Solution Properties of Polygalacturonic Acid

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1. The specimen of polygalacturonic acid used in these studies was shown to contain very little neutral sugar, methyl ester groups or ash, and only residues of galacturonic acid. Its electrophoretic homogeneity was examined in pyridine-acetic acid buffer at pH 6.5 and in borate buffer at pH 9.2. The distribution of effective particle weights was shown to be fairly narrow. 2. The pH-titration curve of the polymer gave a pK value of 3.7. 3. The interaction of the polymer with Ruthenium Red was studied and titration curves were obtained for the spectral shifts associated with the formation of a complex. 4. Optical-rotatory-dispersion studies showed that the Drude constant, λ_c , was dependent on pH. 5. Polygalacturonic acid was shown to display non-Newtonian properties in solution and to have an anomalously high relative specific viscosity at low concentrations. 6. Studies were made of the pH-dependence of the sedimentation coefficient of the polymer. 7. These results are discussed in terms of the structure of the molecule and their relevance to the properties of pectic substances.

Pectinic acids were long considered to be polygalacturonic acids in which a proportion of the carboxyl groups was esterified, and theories of their role in cell development stressed the importance of their free-carboxyl-group content and its effect in binding Ca^{2+} . In particular, Ca^{2+} was thought to form bridges between adjacent chains of low ester content. Studies by Schweiger (1962, 1963, 1964, 1966) have shown that the binding of Ca^{2+} by polygalacturonic acids is intramolecular rather than intermolecular in solution (unlike binding by alginic acid) and involves the hydroxyl groups of the polymer in addition to its carboxyl groups. Moreover, since the demonstration by Aspinall & Fanshawe (1961) of the covalent linkage of neutral sugars to pectinic acids, numerous workers have found neutral sugars in pectinic acids, mostly in the form of neutral 'blocks' (Barrett & Northcote, 1965; Aspinall, Begbie, Hamilton & Whyte, 1967; Aspinall, Cottrell Egan, Morrison & Whyte, 1967; Aspinall, Hunt & Morrison, 1967). The studies by Stoddart, Barrett & Northcote (1967) and Stoddart & Northcote (1967) have further shown that pectinic acids can exist both with and without neutral blocks and that these blocks are metabolically related to the pectic arabinogalactans. Consequently the simple model of the function of pectins is no longer tenable and any new model that is developed must take into account more subtle and complex features of the

physical chemistry of the molecule. The present study of polygalacturonic acid is an approach to an investigation of the properties of these molecules, by way of a simpler molecule related to them.

MATERIALS AND METHODS

Polygalacturonic acid (derived from citrus pectin: mol. wt. range 2×10^4 – 6×10^4) was purchased from Koch-Light Laboratories Ltd., Colnbrook, Bucks. All other chemicals were obtained from British Drug Houses Ltd., Poole, Dorset, or Hopkin and Williams Ltd., Chadwell Heath, Essex, and were of the highest purity available. The molecular weight of Ruthenium Red was calculated from the formula as given in the Merck Index (Stecker, 1968). The techniques for a determination of moisture and ash, the preparation of pectin pectyl hydrolase (EC 3.1.1.11) and enzymic de-esterification have been described by Barrett & Northcote (1965). (Methyl α -D-galactopyranosid)uronic acid was prepared by the method of Morell & Link (1933).

Chromatographic separation of neutral sugars. This was performed on Whatman no. 1 paper with ethyl acetate-pyridine-water (8:2:1, by vol.) (Jermyn & Isherwood, 1949). The sugars were detected by the aniline hydrogen phthalate method (Wilson, 1959).

Zone electrophoresis of the polygalacturonic acid. Electrophoresis was carried out on Whatman GF 81 glass-fibre paper. Separations were performed either in pyridine-acetic acid buffer, pH 6.5 [10% (v/v) pyridine, 0.3% (v/v) acetic acid, + EDTA, final concn. 10 mM] or in sodium tetraborate buffer, pH 9.2 (0.05 M). For separations at pH 6.5 a potential gradient of 44 V/cm. was used for 25 min. at 20° in a tank similar to that described by Michl (1959), with toluene containing 4% (v/v) of pyridine as coolant. For separations at pH 9.2, a potential gradient of 40 V/cm.

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was applied for 30 min. at 20° in a tank of the type described by Durrum (1950), with white spirit as the coolant. Both types of tank were cooled by circulation of water through a cooling coil immersed in the coolant; both had platinum electrodes.

Electrophoretic zones were located by means of the sulphonated 1-naphthol reagent described by Barrett & Northcote (1965).

Detection of amino acids on electrophoretograms and in solution. The presence of amino acids on electrophoretograms was investigated by spraying with indanetrione hydrate and their presence in solutions of polygalacturonic acid was assayed by the method of Stein & Moore (1948).

Hydrolysis of polysaccharides. Hydrolyses were performed with sulphuric acid and the hydrolysates were neutralized with *N*-methyl-*NN*-di-*n*-octylamine (Stoddart *et al.* 1967). Neutralized hydrolysates were analysed by paper chromatography.

Esterification and reduction of polygalacturonic acid. Polygalacturonic acid was esterified with ethylene oxide as described by Barrett & Northcote (1965) and Stoddart & Northcote (1967). The partially esterified product was reduced with KBH_4 (Stoddart & Northcote, 1967).

pH measurements. These were made with a Radiometer type 22 pH-meter. Measurements were made at 18°. Titration curves were determined with a Radiometer TTT1c pH-stat at 18°. The concentration of polygalacturonic acid used was either 0.5% or 1.0% (w/v). NaOH (1.0M) was used as the titrant and was standardized immediately before use. Titration curves were also determined for 1.0% (w/v) polygalacturonic acid in the presence of 1mM-Ruthenium Red, 0.25M-KCl or 0.1M-sodium phosphate. Blank titrations were performed in all cases. For all experiments adjustment of the pH of the polygalacturonic acid solution was made with 0.5M-HCl or 0.5M-NaOH added very slowly in the form of drops to a vigorously stirred solution of the polysaccharide.

Viscosity measurements. These determinations were made at 18° in 0.1M-potassium phosphate buffer, pH 6.5, over the concentration range 0.025–2% (w/v). A horizontal capillary viscometer (Tsuda, 1928; Ostwald, 1933) was used with an adjustable pressure-head. The capillary diameter was 0.042 cm. and the length 24.795 cm. The viscometer was levelled before use and each measurement was made at a constant pressure.

Analytical ultracentrifugation. The sedimentation of a 1% (w/v) polygalacturonic acid solution in 0.2M-potassium phosphate buffer was observed in a Beckman-Spinco model E ultracentrifuge. Schlieren photographs were taken at 16 min. intervals after attainment of top speed (59780 rev./min.). The temperature was maintained at 20°. Runs were performed at various pH values and concentrations. The pH of the solution was adjusted to the required value immediately before the start of the run. Sedimentation coefficients were calculated by the graphical method of Markham (1960).

Partial specific volume. The partial specific volume of the sodium salt of the polygalacturonic acid was measured in a pycnometer of 0.74 ml. capacity at 20°.

Spectrophotometric analyses. Spectra of polygalacturonic acid and Ruthenium Red and the adduct of the two were determined in a Unicam SP.800 recording spectrophotometer at 25°. Comparison of the spectra of the polymer and dye before and after mixing was facilitated by the use of

Fig. 1. Design of the 'split' spectrophotometer cell. A section through the optical plane is shown. Each half of the cell has a 0.5 cm. light-path. The cell is 4.9 cm. high and the central division is 4.0 cm. high. All the silica windows are 1.0 mm. thick.

'split' cells, specially made for one of the authors by Unicam Instruments Ltd., Cambridge. The cell is shown in Fig. 1. After determination of the spectrum with the two components in the separate compartments, they were mixed by covering and inverting the cell and the spectrum of the mixture was determined.

The polygalacturonic acid was made up in 0.1M-sodium phosphate buffer, pH 6.5, to give a final concentration of 1.0% (w/v) and the Ruthenium Red was made up to a concentration of 0.2 mM in the same buffer. The pH values of both components were adjusted to the required value with 0.5M-HCl or 0.5M-NaOH immediately before determinations. Controls in which an equivalent amount of NaCl replaced the acid or alkali were also performed.

*Studies of O.R.D.** Measurements of the O.R.D. of solutions containing 2m-moles of galacturonyl residues/l. in 0.2M-sodium phosphate buffer, pH 6.5, were determined by the use of a Bellingham and Stanley Polarmatic 52 spectropolarimeter. The apparatus was purged with N_2 and measurements were made in a 1.0 cm. sample cell. For studies of the effect of pH on the O.R.D. of polygalacturonic acid, the pH of the samples was adjusted with HCl immediately before the determinations and was checked immediately after. Controls in which HCl was replaced by NaCl were also run.

The O.R.D. results in the wavelength range 500–250 nm. were interpreted in terms of the Drude equation (see Urnes & Doty, 1961):

$$\alpha_\lambda = A/(\lambda^2 - \lambda_0^2)$$

* Abbreviation: O.R.D., optical rotatory dispersion.

where α_λ is the specific rotation at a wavelength λ and A and λ_c are constants. The constants A and λ_c were determined from the slopes of plots of $\alpha_\lambda \lambda^2$ against α_λ .

RESULTS

Characterization of the polygalacturonic acid. The specimen of polygalacturonic acid used was sparingly soluble in water and readily soluble in dilute alkali and phosphate buffer. It showed a specific optical rotation $[\alpha]_D^{20} + 172.5^\circ$. Hydrolysis of the material (12 mg.) with sulphuric acid, followed by neutralization and chromatography of the hydrolysate, failed to show the presence of any neutral sugars and yielded only weakly staining spots, that corresponded in position and colour reaction to galacturonic acid and its oligomers, near the origin.

Zone electrophoresis of the polysaccharide in pyridine-acetic acid buffer, pH 6.5, yielded a single zone of high mobility towards the anode. The zone was stained violet by the sulphonated 1-naphthol reagent. Treatment of a sample of the polygalacturonic acid (4 mg.) with pectin pectyl hydrolase gave a product of the same electrophoretic mobility as the starting material at pH 6.5. A sample of the polysaccharide (10 mg.) was treated with an excess of ethylene oxide and gave a product of greatly decreased electrophoretic mobility.

Zone electrophoresis of the polygalacturonic acid in tetraborate buffer, pH 9.2, yielded a single zone, which was stained violet by sulphonated 1-naphthol.

A portion of the polysaccharide that had been treated with ethylene oxide (as described above) was further treated with an excess of potassium borohydride, and the product was recovered and hydrolysed with sulphuric acid. The hydrolysate was neutralized and analysed by chromatography. In addition to spots corresponding to oligosaccharides, a spot was seen that corresponded to galactose in position and colour reaction with aniline phthalate.

The value obtained for the partial specific volume was 0.65 ml./g. The total ash and moisture content was below 0.2% by weight.

The titration of polygalacturonic acid gave a simple titration curve with a pK value of 3.7 (Fig. 2). Difference titration curves calculated for mixtures of polygalacturonic acid with Ruthenium Red at a final concentration of 1 mM and with phosphate buffer, final concentration 0.2 M, at an initial pH of 6.5 gave similar results.

Studies of the binding of Ruthenium Red. When solutions of polygalacturonic acid and Ruthenium Red at pH 6.5 were mixed, the extinction of the dye increased and the extinction maximum shifted to a higher wavelength. A similar effect was produced when Ruthenium Red was mixed with 3 M-lithium chloride (Fig. 3). Glycerol in aqueous

Fig. 2. Titration curve of a 0.5% (w/v) solution of polygalacturonic acid determined at 18°. A 2.5 ml. sample of the polygalacturonic acid was titrated in a water-jacketed vessel with 1.0 M-NaOH.

Fig. 3. Spectral shifts accompanying the mixing of Ruthenium Red with polygalacturonic acid and with LiCl. The spectra before and after mixing were recorded in a Unicam SP.800 recording spectrophotometer at 25° and pH 6.5 by using 'split' cells (as illustrated in Fig. 1). The spectrum of 0.2 mM-Ruthenium Red is shown before mixing (—), after mixing with 1.0% (w/v) polygalacturonic acid (----) and after mixing with 6 M-LiCl (.....).

solutions at concentrations of up to 25% (by vol.) had no effect on the absorption spectrum of Ruthenium Red. This metachromic effect that occurred on mixture of the dye with polygalacturonic acid was markedly dependent on pH. The shift steadily decreased towards zero with decreasing pH. The hyperchromic effect also decreased with decreasing wavelength and eventually became a hypochromic effect with respect to the free dye. The influences of pH on these shifts are shown in Fig. 4.

Studies of O.R.D. The O.R.D. curves obtained from polygalacturonic acid between pH 2.8 and

Fig. 4. Effect of pH on the spectral shifts that occur when Ruthenium Red is mixed with polygalacturonic acid. The methods used are given in the legend to Fig. 3. The pH of each component was adjusted to the required value with 0.5M-HCl immediately before the spectra were determined. ○, Change in position of absorption maximum after mixing; ●, change in value of extinction after mixing.

Fig. 6. O.R.D. of polygalacturonic acid. Details of the procedures used are given in the legend to Fig. 5. —, Dispersion at pH 3.0; ---, dispersion at pH 6.5.

Fig. 5. Variation of the O.R.D. Drude constant, λ_c , of polygalacturonic acid with pH. The pH of a polygalacturonic acid solution containing 2 m-moles of galacturonyl residue/l. was adjusted to the required value immediately before the determination of the O.R.D. curve between the wavelengths 500 and 250 nm.

Fig. 7. Effect of pH on the sedimentation coefficient of polygalacturonic acid. A solution of 1.0% (w/v) polygalacturonic acid was made up in 0.2M-potassium phosphate buffer, pH 6.5, and the pH was adjusted to the required value immediately before centrifugation. Centrifugation was performed at 20°.

6.5 were plane-positive between 500 and 250nm. and yielded linear Drude plots. The variation of the Drude constant λ_c with pH is shown in Fig. 5.

Studies of the optical rotation in the far u.v. showed a peak at about 211nm. at pH 6.5, which may correspond to the peak of a positive Cotton effect. At lower pH values the maximum of this peak shifted to 217nm. (Fig. 6). (Methyl α -D-galactopyranosid)uronic acid showed a similar peak near 215nm.; the position of this was not found to vary with pH in the range pH 3.3–7.0.

Effects of pH and salt concentration on the sedimentation of polygalacturonic acid. The sedimentation coefficient of polygalacturonic acid decreased with increasing pH between pH 3.0 and pH 6.5 (Fig. 7).

There was no evidence of formation of multiple peaks at low pH values as might have been expected if polymerization had occurred. Plots of sedimentation coefficient versus concentration determined at pH 6.5 and pH 3.2 were linear between 1.0% and 0.2% (w/v) polygalacturonic acid (Fig. 8). The lower concentration represents the limit of detection of the schlieren optical system used. The sedimentation coefficient in 0.25M-potassium chloride for a 1.0% (w/v) solution of polygalacturonic acid was 1.8S, close to the value determined at pH 3.8 in the absence of potassium chloride.

Viscosity of solutions of polygalacturonic acid. At low applied pressures the flow of the solutions of polygalacturonic acid was markedly non-Newtonian, but at higher pressures (above 15mm.

Fig. 8. Effect of concentration on the sedimentation of polygalacturonic acid. The conditions used are given in the legend to Fig. 7. The effects were studied at pH 6.5 (○) and at pH 3.2 (●).

Fig. 9. Dependence of relative specific viscosity ($\eta_{sp.}/c$) in polygalacturonic acid on concentration (c) at 18° of 0.1 M-potassium phosphate buffer, pH 6.5.

Hg) the shear-dependence of the apparent viscosity disappeared. Values of the viscosity under conditions of Newtonian flow were determined for various concentrations of polygalacturonic acid from 0.025

to 2.0% (w/v) in 0.1 M-potassium phosphate buffer, pH 6.5, and from these a plot of $\eta_{sp.}/c$ against c was derived (Fig. 9), where $\eta_{sp.}$ is the specific viscosity and c the concentration of polygalacturonic acid (Huggins, 1942). The relative specific viscosity rose rapidly at concentrations below 0.5% (w/v) polygalacturonic acid.

Since the capillary was long and of fine bore and the volume rate of flow was low, the kinetic corrections were far below the limits of experimental error and were not applied. Likewise the correction for end effects was negligible (Yang, 1961), as were surface-tension and drainage errors.

Molecular weight of the polygalacturonic acid. The apparent molecular weight of the polygalacturonic acid was calculated, from the sedimentation and viscosity data by using the equation of Scheraga & Mandelkern (1953) and assuming a value of 2.5×10^6 for the constant β , to be 31 600.

DISCUSSION

The sample of polygalacturonic acid used in these studies was essentially ash- and moisture-free and also free of detectable neutral sugars, although very small traces of rhamnose could have escaped detection because of the resistance of polyuronide chains to acid hydrolysis (Smidsrød, Haug & Larsen, 1966). It was concluded that the polysaccharide was free from neutral branches, unlike some native pectinic acids (Barrett & Northcote, 1965; Stoddart *et al.* 1967), and was present as the free acid and not as a salt. The high electrophoretic mobility at pH 6.5 and the failure of pectin pectyl hydrolase to increase the mobility further supported the manufacturers' claim that the polysaccharide had a very low ester content. It also indicated again that polysaccharides containing neutral 'blocks' were absent. The decrease in electrophoretic mobility of the polysaccharide after treatment with ethylene oxide indicated the presence of free carboxyl groups. Reduction of the partially esterified acid followed by hydrolysis gave only galactose, again showing the lack of neutral sugars in the material and the probable absence of uronic acids other than galacturonic acid. Electrophoresis of the polygalacturonic acid in borate buffer gave only a single narrow zone indicative of a high degree of configurational homogeneity of the borate complex of the polymer (Northcote, 1954). On analytical ultracentrifugation the polysaccharide showed a single peak, which sedimented slowly. Though the polymer was of heterogeneous molecular weight, these results show that the distribution of effective particle weights about the mean was fairly narrow.

The titration curve of polygalacturonic acid showed a pK value of 3.7, which is close to the value reported by Chowdhury & Neale (1963) for the pK

of galacturonic acid. A similar value was obtained from difference titrations in the presence of phosphate buffer and Ruthenium Red.

Shifts in the absorption spectra of dyes have frequently been observed when the dyes are bound to polysaccharides. Pal & Basu (1958) and Pal (1958) have interpreted the metachromic shifts that occur when Methylene Blue or Toluidine Blue is mixed with acidic polysaccharides as arising from a superposition of neighbouring dye molecules caused by the coiling of the polymer chain. Stone (1967) has also concluded that metachromacy results, in substantial part, from dye-dye interactions. Consideration of the splitting of energy levels of electrons suggests that the enhanced absorption and red shift observed in this system represent a *J*-aggregate and one possible structure of such an aggregate is a helix (Mason, 1964), but Scheibe, Wortz, Haimerl, Seiffert & Winkler (1968) have shown that such spectral shifts can also arise from the adsorption of dyes on surfaces on which a helical structure is impossible. With the Ruthenium Red-polygalacturonic acid system there appears to be no need to postulate an ordered binding on the dye, since the observed shifts can be simulated by high concentrations of salt. The concentration of lithium chloride required to produce effects of similar size to those observed with Ruthenium Red was 3M, which is considerably greater than the concentration of free carboxyl groups in the solution (about 60mM). This discrepancy probably originates from a binding of the Ruthenium Red to the polygalacturonic acid in a highly polar environment.

Both the spectral shift and the hyperchromic effect, caused by the interaction of Ruthenium Red with polygalacturonic acid, were dependent on pH and yielded titration curves (Fig. 4) that resembled the acid-base titration curve of the polysaccharide. Although the spectral shift decreased towards zero as the pH was lowered, the hyperchromic effect gave way to hypochromicity at low pH values. The origin of this hypochromic effect is uncertain, but it suggests that the Ruthenium Red can still interact with the polygalacturonic acid after the carboxyl groups of the latter have been protonated. The failure of glycerol to cause hypochromism in the spectrum of free Ruthenium Red, at concentrations of glycerol up to 25% by volume, makes it unlikely that the observed hypochromicity with polygalacturonic acid arises from a trapping of the molecules of dye in a non-polar environment consequent on a collapse of the polymer molecule caused by neutralization of its carboxyl groups.

The increase in the Drude constant, λ_c , as the pH is lowered gives a titration curve similar to that of the carboxyl groups in polygalacturonic acid. Rao & Foster (1963) observed a sharp fall in the specific rotation of amylose as the pH was increased from

11 to 12. This they interpreted as a transformation of the molecule from an imperfect helical form to a random coil as the pH was raised. In further studies on the effect of pH on amylose, however, Rao & Foster (1965a) interpreted changes in the proton-magnetic-resonance spectra as indicating that the changes in optical rotation at high pH values may be due to an increase in the rotational freedom about the glycosidic bond. With carboxymethyl-amylose, Rao & Foster (1965b) found that both the Drude constant and the specific rotation decreased with decreasing pH in the region of the ionization of the carboxyl groups and also decreased as the pH was raised from 11 to 12. If it is assumed that the ionized carboxyl groups prevent the formation of any ordered structures, these results are difficult to reconcile with the helix-coil transition inferred from the results with amylose. With polygalacturonic acid the pH-dependent changes in the Drude constant can probably be attributed to the change in the position of the peak of optical rotation in the u.v. Stone (1965) observed a peak of optical rotation at 198nm. with heparin and a trough at 188nm. with chondroitin sulphate. Pace, Tanford & Davidson (1964) and Listowsky, Avigad & England (1965) have observed a peak in the O.R.D. of galactose and its derivatives in the region of 210nm. This peak of optical rotation has been interpreted (Listowsky *et al.* 1965) as representing a change of direction of rotation caused by interaction between the substituent on C-5 of the sugar and the ring oxygen atom, giving rise to a negative Cotton effect near 190nm. in the C-1 conformation of the galactose monomers. If this explanation is correct the shift in position of the point of change in direction of the optical rotation for polygalacturonic acid could be caused by either an increase in the proportion of the residues in the C-1 conformation as the pH was lowered, or some further, unidentified, interactions. Hirano, Manabe, Miyazaki & Onodera (1968) have shown that the sugar residues in acetylated polygalacturonic acid are mainly in the C-1 conformation. An increase in the proportion of sugar residues in the C-1 conformation in polygalacturonic acid as the pH is lowered may reflect the formation of some ordered structure in which interaction of sugar residues causes a shift in their conformational equilibria.

The value of 1.83 calculated (at pH values above 5.0) for the sedimentation coefficient, S_{20}^0 , for polygalacturonic acid of this range of molecular weights indicates that the molecules have a high degree of asymmetry.

The sedimentation of polygalacturonic acid was found to be strongly dependent on the pH (Fig. 7). The increases in the sedimentation coefficient as the pH was lowered resembled a titration curve with a point of inflexion near to that of the acid-base

titration curve of polygalacturonic acid. This increase of sedimentation coefficient with lowering of the pH could arise from the molecule's becoming more compact as the carboxyl groups became protonated, or from aggregation. Plots of sedimentation coefficient against concentration were linear both at pH 3.1 and at pH 6.5 (Fig. 8), and gave no sign of the curve that would be expected from an aggregation dependent on concentration. The sedimentation coefficient of the polygalacturonic acid at pH 6.5 was increased by the presence of 0.25 M-potassium chloride, which is consistent with the suggestion that the neutralization of the charge of the carboxyl groups leads to the dependence of the sedimentation coefficient on pH.

Studies of the viscosity of solutions of polygalacturonic acid showed anomalies both in the dependence of the viscosity on the rate of shear and in the dependence of the relative specific viscosity on concentration. The former, non-Newtonian, behaviour could be explained as a charge-repulsion effect between the macroions of the polymer, as an orientation effect of asymmetric molecules or as an effect of the interaction of hydration shells of the polymer. Although all three effects are likely to be involved to some extent the first of them (the 'second electroviscous effect'; Yang, 1961) should be much decreased at high dilution and in the presence of salt; under these conditions the non-Newtonian behaviour was still observed. Possible interactions of hydration shells would also disappear at sufficiently high dilution, and an orientation effect is the most probable explanation.

The value of the partial specific volume was closely similar to those previously reported for neutral polysaccharides (Bell, Gutfreund, Cecil & Ogston, 1948; Olaitan & Northcote, 1962) and the glycopeptide of yeast cell walls (Korn & Northcote, 1960; Sentandreu & Northcote, 1968). It therefore seems unlikely that the hydration of polygalacturonic acid in solution is substantially different from that of neutral polysaccharides.

The first part of the relative specific viscosity-concentration curve shows a linear relationship, which yields a value of the intrinsic viscosity $[\eta]$ of 0.48 ml./g. on extrapolation and a value of 5.4 for the Huggins constant (Huggins, 1942). This intrinsic viscosity corresponded to that given by a prolate ellipsoid of rotation of axial ratio 26, as calculated from the equation of Simha (1940) and Mehl, Oncley & Simha (1940) assuming no hydration. If a 30% (w/v) hydration is assumed the axial ratio is decreased to 21, as calculated from the equations of Oncley (1941). Both values of the axial ratio are very small for a linear polymer of as high a molecular weight as that of polygalacturonic acid. This is explicable if the molecule is flexible, is folded back on itself or is associated to give a rounded or branched

aggregate. The very high value of the Huggins constant is probably caused by the polyionic nature of the molecule and is about ten times larger than a typical value for a neutral flexible polymer. In view of the non-Newtonian behaviour of the solutions the validity of applying the Huggins equation is uncertain and the significance of the value of the Huggins constant is rather doubtful. It is also questionable whether the extrapolation to determine the intrinsic viscosity is valid for the anomalous plots of Fig. 9.

The increase of the relative specific viscosity at low concentrations may be considered to represent an increase in the radius of gyration of the effective hydrodynamic particle equivalent to its becoming an ellipsoid of rotation of axial ratio greater than 50. Such an increase could result from increased rigidity of a flexible molecule, an expansion of a molecule coiled on itself, a large change in hydration, a dissociation of a rounded or branched aggregate into asymmetric subunits or the extension of an asymmetric molecule. It is not possible to distinguish between these, although a sufficiently large change in hydration is unlikely to occur.

The model of polygalacturonic acid in solution that emerges from these studies is that of an extended molecule with some flexibility, which is partially maintained in its conformation by the repulsion of charges on the carboxyl groups of adjacent residues. Consequently effective neutralization of these charges at low pH values or high ionic strength leads to a collapse of the molecule, with possible attendant changes in the conformation of the residues. It seems likely that the hydroxyl groups of polygalacturonic acid play an important part in determining the structure of the molecule, especially under conditions in which charge-repulsion effects are minimal. Hence any future considerations of the role of galacturonans in the plant cell wall must take account, not only of the degree of esterification of the carboxyl groups, but also of the distribution of ester bonds, the degree of substitution of hydroxyl groups and the ways in which ions such as Ca^{2+} may interact with hydroxyl as well as carboxyl groups. The situation *in vivo* will be further complicated by the presence of neutral sugars and branches in the galacturonans, but their general behaviour is likely to approximate to that described here.

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REFERENCES

- Aspinall, G. O., Begbie, R., Hamilton, A. & Whyte, J. N. C. (1967). *J. chem. Soc. C*, p. 1065.
Aspinall, G. O., Cottrell, I. W., Egan, S. V., Morrison, I. M. & Whyte, J. N. C. (1967). *J. chem. Soc. C*, p. 1071.

- Aspinall, G. O. & Fanshawe, R. S. (1961). *J. chem. Soc.* p. 4215.
- Aspinall, G. O., Hunt, K. & Morrison, I. M. (1967). *J. chem. Soc. C*, p. 1080.
- Barrett, A. J. & Northcote, D. H. (1965). *Biochem. J.* **94**, 617.
- Bell, D. J., Gutfreund, H., Cecil, R. & Ogston, A. G. (1948). *Biochem. J.* **42**, 405.
- Chowdhury, F. H. & Neale, S. M. (1963). *J. Polym. Sci. A*, **1**, 2881.
- Durrum, E. L. (1950). *J. Amer. chem. Soc.* **72**, 2943.
- Hirano, S., Manabe, M., Miyazaki, N. & Onodera, K. (1968). *Biochem. biophys. Acta*, **156**, 215.
- Huggins, M. L. (1942). *J. Amer. chem. Soc.* **64**, 2716.
- Jermyn, M. A. & Isherwood, F. A. (1949). *Biochem. J.* **44**, 402.
- Korn, E. D. & Northcote, D. H. (1960). *Biochem. J.* **75**, 12.
- Listowsky, I., Avigad, G. & England, S. (1965). *J. Amer. chem. Soc.* **87**, 1765.
- Markham, R. (1960). *Biochem. J.* **77**, 516.
- Mason, S. F. (1964). *Proc. chem. Soc.* 119.
- Mehl, J. W., Oncley, J. L. & Simha, R. (1940). *Science*, **92**, 132.
- Michl, H. (1959). *Chromat. Rev.* **1**, 11.
- Morell, S. & Link, K. P. (1933). *J. biol. Chem.* **100**, 385.
- Northcote, D. H. (1954). *Biochem. J.* **58**, 353.
- Olaitan, S. A. & Northcote, D. H. (1962). *Biochem. J.* **82**, 509.
- Oncley, H. L. (1941). *Ann. N.Y. Acad. Sci.* **41**, 121.
- Ostwald, W. (1933). *KolloidZ.* **63**, 61.
- Pace, N., Tanford, C. & Davidson, E. A. (1964). *J. Amer. chem. Soc.* **86**, 3160.
- Pal, M. K. (1958). *Makromol. Chem.* **28**, 91.
- Pal, M. K. & Basu, S. (1958). *Makromol. Chem.* **27**, 69.
- Rao, V. S. R. & Foster, J. F. (1963). *Biopolymers*, **1**, 527.
- Rao, V. S. R. & Foster, J. F. (1965a). *J. phys. Chem.* **69**, 636.
- Rao, V. S. R. & Foster, J. F. (1965b). *Biopolymers*, **3**, 185.
- Scheibe, G., Wortz, O., Haimerl, I. F., Seiffert, W. & Winkler, J. (1968). *J. Chim. phys.* **65**, 146.
- Scheraga, H. A. & Mandelkern, L. (1953). *J. Amer. chem. Soc.* **75**, 179.
- Schweiger, R. G. (1962). *J. org. Chem.* **27**, 1789.
- Schweiger, R. G. (1963). *KolloidZ.* **196**, 47.
- Schweiger, R. G. (1964). *J. org. Chem.* **29**, 2973.
- Schweiger, R. G. (1966). *KolloidZ.* **203**, 28.
- Sentandreu, R. & Northcote, D. H. (1968). *Biochem. J.* **109**, 419.
- Simha, R. (1940). *J. phys. Chem.* **44**, 25.
- Smidsrød, O., Haug, A. & Larsen, B. (1966). *Acta chem. scand.* **20**, 1026.
- Stecher, P. G. (1968). In *The Merck Index*, 8th ed., p. 926. Ed. by Stecher, P. G., Windholz, M. & Leahy, D. S. Rahway, N. J.: Merck and Co. Inc.
- Stein, W. H. & Moore, S. (1948). *J. biol. Chem.* **179**, 367.
- Stoddart, R. W., Barrett, A. J. & Northcote, D. H. (1967). *Biochem. J.* **102**, 194.
- Stoddart, R. W. & Northcote, D. H. (1967). *Biochem. J.* **105**, 45.
- Stone, A. L. (1965). *Biopolymers*, **3**, 617.
- Stone, A. L. (1967). *Biochim. biophys. Acta*, **148**, 193.
- Tsuda, S. (1928). *KolloidZ.* **45**, 325.
- Urnes, P. & Doty, P. (1961). *Advanc. Protein Chem.* **16**, 401.
- Wilson, C. M. (1959). *Analyt. Chem.* **31**, 1199.
- Yang, J. T. (1961). *Advanc. Protein Chem.* **16**, 323.