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Shapely Polysaccharides

THE EIGHTH COLWORTH MEDAL LECTURE

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Ever since I was so pleasantly surprised by the letter from Professor Davison that told me about the Committee's decision, it has been natural for me to ponder the rationale of Awards like this. I cannot think that a Society so concerned with righteous standards should perpetuate this Award to encourage young biochemists to vanity rather than productive work. I am sure that it is meant to have a more positive influence and I would like to say first what it means to me.

When I started research, I was very romantic and thought that it would be better to commit myself to a subject that was temporarily unfashionable and that I could believe had jewels concealed behind the grey appearances. A decade later, I see that much of my reasoning was over-optimistic and that my investigations could easily have led nowhere at all. The award of this Medal is a reassurance that I have been lucky enough to avoid disaster and that my research life has, after all, started on the right lines. Perhaps even more, the distinguished men who represent this Society seem to be saying that my work is not only worthy and sound but has some interest; this is a special compliment when one knows from experience that they can quite readily take the reverse view when it is justified. And so this Medal comes as a particularly great reward. I am especially pleased that colleagues in my research field, who committed themselves similarly in faith and hope, should rightly see this Award as a vindication of them as well.

Because of all this, perhaps it is appropriate that this Lecture should have some personal as well as scientific content. My undergraduate training was in the Department of Chemistry at the University College of North Wales, where I was greatly influenced by the research combination of the gentle, rational and philosophical Stanley Peat and that well-known ball of fire, Bill Whelan. It was natural that I should be drawn to this group to do research. By this time, Whelan had moved to the Lister Institute and was replaced by Jim Turvey, who over the subsequent

years provided a background of generosity and sanity without which I might have sunk without trace. Later, I moved to Edinburgh, where carbohydrate research was of course very active already, with David Manners, Elizabeth Percival, Gerald Aspinall, Trevor Greenwood, Peter Schwarz and Douglas Anderson, as well as Professor Sir Edmund Hirst. I began to grow up, under the stern but gentle scepticism that Sir Edmund always shows towards trivial ideas coupled with his great enthusiasm for what he calls the 'big problems', and with day-to-day contacts with Gerald Aspinall in particular and also Peter Schwarz to show up my scientific deficiencies and prod me to do something about them. However, despite all the energy and thought that was going into our work, we all felt that carbohydrate chemistry and biochemistry were running down. Whelan mentions in his CIBA Lecture (Whelan, 1971) that he felt this way about starch and glycogen in about 1957.

Times have changed, however, and great things have happened since then. We have seen a slow unfolding of the story of polysaccharide biosynthesis following Leloir's discovery of sugar nucleotides, which led to his Nobel Prize. Likewise, with great pleasure we have watched Strominger's progress with the relation between antibiotic action and the bacterial cell wall. The three-dimensional structure and probable mode of action of a carbohydrase enzyme has been shown to us by David Phillips and his group. Don Northcote and others have unravelled a great deal about how cytoplasmic organization leads to the structure of plant cell walls. The polysaccharides of mammalian connective tissue, and glycoproteins, begin to make biochemical sense for the first time ever. So many exciting developments have occurred that this period seems to have moved us out of a dark age to see polysaccharides in quite a new light. They have become interesting molecules to contemplate in relation to the life of the cell. The ugly ducklings have begun to look a little more like swans. In this sense, polysaccharides begin to appear attractive molecules, shapely molecules.

Secondly, and somewhat incredibly, my own work has progressed. I say 'incredibly' because at any one moment I usually seem to be beating my head against the same old problems, and it is a mild surprise to see

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(I) [amylose as shown in Haworth formulae; this representation does have advantages that justify its continued use in some circumstances (Rees, 1967), but it also conceals important problems and properties]

that there has been progress over the long term. I don't even call up the same mental image of 'a polysaccharide' that I used to. In the old days this image would have thin flat rings connected by bonds with right-angled bends in them [e.g., structure (I)]. Nowadays the polysaccharide image has a shape—flopping and fluctuating in solution, or reposing as a helix or some other form in the solid state. Polysaccharides are therefore shapely in this second sense also. Since this Lecture is a very special occasion for me, perhaps I can turn aside from the really big advances that others have made over these 10 years and talk about what has happened in my own microcosm.

I have been brought up to have respect for the small details of covalent structure, but, on the other hand, I have always felt that this structure has significance only when related to physical properties or biological function. The difficulties in defining these relationships rigorously are what Sir Edmund usually meant when he spoke of the 'big problems'. When I had the opportunity to formulate an independent research programme of my own, I therefore began by defining a few systems that seemed to offer the opportunity of working from a good foundation of well-characterized covalent structure towards a molecular interpretation of these other aspects. The systems were, first, the remarkable gelling properties of agarose and related seaweed polysaccharides. These properties are well known and much used by biochemists, both for gel filtration and for the culture of micro-organisms. The low concentrations at which these gels form, and the properties that then result, have few parallels that are quite as striking among natural or synthetic polymers. The second system was the plant cell wall; here we have, especially in the young tissue, another type of polysaccharide gel, but one that responds and is changed as part of growth and differentiation. Thirdly, we have looked at some of the polysaccharides on outer layers of bacteria.

Today I shall follow the thread of the investigation of gel systems *in vitro* because I have time for only one topic, and it so happens that this particular thread

has been most productive of clear new ideas. I shall also try to convince you that these systems help us to understand the biological function of polysaccharides; indeed, they do help to make sense of other investigations mentioned in the Citation but that I cannot describe today.

Inside one jelly

Primary structure: defining the molecules. Early work by Araki (1953, 1958) and O'Neill (1955) had partially established the structures of the two gelling polysaccharides, agarose and κ -carrageenan. They showed that residues were arranged alternately, thus:

Residue B is D-galactose in agarose or its 4-sulphate in κ -carrageenan; residue A is 3,6-anhydro-L-galactose in agarose or its D-enantiomer in κ -carrageenan. This is a strange, if not unique, example of enantiomeric residues having almost equivalent occurrence and probably, therefore, almost equivalent function. We were to show later (Rees, 1961b; Lawson & Rees, 1970) that the biosynthetic pathways are equivalent too, and perhaps also the influences on polymer chain conformation (Rees, 1969a; Rees *et al.*, 1969). Indeed, 3,6-anhydrogalactose is an odd sugar altogether and the only sugar anhydride of natural occurrence.

The analytical data, particularly for κ -carrageenan, showed that the polysaccharide could not entirely be described by this simple alternating structure. Our contribution to the understanding of primary structure was to prove that the anomalies are to be explained in terms of a 'masked repeating structure', which is a genuine alternating arrangement modified only in that A and B may each take different forms within the same chain. The archetype of this structure is porphyran (Anderson & Rees, 1965; Turvey & Christison, 1967), a polysaccharide in which there is a strictly alternating arrangement of D-galactose and L-galactose residues:

but with partial and rather irregular 6-*O*-methylation of D-residues and with occurrence of L-residues as the 3,6-anhydride and as 6-sulphate. The sample-to-sample variation can be almost continuous, despite the fundamental regularity of structure (Rees & Conway, 1962). We showed further that this idea of a 'masked repeating structure' applies to a large number of seaweed polysaccharides (Anderson *et al.*, 1965; Rees, 1965, 1969*b*). More recently it has become evident that many mucopolysaccharides can also be described in these terms (see, e.g., Fransson, 1968; for a review see Rees, 1969*b*).

Secondary and tertiary structure: a polysaccharide double helix. These seaweed polysaccharides led to our first adventures in polysaccharide shape. By 1965 we had characterized a family of seaweed polysaccharides; they all share this alternating arrangement of α -(1 $\rightarrow$ 3)- and β -(1 $\rightarrow$ 4)-linked galactose residues, but can otherwise differ very much. The 4-linked residue can be D or L in configuration, and can be a 3,6-anhydride or not; sulphate may occur on either residue or both in a variety of positions, or on none at all. Certain members show physical similarities, especially in their remarkable ability to form reversible gels, whereas others do not gel at all. After all the developments that had occurred in protein and nucleic acid biochemistry, it was not difficult to hope that the physical similarities and differences could be explained in terms of the similarities and differences in primary structure—if only we had some information about molecular shape.

I was prodded and helped through the next stage in this story. First, my publications had been noticed by an Industrial Company, Marine Colloids Inc., of Rockland, Maine, U.S.A., which manufactures these polysaccharides for food and biomedical uses. The research staff supported me with advice and discussion, samples and a generous research grant. Secondly, I had to turn to the only experimental method that is capable of giving detailed and unambiguous information about the shapes of complex molecules, namely X-ray diffraction, and I had no experience of its use; however, I had fortunately met Professor Struther Arnott, then of King's College, and he helped a great deal with the theory and practice of X-ray diffraction by fibres. My colleague at Edinburgh, Marjorie Harding, helped with X-ray methods in general and indeed participated in the work. Nevertheless it was 2 years before we found a polysaccharide and the conditions for a crystalline fibre that gave enough diffraction data. This was ι -carrageenan, a co-polymer of D-galactose 4-sulphate and 3,6-anhydro-D-galactose 2-sulphate (II) with 'masking' by replacement of about one-tenth of the anhydride by D-galactose 2,6-disulphate (III) and a further much smaller proportion by D-galactose 6-sulphate (IV). For analysis by X-ray diffraction by fibres a polymer is required that has periodicity of

chain conformation, and this requires a periodicity of covalent structure. Fortunately it was known from earlier work that the masking features could be removed without serious breakdown of the chain; the 6-sulphate is eliminated to form 3,6-anhydride by use of alkaline borohydride (Rees, 1961*a*, 1963). Without this pretreatment the diffraction photographs were almost featureless, but afterwards it was possible to get very good results for a variety of salt forms.

The argument that led to the correct structure was long and tortuous, but, looking at the accumulated evidence as we now can, there are one or two facts that are particularly compelling, and I shall only mention these. First, with the magnesium salt it is possible to obtain diffraction diagrams (S. Arnott, C. G. M. McNab, D. A. Rees & W. E. Scott, unpublished work) that show an axial periodicity of 2.60 nm (26.0 Å). During experiments in which the relative humidity was increased there was spectacular change in the diffraction pattern. The weaker, odd-numbered, layer lines disappeared, to indicate a new structure with axial spacing of 1.30 nm (13.0 Å), whereas the even-numbered layer lines showed virtually no change in spot positions or intensity distribution. In other words something happened to the structure to cause elimination of odd-numbered layer lines with little effect on conformation or packing. This is easily explained on a double-helix model, but is otherwise difficult. Consider two parallel threefold helical chains, each twisted around the other and having a rise per turn of 2.6 nm (26 Å). If now, like two serpents in an embrace, the two chains move past each other from relative positions that are perfectly general to form a special arrangement that is exactly

Fig. 1. Readjustment of a schematic double helix to halve the translation period

Filled circles represent any set of chemically identical atoms or groups.

staggered (Fig. 1), extinction will occur of the diffraction on certain layer lines because identical groups have moved to half the initial spacing.

Of course, this evidence does not specify the conformation in detail, but it was possible to devise two independent mathematical model-building methods that start from the known covalent structure and search for sterically possible ways in which the chain might be draped to give a double helix of this sort having the dimensions and symmetry required by the X-ray-diffraction evidence. It was satisfying that both methods led to essentially the same double helix (Anderson *et al.*, 1969); a projection and photographs of this have been published (Anderson *et al.*, 1969; Rees *et al.*, 1969; Rees, 1969*b*, 1970*b*).

When a molecular model was actually set up we were delighted to see that both hydroxyl groups in the repeating unit [see structure (II)] were positioned to engage in hydrogen-bonding between the stands. The hydrogen bond is buried in the helix interior and should therefore be resistant to deuterium exchange; it is also transverse to the helix axis and should show perpendicular dichroism in the i.r. spectrum when the sample is oriented and the beam is polarized. Both these properties were verified (D. A. Rees & F. B. Williamson, unpublished work). All this and other evidence (Anderson *et al.*, 1969; Rees, 1969*b*) confirms that the model is essentially correct. Further work is required to locate counterions and refine the detailed geometry and packing. This is in progress in

(V) (masking features are not shown)

collaboration with Bill Scott and Struther Arnott, both of whom are now at Purdue University.

Helices in gels? This double-helix model was actually derived by investigation of ι -carrageenan (II), but there is enough evidence now to suggest that a similar conformation occurs in κ -carrageenan fibres, and perhaps (Rees, 1969*a*) for certain polysaccharides in the diastereomeric series, such as agarose (V). This immediately suggests a very attractive mechanism for the property of gel formation that we are seeking to explain. These gels liquefy when heated and set when cooled, and it is tempting to suggest that this occurs by the process shown schematically in Fig. 2; when the solution is cooled, the chains link by double-helix formation to give a three-dimensional framework, the interstices of which are occupied by water and sometimes other small molecules and ions. This framework is swollen by osmosis and 'props up' the solution to give some

Fig. 2. Schematic representation of the mechanism proposed for gelation of carrageenans and some related polysaccharides

Fig. 3. Changes in optical rotation that accompany the sol-gel transition in κ -carrageenan

Heating and cooling curves are distinguished by the arrows.

of the properties that we would normally expect of a solid, such as an ability to hold its own shape and an elastic response to applied stress.

Although the mechanism is speculative at this

stage in the argument, the system does behave in several ways that recall known helix $\rightleftharpoons$ coil transitions. First, as all bacteriologists know, there is hysteresis in the setting and liquefaction of many gels of this type. This would mean that, after heating to 'melt' the helices, cooling to just below the 'melting' temperature does not cause them to re-form, as we might expect. It follows from thermodynamics that, without further cooling, re-formation must be too slow to be observed on our usual time-scale. Such an increase in rate with decrease in temperature is unusual for chemical processes in general, but is characteristic of those that involve a nucleation step. The renaturation of DNA, for example, shows this effect (Marmur & Doty, 1961; Wetmur & Davidson, 1968). A further analogy with helix formation in other biopolymer systems is that the process shows a sharp change in optical rotation, and, indeed, optical-rotation measurements provide a good method for characterizing the hysteresis, as shown in Fig. 3 (Rees *et al.*, 1969).

Of course, the existence of carrageenan helices in the gel is a mere extrapolation from the X-ray-diffraction evidence for fibres. Confirmation was obtained by use of the masking features in a way that can be explained after we have examined the influence of these features on molecular shape.

Masking is kinking. At the start of the X-ray-diffraction work, masking features were eliminated by chemical modification. To examine their influence had we not removed them, we used the method of model-building in the computer that is now very well

known. The first systematic applications of this method to polymers were of course by Ramachandran and Liquori and their groups, and Ramachandran included polysaccharides in an early investigation (Ramachandran *et al.*, 1963). The starting assumption is made that sugar residues are so constrained in their shapes that they can as a good approximation be treated as rigid. This is supported by all the evidence available from monosaccharide chemistry. The shape of the backbone then depends on torsion angles about the bonds that connect the rings; by convention, these are known as ϕ and ψ , as shown in structure (VI) for the simple example of cellulose.

This simplification is useful because so many conformation questions in any branch of chemistry or biochemistry reduce to the problem of finding a sterically possible (or, better, energetically favoured) conformation that is consistent with a particular set of experimental measurements, and it can now be handled as a computer search. The polysaccharide is put in some arbitrary starting conformation and this is expressed as a set of atomic co-ordinates. The computer is programmed to generate all other conformations from this, also as sets of co-ordinates, by systematic variation of ϕ and ψ . From the co-ordinates at each stage we can in principle calculate all

(VI) (skeletal bonds only are shown)

(VII) (skeletal bonds only are shown)

(VIII) (idealized representation)

we want to know, such as particular properties to compare with experimental observation and the conformational energy.

For more complicated polysaccharides such as carrageenans we must consider at least four conformation angles, as shown in structure (VII). We have examined a variety of polysaccharides of the simple (Rees & Skerrett, 1968) and alternating (Rees, 1969a; Rees *et al.*, 1969) types, including the important animal polysaccharides with a backbone structure that is rather similar to agar and carrageenan [e.g., chondroitin 4-sulphate (VIII)]. The conformation similarities within the alternating group were striking and led us to suggest that double-helical forms might be rather widespread. However, X-ray evidence that has since become available does not favour this possibility for hyaluronic acid fibres (Atkins & Sheehan, 1971).

The model-building method showed that the 'masking' residues must cause kinking of the helical carrageenan chain. This conclusion might have been expected on intuitive grounds because the masking residue has an inverted ring conformation [Reeves C1 (III)] relative to the 3,6-anhydride that it replaces [Reeves 1C (II)]. Thus it is likely that the physical and biological function of these residues is to serve as helix-breaking interruptions that cause each chain to enter into double-helical association with more than one partner. This is necessary if a three-dimensional network is to be formed rather than a collection of isolated chain pairs.

Conformational kinking would seem to be widespread in other polysaccharides that may be called 'network polysaccharides', namely those that are organized in a loose, hydrated, manner in biological

tissues. Examples are the mucopolysaccharides of connective tissues in which glucuronic acid and iduronic acid residues 'mask' for each other and are conformationally inverted with respect to each other (Perlin *et al.*, 1970), and pectic substances of plant cell walls in which the rhamnose insertions must have a kinking effect on chains of galacturonic acid residues (Rees & Wight, 1971).

Chemical and biological reactions at the kinks. It is now clear that the chemical modifications that removed the masking features in carrageenans are to be seen as a chemical 'dekinking' (Fig. 4). More significantly, enzymes are known that catalyse the same reaction both in the L-series (Rees, 1961*b*) and the

D-series (Lawson & Rees, 1970), and these co-occur with the polysaccharides themselves. I like to call these enzymes 'dekinkases', although they do have longer names [galactose 6-sulphate alkyltransferases (cyclizing), EC 2.5.1.5]. Thus the algae have machinery to regulate the distribution of helix-breaking residues at a very late stage in polysaccharide biosynthesis—perhaps even after deposition in the tissue. This could have adaptive value for the organism and, to support this possibility for at least one species, there is a correlation between the incidence of 6-sulphate in *Porphyra umbilicalis* and the growth habitat (Rees & Conway, 1962).

ι -Carrageenan has kinks of two types [structures (III) and (IV)], of which one [structure (IV)] should be vulnerable to periodate oxidation at the carbon-carbon bond which carries two unsubstituted and adjacent hydroxy groups. There are no other oxidizable groups in the molecule, and this reaction can therefore be used as the starting point for Smith degradation (Goldstein *et al.*, 1965) to split the chain selectively at this residue. Subsequent treatment with alkaline borohydride to 'dekink' at the 2,6-disulphate (III) should then yield short chains capable of forming regular helical strands, rather than long chains that must form kinky strands. The two steps are shown schematically in Fig. 5.

The product of these reactions was found to have very interesting properties indeed (McKinnon *et al.*, 1969); solutions do not gel when cooled, but they do show the optical-rotation shift that is a normal accompaniment of gelation.

Sign and magnitude of the optical-rotation shift. It is an important advantage to have this system in which the optical-rotation shift is observed without the complication of gel formation. It allows an

Fig. 4. Schematic representation of chemical or enzymic dekinking of carrageenan

Note that the chain is believed to be helical only when combined in the double helix.

Fig. 5. Conversion of ι -carrageenan into short, non-kinked, chains

Note the same reservations as for Fig. 4.

attempt at interpretation of the sign and magnitude of the shift without fear of artifacts of the bulk change, for example from stress birefringence.

In the search for a basis on which to predict the optical rotations we re-examined the empirical treatments that have been proposed by Kauzmann *et al.* (1961), by Whiffen (1956) and by Brewster (1959). In principle, the treatment proposed by Kauzmann's group is rigorous because their only assumption is that optical rotation is an interaction effect between atoms and all quantum mechanical approaches agree that this is valid. On the other hand, Whiffen's and Brewster's methods make further assumptions about which types of interaction are dominant. They do, however, give remarkably good estimates of the optical rotations of many saturated natural products, including the whole monosaccharide family. Their value is also demonstrated by recent applications, in modified form, to conformational problems in monosaccharide chemistry that were not amenable to other spectroscopic methods (Lemieux *et al.*, 1969; Lemieux & Martin, 1970). None of these treatments is, as it stands, directly applicable to our polysaccharide problem, but it was possible to combine basic ideas inherent in all of them to derive an expression (Rees, 1970a) to evaluate a conformational contribution to optical rotation, $[A]$:

$$[A] = A - B(\sin \phi + \sin \psi) \quad (1)$$

The parameters A and B are empirical constants related to those of Brewster and are not adjustable in our work. It is very convenient that this expression is based on the variables that are most fundamental to the exploration of conformations by computer, namely ϕ and ψ . Because optical rotations are measured in solution, $\sin \phi$ and $\sin \psi$ are usually average rather than unique values. The expression was checked with di- and oligo-saccharides for which there was information from crystal-structure determinations or n.m.r. to indicate likely solution conformations, and the agreement was found to be very good (Rees, 1970a). In a more extensive test we took all the recorded optical rotations for β -glucosyl, β -galactosyl and α -glucosyl disaccharides (over 42 compounds) and tested the equation by attempting to deduce the change in conformation angles that occurs from each compound to another; these predictions were compared with those made by conformational analysis and, again, the internal consistency was excellent even in fine detail (Rees & Scott, 1971).

Thus it seemed worth while to attempt to estimate the optical rotation of the carrageenan double helix from the values of the conformation angles derived from diffraction evidence for the solid state, and to compare this with measurements on the solution in which the transition is supposed to occur. We merely substitute the values of ϕ^{AB} , ψ^{AB} , ϕ^{BA} and ψ^{BA}

[structure (VII) (Anderson *et al.*, 1969)] into eqn. (1). For the random coil we assume that the conformational terms are the same as in the corresponding disaccharides. Thus we have (Rees *et al.*, 1970):

Shift in $[\alpha]_D$ observed on cooling: $+38^\circ \rightarrow +64^\circ$

Shift in $[\alpha]_D$ calculated for coil to helix: $+34^\circ \rightarrow +63^\circ$

This agreement is very good, and gives us further encouragement to believe that our account of carrageenan conformation and its relation to properties is essentially correct.

Quaternary structure and ligand-induced changes

Gels from mixed polysaccharides. I like to regard the carrageenan gel as a primitive model for biological tissue because it is an assembly of macromolecules that has some organization and is highly hydrated. Now we progress to a system that is more elaborate in that it involves the organization of two distinct polysaccharide components, rather than merely one type.

I have already mentioned how promptings from Industry were involved in our first plunge into polysaccharide conformations. As we neared the end of that investigation, similar proddings began to nudge me in the direction that I shall now describe. These came from several sources, chiefly from Marine Colloids, who again provided funds to initiate the work, and also from Bulmers of Hereford, and more latterly from within Unilever. This pressure took the form of repeated comments such as 'funny things happen when carrageenans are mixed with certain galactomannans—I bet you can't explain them'. When I was a University teacher I came to feel very strongly that, within Industry, there is a fund of intelligent observation of polysaccharide behaviour that contains many clues to the next fundamental advances. Since these observations are often made by investigators with no primary interest in making academic advances, it is important for the rest of us to take advantage. I will give more examples later, but I would like to say that this conviction has been one influence that has made me a new convert to basic research in Industry.

Funny things do happen when certain galactomannans are mixed with some other polysaccharides. I shall not go into all the details because they are of little interest to this audience, but they can be found in handbooks of Food Technology (e.g., Glicksman, 1969). It is only important here to mention that these polysaccharide mixtures can form gels under unexpected conditions. For example, when a carrageenan solution is diluted until it does not itself gel and then mixed with a suitable non-gelling galactomannan, the mixture may gel even though this is not to be expected from the properties of the individual

components. Similar observations are made for agarose-galactomannan mixtures and other mixtures to be mentioned later. A simple interpretation of these systems would be that, although neither polysaccharide species can itself form the continuous network that is necessary for gel properties, the two types can interact to form such a network.

This interpretation is reinforced by the behaviour of chain segments from κ -carrageenan, prepared by the kink-splitting procedure described earlier. No gel is observed with these segments in aqueous solution because, although double helices can exist, they do not connect in a network. With a suitable galactomannan, however, a gel is formed. Again there would appear to exist an interaction between unlike polysaccharides to give a network that is not observable otherwise.

The setting and liquefying behaviour of these mixed gels and the variations with concentration are as expected if they depend on helix formation and 'melting' by the carrageenan component. This suggests that any interaction involves κ -carrageenan in the helical form.

Pharaoh's polysaccharide. The galactomannans that I mentioned occur as reserve materials in seeds of many leguminous plants, and different species yield polysaccharides having the same component sugar residues but in different proportions. Powdered seeds consisting largely of these galactomannans were used to make a paste for binding mummies in Ancient Egypt; I find it a fascinating thought that the chemistry that I shall describe was manipulated, even unknowingly, 5000 years ago. The gross structures of such important examples as locust-bean gum (carob gum) and guar gum have been known for many years to be based on a linear chain of β -(1 $\rightarrow$ 4)-linked D-mannose units to which D-galactose residues are attached by α -(1 $\rightarrow$ 6)-linkages (Smith & Montgomery, 1959).

Information on the way these side chains are arranged has only become available recently (Courtois & Le Dizet, 1966, 1970) by analysis of the products of action of α -galactosidase and β -mannanase. There is a tendency for the galactose substituents to occur in blocks, as shown in structure (IX).

The stable conformations of these polysaccharides are not characterized, but we do know enough about them to be useful. In a systematic survey of the conformations of all homopolysaccharides of natural occurrence (Rees & Scott, 1971) we showed by model building in the computer that regular conformations are always constrained by steric repulsion to one of several distinct types, depending on the sugar residue and linkage. The mannan backbone is of a type that is restricted to a range of extended ribbon-like conformations. The X-ray-diffraction evidence for related polysaccharides such as mannan (Frei & Preston, 1968), guar galactomannan (Palmer & Ballantyne,

Fig. 6. *Schematic representation of galactomannan conformation*

Each bend in the backbone represents a connexion between β -D-mannopyranose residues, and each 'T-junction' represents the attachment of an α -D-galactopyranose residue.

Fig. 7. *Possible model for the interaction between carrageenan and locust-bean galactomannan, resulting in gel formation*

Chain contours only are shown; the double-helical carrageenan binds the 'smooth' regions (see Fig. 6) of galactomannan.

Fig. 8. Optical-rotation changes on heating and cooling solutions of κ -carrageenan and locust-bean galactomannan separately and together

The figure shows the summed optical-rotation curves observed on heating and cooling separate aqueous solutions of non-gelling κ -carrageenan segments (4%, w/v) and locust-bean galactomannan (1%, w/v) (—) and the behaviour of a solution in which these components are mixed (— · —); gelation occurs in this second system.

1950), poly(mannuronic acid) (Atkins *et al.*, 1971) and mannan acetate (Bittiger & Marchessault, 1971) shows that they are indeed of this form. From this and the covalent structure, any regular galactomannan conformation is likely to be a ribbon that is alternately 'smooth' and 'hairy', as shown in Fig. 6. Galactomannans that are most effective in forming gels in interacting mixtures with other polysaccharides have lower proportions of galactose residues. Therefore we suspect that 'smooth' rather than 'hairy' regions are predominantly involved in the interactions.

This thread of part-reasoning, part-speculation, leads to a working hypothesis for the interaction that is shown in Fig. 7, and that will now be tested against evidence from optical-rotation measurements on setting and liquefying gels.

Influence of association on optical rotation. The effect of galactomannan on the optical-rotation behaviour of κ -carrageenan segments is shown in Fig. 8 (I. C. M. Dea, A. A. McKinnon & D. A. Rees, unpublished work). The shift in optical rotation has a sign and magnitude that is characteristic of the carrageenan double helix, and it occurs at about the usual temperature and with the usual hysteresis. This confirms that carrageenan helices are formed as in the absence of galactomannan. However, galactomannan evidently causes the helix to form a little earlier on the cooling curve (Fig. 8), and this is exactly as we might expect from our model (Fig. 7), since binding energy should lower the free energy of the critical nucleus. Similarly, binding energy stabilizes the helix as a whole to raise the temperature at which it 'melts' (Fig. 8).

The influence of galactomannan on the optical-rotation behaviour of agarose is more spectacular. Unfortunately, the conformation of agarose is not known for either the solid or gel state, although we strongly suspect that, like carrageenan, it is a multiple-stranded helix (Rees, 1969a,b; Rees *et al.*, 1969). For simplicity in this discussion I shall use a carrageenan model, but this is not essential to the general validity of the conclusions. Agarose alone shows a negative shift in optical rotation when it gels rather than a positive one as for carrageenans. There is an enormous hysteresis loop (Fig. 9; I. C. M. Dea, A. A. McKinnon & D. A. Rees, unpublished work) to match the well-known difference between liquefaction and setting temperatures. In the presence of galactomannan the shift becomes positive and the hysteresis assumes a butterfly-shaped form (Fig. 9; I. C. M. Dea, A. A. McKinnon & D. A. Rees, unpublished work). Odd though it is, this behaviour is explained very easily in terms of our model (Fig. 7). The properties of the mixture do not change with cooling until about the temperature at which transition to agarose helices usually occurs, as expected if the mechanism is similar to that for carrageenans (Fig. 7). The reversal in sign of the shift suggests an extra contribution that overwhelms the usual agarose shift. This could arise from a conformation change of the mannan backbone when it binds to agarose, and, in support of this possibility, we find that interpretation in terms of eqn. (1) leads to a mannan conformation that is sterically possible and has a translation period matching that derived from diffraction diagrams for agarose fibres (I. C. M. Dea, R. Moorhouse & D. A. Rees, unpublished work); this is consistent with a 'meshing' of the two polysaccharides, as in our hypothesis (Fig. 7).

On the reheating curve, the decrease in optical rotation that crosses the cooling curve (Fig. 9) is interpreted as the 'melting off' of galactomannan to leave agarose helices naked and free to behave as in the absence of galactomannan; this is confirmed by the way in which the tails of both heating curves (Fig. 9) coincide when allowance is made for the optical-rotation background in the mixture. This also confirms that the initial positive shift did indeed conceal the formation of agarose helices. Further support is given by the behaviour of short segments of agarose chains prepared by the 'kink-splitting' sequence devised for carrageenans. They do not gel alone in aqueous solution, but do so when galactomannan is added. In terms of our model, these gels contain agarose helices connected only by galactomannan chains, and their liquefaction temperature should be a guide to the temperature at which galactomannan dissociates from agarose; it does indeed occur at about 45°C, as required for consistency with our explanation of the form of the heating curve for the mixture in Fig. 9.

Fig. 9. Optical-rotation changes on heating and cooling a solution of agarose in the absence and in the presence of locust-bean galactomannan

The figure shows the optical-rotation curves observed on heating and cooling a solution of agarose (0.05%) in the absence (a) and in the presence (b) of locust-bean galactomannan (0.1%). The optical rotation-temperature profile is almost linear for the galactomannan alone.

Conclusions and implications. This all-polysaccharide system can be described in the language of protein biochemistry as showing secondary, tertiary and quaternary structure. The secondary structure is the shape of the single polysaccharide strand (compare the polypeptide α -helix); the tertiary structure is the way the two chains fit together in the double helix (compare collagen, DNA or the arrangement within one subunit of a globular protein); and the quaternary structure is the association of unlike polysaccharide chains in co-operative binding of galactomannan to the helix, which can be compared with the binding between different protein subunits as, for example, in the haemoglobin structure, and is the first example of this type of organization for polysaccharides.

It is an important property of these systems that the helix can be stable with bound galactomannan under conditions in which it would not be stable alone. Thus carrageenan is induced to change its shape by interaction with another molecule and therefore represents an example of ligand-induced change of polysaccharide conformation—almost of ‘polysaccharide allostereism’.

Finally, we know that polysaccharides generally have their biological importance when built up and organized in tissues by associations with each other and with other molecules. The galactomannan-helix systems show one way in which unlike polysaccharides could cohere to contribute to biological integrity. Indeed, there are stereochemical analogies between the galactomannan and important components of, for example, plant and bacterial cell walls (particularly with cellulose and peptidoglycan) to suggest that our systems may have a very direct relevance to the understanding of supermolecular structure in these walls.

Another kind of jelly

Structure, shape and properties of alginates. I shall now discuss an example of polysaccharide conformation and association that illustrates rather different principles from those involved in carrageenans and galactomannans. This is seen in the behaviour of alginates, a group of polysaccharides that also gel reversibly and that are therefore suspected of an ability to form an ordered conformation when stabilized by cohesion with other chains (Rees, 1969b). Unlike the gels already discussed, they are not ‘melted’ and re-formed by heat, but are formed by controlled mixing of the polysaccharide with the salt of a suitable cation, often Ca^{2+} , and liquefied by ion exchange with an alkali-metal cation such as Na^+ or by sequestering the gelling cation.

The covalent structure is again based on two sugar residues, which in this instance are β -D-mannopyranosyluronic acid (X) and α -L-gulopyranosyluronic acid (XI), each of which is linked through C-4 (Hirst & Rees, 1965; Rees & Samuel, 1967), and the ring conformations are known to be as shown for both solid state (Atkins *et al.*, 1970) and solution (A. Penman & G. R. Sanderson, unpublished work). These residues are arranged in blocks of three types (Haug *et al.*, 1966, 1967a, 1969; Larsen *et al.*, 1969), which we call poly-M (XII), poly-G (XIII) and poly-MG (XIV). The chain conformations of structures (XII) and (XIII) have been established for the acid forms by fibre diffraction, with supporting evidence from i.r. dichroism (Atkins *et al.*, 1970, 1971). The chains are ribbon-like and extended, and they pack like planks in a timber-yard rather than by twisting around each other in the manner of carrageenans. The chains have twofold screw symmetry. Of course, any ordered conformation in a solution or gel need

[symbols M and G represent the residues shown as (X) and (XI) respectively]

Fig. 10. *Proposed structure of an alginate gel*

Chain contours only are shown, and the different types of block present in each chain are not distinguished.

not necessarily correlate with that in a fibre or film, but model-building calculations show that the only ordered conformations that are stable enough to exist are of the extended type (Rees & Scott, 1971). From the properties of the gels it can be deduced that co-operative association of chain segments is involved in formation of the network (Rees, 1969*b*), and the mechanism can thus be shown schematically as in Fig. 10. Ca^{2+} ions are bound into the chain bundles, and their role is no doubt to contribute to the packing energy that holds the assembly together. It is likely, though not necessary, that each bundle is formed from

covalent blocks of a single type, and that each chain is caused to leave the association at the appropriate block terminus. These termini would then be equivalent in their role to the kinks in carrageenan. To understand the physical and biological properties of the gel, we must test this picture, and, if it is true, determine the conditions under which each type of block tends to associate, and whether the properties of the gel are affected by the block type that is predominant in forming bundles.

Structure and associations from circular-dichroism evidence. In the examination of carrageenan and

related gels we used optical rotation to follow the conformation changes of chains. Although it is generally agreed that monochromatic optical rotation

Fig. 11. Circular-dichroism spectra for (methyl β -D-mannopyranosid)uronate (—) and (methyl α -L-gulopyranosid)uronate (---), each as the sodium salt.

The figure shows the circular-dichroism spectra for (methyl β -D-mannopyranosid)uronate (—) and (methyl α -L-gulopyranosid)uronate (---), each as the sodium salt.

is the least powerful of chiroptical techniques in principle, the other methods (optical rotatory dispersion and circular dichroism) require at least one chromophore with an absorption band above 185 nm. No such chromophores are present in carrageenan, agarose or galactomannan, and for these polysaccharides we must be content for the moment with straightforward optical-rotation measurements. In alginate, however, the carboxylate group has an $n \rightarrow \pi^*$ band at about 215 nm and there is the opportunity to use more expensive machinery.

The methyl glycosides corresponding to the two sugar residues [structures (X) and (XI)] gave quite different circular-dichroism spectra (E. R. Morris, D. A. Rees & G. R. Sanderson, unpublished work), as shown in Fig. 11. By partial fragmentation of seaweed alginate (Haug *et al.*, 1966, 1967a; A. Penman, unpublished work) and other means (C. J. Lawson, unpublished work) it is possible to prepare blocks of residues that approximate in their arrangements to structures (XII), (XIII) and (XIV). The spectra (E. R. Morris, D. A. Rees & D. T. Thom, unpublished work) approximate only roughly to expectations based on linear combination of glycoside spectra (Fig. 12). The

Fig. 12. Circular-dichroism spectra for poly-M, poly-G, poly-MG and a mixture of poly-M and poly-G

The figure shows the circular-dichroism spectra (a) for poly-M blocks (—) and (methyl β -D-mannopyranosid)uronate (---), (b) for poly-G blocks (—) and (methyl α -L-gulopyranosid)uronate (---), and (c) for poly-MG blocks (—) and a mixture of equal proportions of poly-M and poly-G blocks (---). All substances were examined as sodium salts, and the spectra are normalized to the same peak height for each comparison.

Fig. 13. Circular-dichroism spectra for solutions of alginates before and after gelation

The figure shows circular-dichroism spectra for solutions of alginates (0.1%) before (—) and after (----) gelation by diffusion against a large volume of 6M-CaCl₂ solution for 10 days: (a) alginate having guluronate and mannuronate residues in the ratio 1:0.40; (b) another sample having these residues in the ratio 1:1.29.

spectrum for poly-MG also differs from that of a mixture of poly-M and poly-G (Fig. 12). In other words the spectrum is sensitive, not only to the particular uronic acids present, but also, to a smaller extent, to the way they are arranged. The 'block composition' of an alginate can therefore be characterized by measurement of the circular-dichroism spectrum and then use of the computer to find the combination of poly-M, poly-G and poly-MG spectra that gives the best fit.

When this technique is used to follow the gelation of alginate, exceedingly interesting results are obtained (E. R. Morris, D. A. Rees & D. T. Thom, unpublished work). The gels are made to form by slow diffusion of a low concentration of Ca²⁺ ions into the spectropolarimeter cell. An alginate that is rich in poly-G shows a marked change as it gels, the nature of which suggests that poly-G blocks are being 'removed' from the system (Fig. 13). An alginate that is rich in poly-M also shows the 'disappearance' of poly-G blocks, probably followed by progressive 'disappearance' of poly-M blocks (Fig. 13). Putting aside the spectroscopic mechanism for the moment, it seems that diffusion of Ca²⁺ affects poly-G blocks first, and only when these are exhausted can the concentration of Ca²⁺ rise to affect poly-M blocks. Poly-MG blocks evidently have the least tendency to be affected, if indeed they ever are. Since we know that

chain association is proceeding in parallel, it is likely that this association is the primary cause of the spectroscopic changes. This would agree with equilibrium-dialysis measurements, which show that alginates having high contents of guluronic acid have a correspondingly high affinity for the binding of Ca²⁺ ions in micro-gel particles (Smidsrød & Haug, 1968; cf. Haug *et al.*, 1967b).

We do not know yet why the association of alginate blocks should cause disappearance of their circular-dichroism spectrum in the $n \rightarrow \pi^*$ region, although several possibilities can be suggested. These include interactions in the associated form that influence the electronic transition (cf. McClure, 1959) and various types of 'concentration obscuring' effects that often happen with systems that contain particles larger than the usual molecular dimensions, such as ribosomes and membrane fragments.

Why do uronic acid residues differ? Finally, we may ask why poly-G blocks should associate more strongly with Ca²⁺ ions. Monosaccharide derivatives bind Ca²⁺ ions weakly, although there is some enhancement with increasing number of axial oxygen substituents (Gould & Rankin, 1970); on these grounds alone α -L-gulopyranosiduronate derivatives should bind more strongly than β -D-mannopyranosiduronate derivatives. However, much stronger binding is observed in oligosaccharide derivatives

Fig. 14. Stereochemical relationships in the crystal structure of calcium galacturonate

(Kohn *et al.*, 1968), especially for guluronic acid oligomers, showing that there is an important influence from the joining of residues in the chain. When binding occurs with chain association there is likely to be a third level of influence from the mode of chain packing.

Some clue to the nature of the second influence, that of chain structure, seems to have emerged from the crystal structure of the calcium salt of another uronic acid, galacturonic acid (R. O. Gould, S. E. B. Gould, D. A. Rees & W. E. Scott, unpublished work). It turns out that two-thirds of the Ca^{2+} ions are 9-coordinate, occupying the centre of a trigonal prism of oxygen atoms with three further oxygen atoms beyond the centre of each face. This geometry is also found in calcium chloride hexahydrate (Jensen, 1940) and in the bone mineral apatite (Beevers & McIntyre, 1946). In calcium galacturonate the bases of the prism are formed by three molecules of water of crystallization and the O-6 atoms of three galacturonate ions, while the facial positions are occupied by the O-5 atoms of the same three ions. The distances are such that an approximately equilateral triangle with an edge of about 0.3 nm (3 Å) is defined by O-5 and O-6 of one residue and one of the water molecules (Fig. 14). In a disaccharide or oligosaccharide the third corner could be formed by using a hydroxyl group of an adjacent residue. The extent of chelation and thus of association would then be enhanced. Molecular models suggest that this is possible in stable conformations of buckled axially-axially linked disaccharides of guluronic acid and galacturonic acid (Fig. 15), but not in the more extended mannuronic acid dimer. This conclusion corresponds exactly to the results of an experimental comparison of the three oligosaccharide series (Kohn *et al.*, 1968). This geometrical compatibility with Ca^{2+} could also help chain association by guiding the chains into relative positions that allow good overall packing.

A further cause of enhanced binding by oligosaccharides relative to monosaccharides is that the former would show polyelectrolyte properties, the more so for the guluronic acid and galacturonic acid series because charges are closer than in the mannuronic acid series.

Fig. 15. Conformation of *O*-α-L-gulopyranurosyl-(1→4)-L-gulopyranuronate in which O-6, O-5 and O-2' could form a triangle of the type that is suggested to facilitate binding of Ca^{2+}

The figure shows only one of the possible conformations that can be proposed to provide such geometry for binding.

Bacteria can do it too

A viscostatic polysaccharide. Having discussed conformation and interaction effects in some polysaccharide systems from seaweeds and higher plants, with brief reference to the animal kingdom, I conclude with an example from bacteria. This is yet another commercial system. The extracellular polysaccharide from *Xanthomonas campestris* was discovered to have unusual and potentially useful physical properties in a survey at the Northern Regional Laboratory of the U.S. Department of Agriculture (Jeanes *et al.*, 1961). Subsequently it was approved for use in foods in the U.S.A., where it is used increasingly widely. One strange property is that, compared with other polysaccharides, the thickening and suspending abilities are not lost rapidly with increased temperature. Statements are sometimes made in the trade that 'viscosity is constant over a wide temperature range', but this is not strictly true because careful measurements (Jeanes *et al.*, 1961) show that viscosity first diminishes with increased temperature, then rises sharply and finally falls again. This suggests a co-operative process, perhaps a conformation change. When we examined the process by optical rotation (E. R. Morris & D. A. Rees, unpublished work) there was a shift that coincided exactly with the viscosity change (Fig. 16), thus supporting our interpretation.

This polysaccharide contains several carboxylic acid derivatives (Sloneker & Jeanes, 1962; Sloneker & Orentas, 1962), which act as chromophores and

Fig. 16. Comparison of the changes in viscosity and optical rotation when a solution of *Xanthomonas* polysaccharide is heated and cooled

The figure shows the viscosity change (—) and optical-rotation change (----) when a solution of *Xanthomonas* polysaccharide (1.0%) is heated and cooled.

again make possible the use of circular dichroism; the results confirm the conformation transition (E. R. Morris & D. A. Rees, unpublished work). The transition is not very sensitive to polysaccharide concentration, which suggests that one chain only is involved. The 'melting' of this conformation presumably causes an increase in hydrodynamic volume that causes the viscosity change.

This system is still poorly characterized, both in terms of primary structure (cf. Sloneker *et al.*, 1964; Siddiqui, 1967) and the precise nature of the ordered conformation.

Shapes and interactions. The *Xanthomonas* polysaccharide does not form gels by itself, but, when mixed with locust-bean galactomannan, which also does not gel alone, a stiff rubbery gel can be formed. Optical-rotation changes accompany this gelation and are shown in Fig. 17. The cooling curve follows the curve that is observed when galactomannan is absent except that, when conformation conversion is almost complete, there is a positive shift that leads to gel formation. These observations, when taken with evidence of the type outlined earlier for the carra-

Fig. 17. Comparison of the temperature-induced changes in the optical rotation of *Xanthomonas* polysaccharide in the presence and in the absence of locust-bean galactomannan

The figure shows the temperature-induced optical-rotation changes observed for *Xanthomonas* polysaccharide solution (0.25%) in the presence (—) and in the absence (----) of locust-bean galactomannan (0.50%); the constant background rotation of galactomannan is added to the latter.

geenan and agarose systems, indicate that the 'smooth' sites of galactomannan can attach to form a stable association (compare Fig. 7) when the *Xanthomonas* polysaccharide is converted into an ordered conformation by cooling. The steeper slope during the first stage of cooling (Fig. 17) provides evidence for some ligand-induced increases in co-operativity.

Conclusion

This Lecture has been more of a sketch than a detailed argument because I wanted to try to put polysaccharide conformation in some sort of overall perspective rather than to establish the truth of any specific facts. I have contended that, as with other biopolymers, if polysaccharides exist in Nature for any purpose then this is based on an ability to interact co-operatively. Sometimes this means that conformations are switched rather sharply from one state to another. I have given examples from higher plants, algae and bacteria, and I am sure that similar developments with animal polysaccharides are just around the corner.

Polysaccharides are unlike many other biopolymers in that they have long pieces of repeating pattern in their covalent structure. These seem to represent the sites of co-operative interaction, both within and between molecules. They are 'marked off' by changes

in sequence such as the insertion of a kink in carrageenan or the change to a different type of block in alginate.

However, polysaccharides are like other biopolymers in that they show a hierarchy of organization that ranges from the interactions within a particular chain, through interactions between like chains, to interactions between unlike chains. Beyond this hierarchy lie 'mixed' interactions such as between carbohydrates and proteins, and these are great challenges for the future.

I have spoken a great deal about shapes in this Lecture and, of course, we value the shapes of things in human experience not only when they are functional but also when they please the senses. Whichever of these two ways you like to take it, my theme has been that polysaccharides are beginning to reveal themselves as very shapely molecules.

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