some K. aerogenes polysaccharides, its presence in this group of exopolysaccharides has now been discounted after re-examination with fast atom bombardment spectroscopy.

The acyl content of some polysaccharides can be relatively high and, as well as increasing the lipophilicity of the molecule, may affect the capacity to interact with other polysaccharides and with cations.

In many exopolysaccharides the acyl groups are found in stoichiometric ratios relative to the monosaccharides present, but this is not always the case. Several polymers contain acyl substituents on alternate repeat units. Other polymers may be more random in their acylation and it should be remembered that most commercial preparations of xanthan only contain pyruvate on about 30% of the terminal side-chain mannosyl residues. In bacterial alginates, which lack any regular repeat structure, the acetyl content may be up to 15–20%, only the D-mannuronic acid residues being acetylated. Bacterial alginates also belong to the small group of polymers in which multiple acetylation of single sugar residues has been detected.

The presence of several amino acids in bacterial exopolysaccharides has recently been reported. Serine is found in the exopolysaccharide from E. coli K40; L-glutamic acid has been detected during a re-examination of Klebsiella aerogenes type 82 polysaccharide. Taurine is found as an ester-linked substituent in some other polysaccharides.

1.5. Inorganic substituents

It was long thought that sulphate was limited to eukaryotic polysaccharides and proteoglycans, but it now appears that sulphate is present in some prokaryotic polymers. As yet, these are confined to species of Cyanobacteria. The extracellular sheath or flocculant produced by the cyanobacterial *Phormidium* species is one such sulphated heteropolysaccharide.

Phosphate is of much more widespread occurrence and is frequently found in bacterial polysaccharides of immunological significance. Many of the phosphorylated exopolysaccharides resemble the teichoic acids present in the walls of Gram-positive bacteria; indeed, many strains produce phosphate-containing wall polymers and extracellular polysaccharides. Although phosphate is absent from many of the Gram-negative bacterial polysaccharides investigated so far, including those from genera such as *Klebsiella*, *Rhizobium*, *Xanthomonas* and *Pseudomonas*, it has been identified in the products of a number of *E. coli* strains. As well as the inorganic components which form part of the structure of exopolysaccharides, it has to be remembered that all polyanionic polymers will normally be obtained in the salt form. These will represent a mixture of cations, although some may be bound more firmly than others. Thus some alginates bind the divalent cations calcium, barium and strontium very strongly, whereas polysaccharide XM6, from a strain of *Enterobacter*, favours the monovalent and divalent cations with ionic radius of about 1.0, i.e. Na^+ and Ca^{2+}. The ions associate with the polymer during its production but can be displaced by appropriate procedures (ion exchange, electrodialysis, etc.) to enable conversion into a free acid or uniform salt form. This is important in determining the physical properties of some of the exopolysaccharides but is not normally needed for commercial products. The range of non-carbohydrate substituents found in microbial exopolysaccharides is indicated in Table 1.1.

1.6. Homopolysaccharides

Many microbial exopolysaccharides, including several of potential industrial importance, are homopolymers. These include a number of glucans, which, because of their different structures, possess significantly different properties even though the sole monosaccharide component is D-glucose. The fungal product scleroglucan has high viscosity whereas curdlan, a product of several bacterial species, is gel-forming, and bacterial cellulose is microcrystalline and insoluble. Curdlan and cellulose are composed of a single linkage type, but several other homopolysaccharides, including scleroglucan and pullulan, possess two types of glucosyl linkage. Dextran, also a glucan, is more complex and contains three different types of linkage

Table 1.1. *Non-carbohydrate substituents in exopolysaccharides*

Substituent	Linkage	EPS-producing bacterium
Organic acids		
Acetate	ester	very common
Glycerate	ester	*Pseudomonas elodea*
Hydroxybutanoate	ester	*Rhizobium trifolii, R. leguminosarum*
Propionate	ester	*Escherichia coli*
Pyruvate	ketal	very common
Succinate	ester	*Rhizobium* spp, *Agrobacterium* spp
Amino acids		
L-glutamate		*Klebsiella aerogenes* K82
Serine		*E. coli* K40
Inorganic acids		
Phosphate		common
Sulphate		cyanobacteria

(Table 1.2). Although most homopolysaccharides are composed of neutral sugars, a small number are polyanionic. Alginates composed solely of D-mannuronic acid can be obtained, and some bacterial homopolysaccharides are polysialic acids (Chapter 11).

1.7.   Heteropolysaccharides

The majority of microbial polysaccharides are probably heteropolysaccharides. These range from polymers with two sugar components to others with four or five monosaccharides. The possible range of structures and of resultant differences in properties is very great indeed because of the number of possible linkages and configurations. Each hexose can be α- or β-linked; in the pyranose or furanose form; and linked through the 2, 3, 4 or 6 position. However most of the polysaccharides that have been discovered so far are formed from two or three sugars and various acyl substituents. It is also probable that most of the polymers with industrial potential will contain certain types of linkage, at least in the main chain, as these will confer the desired physical properties (Chapter 8). However, a much wider range of structures can be expected among polysaccharides of medical interest.

1.8.   Visualisation of polysaccharides

Several methods of examining exopolysaccharides take advantage of the polyanionic nature of many of them. One such method involves cytochemical treatment with cationic ferritin. The complexes formed with the acid

Table 1.2. *Properties of dextrans*

| Group	Linkage class	Intrinsic viscosity at 25°C	Nature of polymer	Appearance of 1–2% aqueous solution
1	A,B	1.2–0.6	very cohesive, tough gum or flocculant	very turbid
2	A,C	0.5–0.2	fine or flocculant precipitate	opalescent solution
3a	A,B	0.9–0.5	fine or flocculant precipitate or dense gum	very turbid
3b	C	1.4–0.5	flocculant precipitate or dense gum	very turbid
4a	A,B	1.3–1.0	soft gum	slightly opalescent
4b	A,B	2.0–1.6	cohesive, stringy gum	slightly opalescent
4c	A,C	1.4–0.4	stringy or fluid gums	clear or slightly turbid
5a	A,B	1.2–0.6	short or stringy gums	turbid or slightly opalescent
5b	A,B	1.0–0.9	flocculant precipitate or short gum	slightly to very turbid
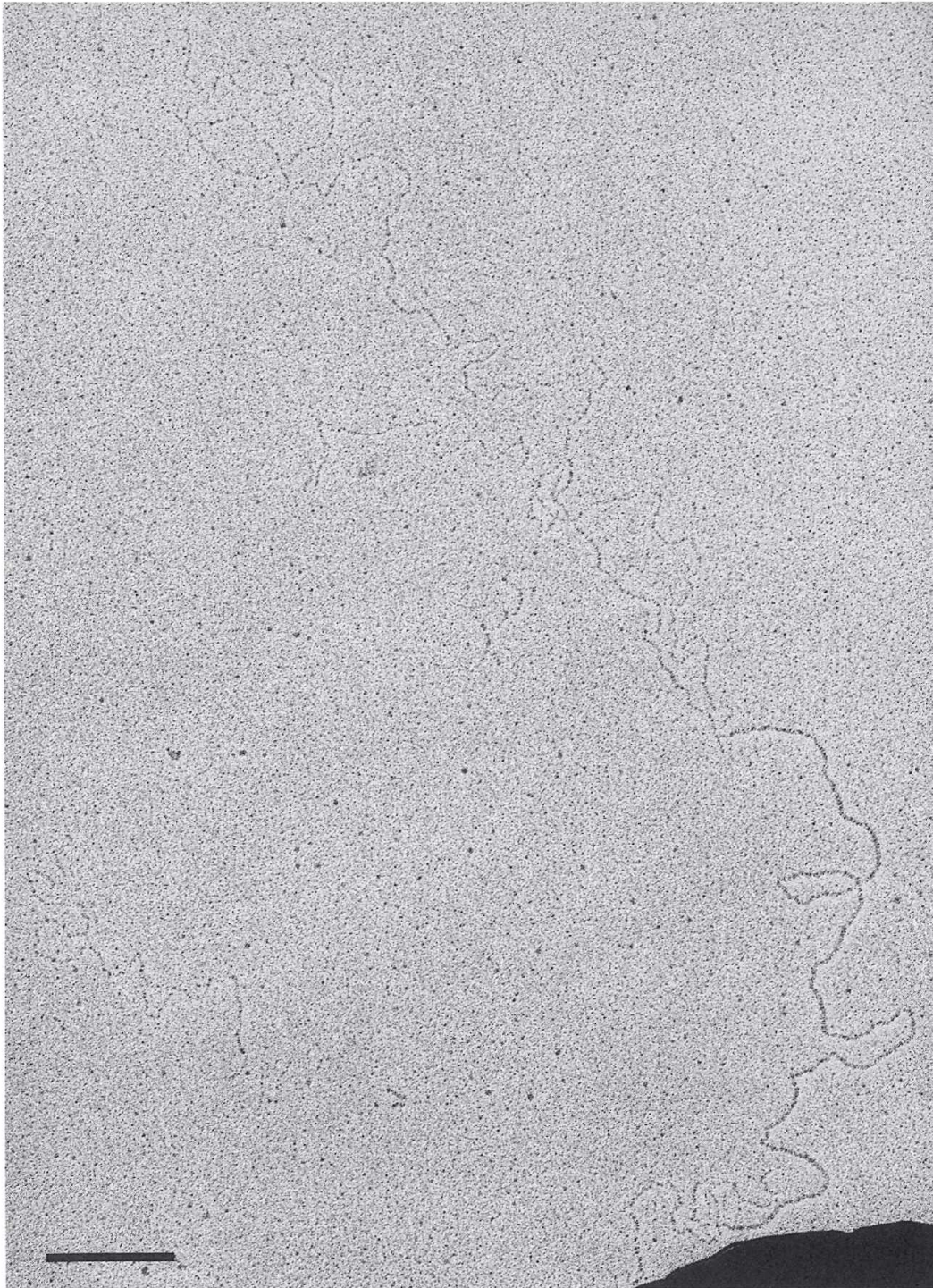
Fig. 1.3. Electron micrograph of a xanthan preparation showing the apparently double-stranded chain separating into two thinner strands. Prepared in 50% glycerol, 2 mM ammonium acetate, pH 7.0. Bar, 200 nm. (From Stokke *et al.* (1986) with permission.)

polysaccharides of *Klebsiella* species can then be readily seen. If the preparations are first interacted with homologous anticapsular serum and fixed with glutaraldehyde, the polymeric material is cross-linked and the polycationic ferritin cannot penetrate the complex but is seen on the capsular surface.

Recent studies have shown that xanthan, vacuum dried from a glycerol-containing solution, can be rotary shadowed and examined by electron microscopy. The polysaccharide can be seen as single- and double-stranded chains, together with partly dissociated double-stranded structures (Fig. 1.3). From the electron microscopic data and intrinsic viscosity measurements, the average relative molecular mass can be calculated.

Further reading

Sutherland, I. W. (1988). *Bacterial surface polysaccharides: structure and function. International Review of Cytology* **113**, 187–231.