The Degree of Polymerization and Polydispersity of Mannan from the Cell Wall of the Green Seaweed Codium fragile

W. MACKIE and D. B. SELLEN

Mannan was extracted from the cell wall of the green seaweed Codium fragile *using a very mild procedure. Three mannan trinitrate samples were prepared using different times of nitration. These samples together represented 95 per cent of the cell wall mannan. Degree of polymerization determinations were made using light scattering, osmometry, gel permeation chromatography and viscosity techniques, and frequency distribution curves obtained. Comparison of these curves showed that there was little degradation during the nitration procedure. The results taken together show that the mannan in the native cell wall has degrees of polymerization ranging from below 20 to above 10 000. 90 per cent of the mannan has degrees of polymerization between 100 and 2 500 and the peak in the logarithmic distribution curves occurs at about 600. Thus most of the mannan chains have degrees of polymerization much higher than those found for other similar mannans. From the light scattering results the chain statistics of mannan trinitrate in solution appear to be identical with those of cellulose trinitrate.*

POLYMERS consisting largely of β-1,4 linked D-mannose residues are known to form a large proportion of the endosperms of date[1], palm[2] and coffee beans[3]. Similar polysaccharides also occur as the major constituents of the cell walls of certain green algae[5–8] and in the surface layers of some red algae[4,5]. Only the endosperm ('ivory nut') mannans have been the subject of extensive degree of polymerization (DP) investigations, although one estimate of the DP of algal mannan has been made. Some of the mannans have been fractionated into two main components, designated mannan A and mannan B, which differ significantly in chain length. Some published estimates of average DP of mannans A and B together with the method of determination are given in *Table 1.*

Table 1. Degrees of polymerization of mannans from various sources

Source	*Degree of polymerization*		*Method of determination*
	Mannan A	*Mannan B*	
Palm seeds	10–13	40	end-group analysis[2]
(*Phytelephas*	16–21	80	osmometry[1]
macrocarpa)	5–7	300–1200	viscometry[9]
Coffee bean (*Coffea arabica*)	45 (not fractionated)		ultracentrifugation[3]
Greeen seaweed (*Codium fragile*)	16 (not fractionated)		end-group analysis[7]

It is not easy to compare these values directly with each other or even to assess their individual significance, since samples were variously treated

with sodium chlorite and/or alkali, which may have degraded the mannan chains, or else were contaminated with considerable proportions of high molecular weight cellulose.

It was decided to re-investigate the DP of mannan from the cell wall of the green seaweed, *Codium fragile*. This species was the most suitable for investigation, because of its ready availability, because cellulose is absent and because it already had been the subject of a thorough chemical study which indicated that the mannan possessed an esssentially linear structure of β-1,4 linked D-mannose units[6].

It has been shown by physical methods that mannan occurs in green algae as partly crystalline associations of chains in well defined orientations within the cell walls[8]. It has also been observed by electron microscopy that the cell walls of *C. fragile* contain a microfibrillar mannan component[10], in addition to a more abundant granular mannan in which the microfibrils appear to be embedded. This provided an additional point of interest since it was felt that the granular mannan might well differ from the fibrillar mannan in degree of polymerization.

In order to determine degrees of polymerization of insoluble polysaccharides such as mannan it is necessary to prepare a soluble derivative without at the same time degrading the mannan chains. It was decided to prepare the trinitrate because many workers have investigated cellulose in this way[9, 11–13] and the nitration conditions are thought not to cause severe degradation. This derivative has also been used in an earlier investigation of mannan[1, 9]. The properties of cellulose trinitrate in solution have been studied extensively[14–18] and it was thought that a comparison with mannan nitrate would be interesting.

PREPARATION OF MANNAN NITRATE FROM THE CELL WALL

Fresh fronds of *C. fragile* were successively treated in a blender with aqueous solutions of *n*-butanol (10 per cent v/v), sodium lauryl sulphate (0·3 per cent w/v), urea (50 per cent w/v) and sodium chloride (5 per cent w/v). This procedure provided a fibrous white cell wall powder (25 per cent of the dry weed) which contained carbohydrate (*ca.* 75 per cent) and protein (about five per cent). Acid hydrolysis produced three monosaccharides; galactose (two per cent), mannose (97 per cent) and a trace of glucose ($< 0{\cdot}2$ per cent). For molecular weight studies the mannan trinitrate was prepared by treating the cell wall powder (2 g) with a solution (200 ml) containing 40·4 g of phosphorus pentoxide per 100 g of 90 per cent fuming nitric acid[11] (sp. gr. 1·483 at 20°C) at +18°C. The product was washed free from acid with ice-water, stabilized in hot water and then rinsed with methanol, ether and dried (3·1 g). It was dispersed (30 minutes) in acetone and the suspension centrifuged (2 min/2 000 *g*). The acetone-insoluble residue was washed with methanol and ether and dried. It was subsequently re-nitrated in the same way for a longer period of time to yield an additional quantity of acetone-soluble mannan nitrate. The nitrated mannan was recovered from the supernatant by adding the acetone solution (1 per cent w/v) to cold water (10 volumes) to precipitate

(85 to 95 per cent recovery) the mannan nitrate as a fibrous white solid.

The nitration (i.e. the proportion of product soluble in acetone) was found from small scale experiments to follow the course shown in *Figure 1*. It is evident that the nitration proceeds rapidly for the first hour during which 40 to 50 per cent of the crude product becomes soluble in acetone.

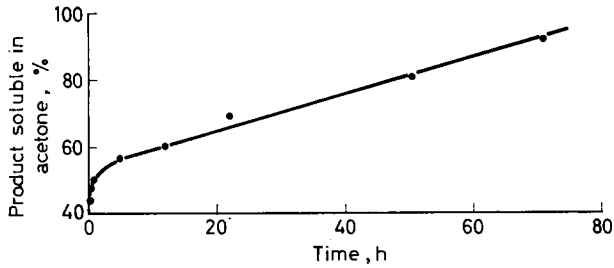

Figure 1—Nitration of *Codium fragile* cell wall

Thereafter the nitration continues at a diminished rate until after 70 hours about 90 per cent of the product is soluble in acetone. The samples used for degree of polymerization experiments were obtained in larger scale experiments, details of which are given in *Table 2*. It is estimated that these samples taken together represent some 95 per cent of the cell wall mannan.

Table 2. Mannan trinitrate samples derived from the cell wall of *Codium fragile* used for degree of polymerization determinations

Sample	*Description*	*Estimation of percentage of total mannan in the cell wall*
I	nitrated for one hour, dissolved in acetone and precipitated with water	50%
II	acetone-insoluble residue from I nitrated for a further 20 hours, dissolved in acetone and precipitated with water	24%
III	acetone-insoluble residue from II nitrated for a further 24 hours, dissolved in acetone and precipitated with water	21%
		—
		95%

The nitrogen content of the samples used was determined using material recovered from solution after the DP determinations and found to be 13·6 per cent in all cases (Kjeldahl determination). (Theoretical is 14·1 per cent for a trinitrated anhydrohexosan.) Portions of the recovered materials were also denitrated with ammonium hydrosulphide and after acid hydrolysis found to consist of 96 or 97 per cent carbohydrate in the form of mannose with only traces ($< 0{\cdot}2$ per cent) of glucose.

Table 3. Degrees of polymerization of mannan trinitrate samples derived from the cell wall of *Codium fragile*

Sample	*Solvent*	*Light scattering*		*Osmotic pressure*		*Gel permeation chromatography*			*Viscosity*
		$(DP)_W$	$B(10^{-4}$ cm^3 g$^{-2})$	$(DP)_N$	$B(10^{-4}$ cm^3 g$^{-2})$	$(DP)_N$	$(DP)_W$	$(DP)_V$†	$(DP)_V$†
I	acetone	1 400 + 100	6·5 ± 1·0						650 ± 50
	ethyl acetate	1 400 ± 100	6·0 ± 0·5	210 ± 15	14 ± 2				
	n-butanone			195 ± 15	14 ± 2				
	tetrahydrofuran					270 ± 20	1 300 ± 100	1 200 ± 100	
II	acetone								1 200 ± 100
	ethyl acetate	4 000 ± 1 000	6·5 ± 0·5	460 ± 40	12 ± 1				
	tetrahydrofuran					670 ± 40	3 000 ± 500	2 500 ± 500	
III	acetone								600 ± 50 } *
	ethyl acetate	4 500 ± 1 000	3·6 ± 0·5	360 ± 30	12 ± 1				1 100 ± 100 }
	tetrahydrofuran					510 ± 30	1 400 ± 300	1 300 ± 300	

*For explanation see text.

†Calculated using the expression of Huque *et al.*[11].

DEGREE OF POLYMERIZATION DETERMINATIONS

Degrees of polymerization (DP) were obtained as described below. The results are summarized in *Table 3*.

(a) *Light scattering*

All the samples were measured in ethyl acetate and sample I was also measured in acetone. Stock solutions were made up at concentrations of

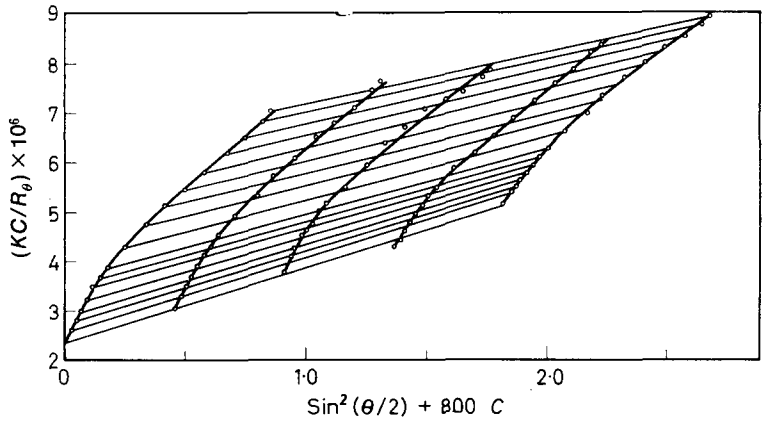

Figure 2—Zimm plot for mannan nitrate derived from the cell wall of *Codium fragile*. Sample I (see *Table 2*) in ethyl acetate, 4 358Å

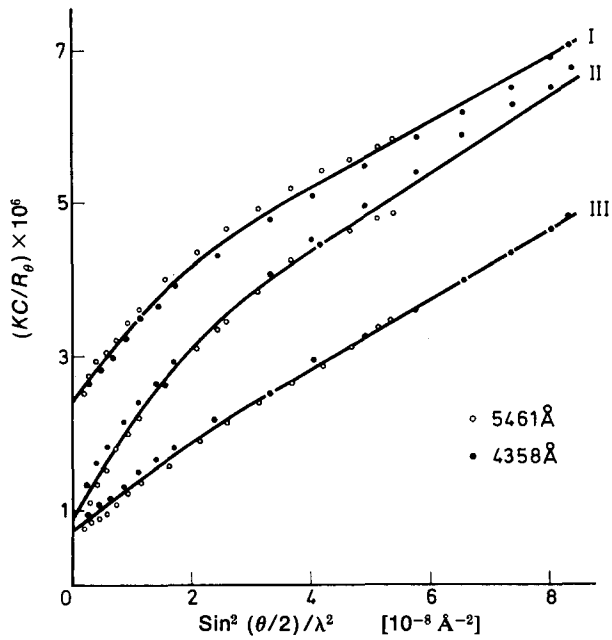

Figure 3—Light scattering of three mannan nitrate samples (see *Table 2*) derived from the cell wall of *Codium fragile*. Ethyl acetate as solvent

about 0·3 per cent and dispersed in the solvent by slowly agitating for three days. Undissolved material was removed by centrifugation (25 000 $g/2$ h), dried and weighed and the exact concentration of the solution calculated. Solubilities were found to be 85 to 90 per cent for all samples in each solvent.

Light scattering measurements were made with the Aminco* apparatus tested and calibrated as already described[18]. The mannan nitrate was handled in a manner similar to that previously described for cellulose nitrate[18], clarification being achieved by centrifuging for two hours at 30 000 *g*. *Figure 2* shows the Zimm plot at 4 358Å for sample I in ethyl acetate and *Figure 3* the zero concentration lines for all three samples at 4 358Å and 5 461Å. For measurements at 4 358Å a small fluorescence correction was made in the manner previously described[18]. It amounted to about ten per cent of the scattered intensity at 90°.

The refractive index increment dn/dC was measured in a Rayleigh differential refractometer and found to be $0{\cdot}102 \pm 0{\cdot}004$ cm^3 g^{-1} in acetone and $0{\cdot}101 \pm 0{\cdot}002$ cm^3 g^{-1} in ethyl acetate; the same values being obtained within experimental error at both wavelengths. These values are identical with those found for cellulose nitrate[14-18].

(b) *Membrane osmometry*

Osmotic pressure measurements were made in the Mechrolab† apparatus (Model 503). All three samples were measured in ethyl acetate and sample I was also measured in *n*-butanone (acetone was found to be unsuitable for instrumental reasons). The relevant graphs for ethyl acetate as solvent are

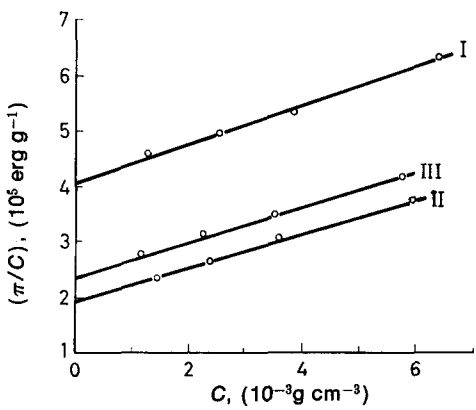

Figure 4—Osmotic pressure of three mannan nitrate samples (see *Table 2*) derived from the cell wall of *Codium fragile*. Ethyl acetate as solvent

shown in *Figure 4*. Measurements were made at 30°C using type 08 membranes. Stable pressures were usually obtained within ten minutes and no permeation of the membrane by the solute was observed.

(c) *Viscosity*

Viscosity measurements were made at 25°C in acetone. Two Cannon–

*American Instrument Co. Inc., Silver Spring, Maryland, U.S.A.
†Hewlett-Packard Ltd, Slough, Buckinghamshire.

Fenske viscometers were used each having two bulbs, thus giving four rates of shear in the range 500 sec^{-1} to 2 000 sec^{-1}. The data were extrapolated to zero rate of shear and zero concentration to give the intrinsic viscosity

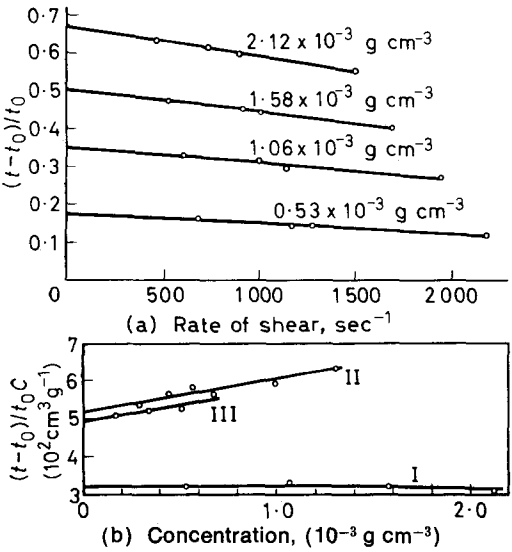

Figure 5—Viscosity of mannan nitrate in acetone derived from the cell wall of *Codium fragile*: (a) Extrapolation to zero rate of shear for sample I (see *Table 2*); (b) Extrapolation to zero concentration for all three samples

as shown in *Figure 5*. The intrinsic viscosities were corrected for degree of nitration by using the expression of Lindsley and Frank[19] and converted to degrees of polymerization using the empirical relationship of Huque *et al*[17]. Both of these relationships were originally obtained for cellulose nitrate in acetone and this will be discussed in more detail.

(d) *Gel permeation chromatography*[20,21]*

All three samples were measured in tetrahydrofuran (this solvent was used because of its low refractive index which for instrumental reasons

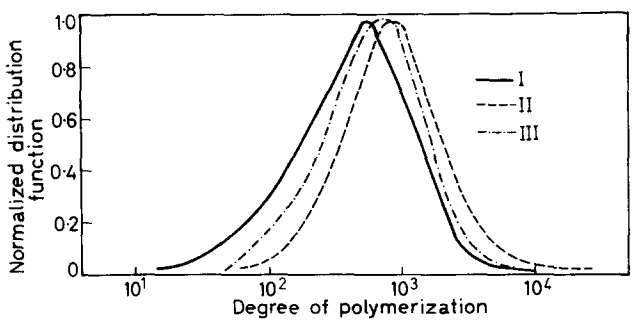

Figure 6—Degree of polymerization normalized distribution curves calculated from gel permeation chromatography measurements upon three mannan nitrate samples (see *Table 2*) derived from the cell wall of *Codium fragile*

*These measurements were carried out for us by Water's Associates Analytical Service Laboratory, Water Street, Stockport, Cheshire.

renders it most suitable). The elution diagrams obtained from the instrument were converted into normalized distributions of DP on a logarithmic scale (*Figure 6*) by differentiating cumulative plots. The elution volume 'counts' were converted to DP by making use of a chain length calibration obtained with polystyrene standards. The validity of this procedure is discussed below. Number and weight average DPs were calculated together with viscosity averages based on the Mark–Houwink equation of Huque *et al.*[17] for cellulose nitrate in acetone.

(e) *Comparison of degrees of polymerization obtained by different methods*

Table 3 shows that, with the exception of sample III, data obtained by the various methods are in fairly good agreement. In view of the method of calibration, the gel permeation chromatography (GPC) values cannot be regarded as absolute, and the number average values are in fact consistently higher than those obtained by osmotic pressure by a factor of 1·4. The agreement of the weight average values obtained by GPC and light scattering for sample I may then be fortuitous as it is not possible to ensure the complete absence of microgel which would give rise to a high light scattering value, although the same result was obtained for both solvents. The viscosity average DPs derived from the GPC data are all greater than those actually obtained from viscosity measurements by a factor of two. This may in part be due to an error in the GPC calibration but it may also be due to the method of calculation. In the Mark–Houwink equation of Huque *et al.*[17] the index, α, is 0·91. This makes the viscosity average about ten per cent lower than the weight average (not experimentally significant here). However, there is some evidence in the case of cellulose nitrate[17,18] that the larger molecules adopt a more coiled configuration, thus reducing the effective value of α and the viscosity average DP. The same may be the case for mannan nitrate. It must be remembered that DPs have been calculated from intrinsic viscosities using relationships obtained empirically for cellulose nitrate. This assumes that the chain statistics are the same for both substances and this is discussed further below.

The weight average values of sample III by GPC and light scattering disagree considerably. It was, however, noted that although GPC measurements for samples II and III were carried out under identical conditions the area under the elution curve was 20 per cent less for sample III. This is greater than the random error to be expected in measurements of this kind (ten per cent quoted by the makers of the instrument), and indicates that some material may have been left behind in the gel. Viscosity measurements were made both on the original sample III and on material recovered from solution after the GPC measurements, and a discrepancy factor of two was found (*Table 3*), indicating that some of the higher molecular weight material originally present in the sample III was not present after gel permeation chromatography.

Values of the second virial coefficient B obtained by osmotic pressure and light scattering differ widely (*Table 3*). This may be due to the high polydispersity of the samples but Holtzer *et al.*[15] report similar discrepancies for cellulose nitrate using fractionated material.

CHAIN STATISTICS OF MANNAN TRINITRATE IN SOLUTION

The weight average DPs obtained from light scattering are derived from the intercepts of the curves in *Figures 2* and *3*. If Gaussian statistics are assumed then it is possible to compare the rest of the curves with those that would result from any given distribution of degrees of polymerization. This may be done numerically from the GPC data by using an adaptation of the formula for the particle scattering factor $P(\theta)$ due to Zimm[22], viz.

$$P(\theta) = \Sigma (DP) \cdot H P(x) / \Sigma (DP) \cdot H \tag{1}$$

where H is the height at each count on the elution curve and DP is the corresponding degree of polymerization. $P(x)$ is the particle scattering factor for a monodisperse Gaussian chain having this degree of polymerization and is given by

$$P(x) = (2/x^2)[e^{-x} - (1-x)] \tag{2}$$

where

$$x = (8/3)\pi^2 b^2 \{\sin^2(\theta/2)/\lambda^2\} (DP) \tag{3}$$

b is the effective bond length given by:

$$b^2 = \overline{r_N^2}/(DP)_N = \overline{r_W^2}/(DP)_W \tag{4}$$

where $\overline{r^2}$ is the mean square end to end distance of the chain. In order to calculate $P(\theta)$, b must be known. According to the theory of Benoit[23,24] $(DP)_N$ and $\overline{r_N^2}$ may be calculated from the intercept of the asymptote to the curve at high angles, and the ratio of slope to intercept of asymptote respectively. b is therefore given by the slope of the asymptote

$$b = (\lambda/2\pi)(3M_R \times \text{slope of asymptote})^{\frac{1}{2}} \tag{5}$$

where M_R is the molecular weight of the repeat unit. However, the number average values of DP obtained here are much too low for the curves to be asymptotic within the experimental range of $\sin^2(\theta/2)/\lambda^2$ although all the curves exhibit 'apparent asymptotes'. Calculations made by Krahtochvil[25] upon a wide variety of types of polydispersity show that the true asymptote is displaced upwards from the apparent asymptote but that the slopes are virtually the same. Thus equation (5) can be used to calculate b as it only involves the slope of the asymptote.

It was decided to fit the GPC data of sample I to the light scattering data obtained with acetone as solvent. The data in acetone were used because it has been shown that the chain statistics of cellulose nitrate in acetone are less dependent upon molecular size than they are in ethyl acetate[17]. *Figure 7* shows the result of this calculation. The agreement is as good as can be expected in view of the accuracy of the data. The dotted line is the true asymptote to the curve, the slope of which is indistinguishable from that of the apparent asymptote, thus justifying the original calculation of b. The value of b was found to be 29 ± 3Å, and is identical within experimental error with the values obtained for cellulose nitrate[18,24]. It should be noted that the curvature at low angles is predicted from the polydispersity given

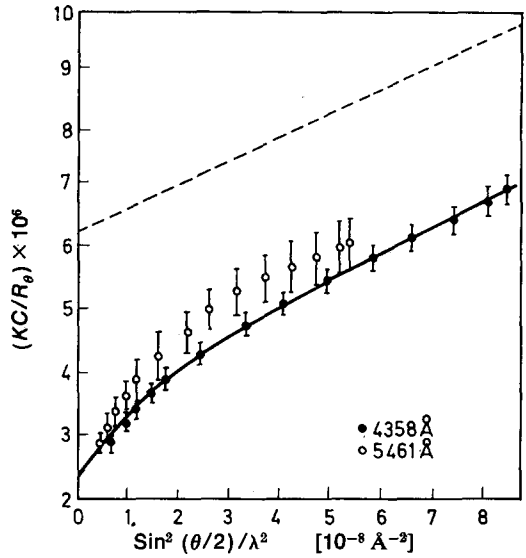

Figure 7—Light scattering of mannan nitrate in acetone derived from the cell wall of *Codium fragile*, sample I (see *Table 2*). Solid line is the theoretical curve calculated from gel permeation chromatography data (*Figure 6*). Broken line is the asymptote to this curve

by the GPC data and is therefore not merely a result of spurious scattering due to microgel or incomplete clarification. To some extent the fit of the GPC to the light scattering data justifies the GPC calibration. This is not true for the lower DPs, however, as during the calculation it becomes apparent that molecules having DP below 300 have no influence on the particle scattering factor within the experimental range.

The light scattering curves for sample I are almost the same for both solvents indicating that mannan nitrate has similar chain statistics in ethyl acetate and acetone. The apparent asymptotic slopes are also nearly the same for the three samples.

DEGREE OF POLYMERIZATION AND POLYDISPERITY OF MANNAN IN THE CELL WALL

Although there is some degree of disparity between the GPC data and DPs obtained by absolute methods, these may be regarded as minor in view of the high degree of polydispersity and will make virtually no difference when the distributions are plotted on a logarithmic scale. *Figure 6* may therefore be taken as a fair description of the DP distributions for the three samples.

It is possible that the nitration procedure causes severe degradation so that degrees of polymerization obtained are not representative of the mannan in the cell wall. However, *Figure 6* shows that there are far less short chains in sample II than in sample I even though sample II consists

only of material obtained after a longer period of nitration. This indicates that the nitration procedure has not caused severe degradation in samples I and II. Sample III may, however, contain some degraded material, although this sample represents only 21 per cent of the total cell wall mannan.

Another complication arises from the fact that not all of sample III is represented in the GPC curve as some of it may have been left behind in the gel. One possible explanation is that some of the material is branched and forms a complex with the gel. This is supported by the shape of the light scattering curve which is far less curved than would be expected for the degree of polydispersity. Chain branching would cause an upward curvature which would tend to cancel out the downward curvature due to polydispersity. Unfortunately it was not possible to repeat the GPC analysis so that this is only a very tentative suggestion. It should, however, be emphasized that the amount of material involved is about 20 per cent of sample III and therefore only about four per cent of the total mannan investigated.

Degradation of a purely mechanical nature could also arise during the initial grinding process but this is not thought likely to be serious in view

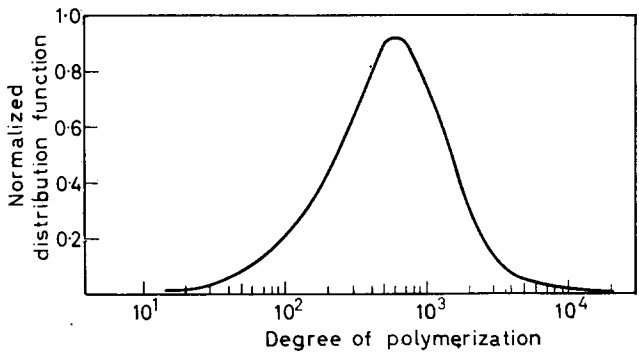

Figure 8—Degree of polymerization normalized distribution curve of mannan in the cell wall of *Codium fragile* (see text)

of the DPs involved. It is hoped, however, to investigate this further by means of small scale preparations.

It is therefore thought that the GPC curves are a justifiable representation of the mannan in the native cell wall. The DP distribution curve for all the mannan can be found by adding the curves for the three samples in the appropriate proportions and the resultant normalized curve is shown in *Figure 8*. There is no real evidence that there are two distinct components corresponding to granular and fibrillar mannan but the range of DP is great enough to account for both types of ultrastructure. Most of the mannan chains have DPs much higher than those found for other similar mannans (*Table 1*). DPs range from below 20 to above 10 000 with 90 per cent of the material having DP between 100 and 2 500. The peak in the logarithmic distribution occurs at about 600.

CONCLUSIONS

Mannan from the cell wall of the green seaweed *Codium fragile* has degrees of polymerization ranging from below 20 to above 10 000. Of the material, 90 per cent has DPs between 100 and 2 500 with a peak in the logarithmic distribution at about 600. Thus most of the mannan chains have DPs much higher than those found for other similar mannans.

The chain statistics of mannan trinitrate in solution appear to be identical with those of cellulose trinitrate.

Astbury Department of Biophysics,
University of Leeds

(*Received January 1969*)

REFERENCES

[1] MEIER, H. *Biochim. Biophys. Acta,* 1958, **28,** 229
[2] ASPINALL, G. O., HIRST, E. L., PERCIVAL, E. G. V. and WILLIAMSON, I. R. *J. chem. Soc.* **1953,** 3184
[3] WOLFROM, M. L., LAVER, M. L. and PATIN, D. L. *J. org. Chem.* 1961, **26,** 4533
[4] JONES, J. K. N. *J. chem. Soc.* **1950,** 3292
[5] FREI, E. and PRESTON, R. D. *Nature, Lond.* 1961, **192,** 939
[6] LOVE, J. and PERCIVAL, E. E. *J. chem. Soc.* **1964,** 3345
[7] IRIKI, Y. and MIWA, T. *Nature, Lond.* 1960, **185,** 178
[8] FREI, E. and PRESTON, R. D. *Proc. Roy. Soc. B,* 1968, **169,** 127
[9] TIMELL, T. E. *Canad. J. Chem.* 1957, **35,** 333
[10] MACKIE, W. and PRESTON, R. D. *Planta.* 1968, **79,** 249
[11] ALEXANDER, W. J. and MITCHELL, R. L. *Analyt. Chem.* 1949, **21,** 1497
[12] TIMELL, T. E. *Pulp Pap. (Mag.) Can.* 1955, **56,** 104
[13] MARX-FIGINI, M. and SCHULZ, G. V. *Biochim. Biophys. Acta,* 1966, **112,** 81
[14] BADGER, R. M. and BLAKER, R. H. *J. phys. Chem.* 1949, **53,** 1056
[15] HOLTZER, A. M., BENOIT, H. and DOTY, P. *J. phys. Chem.* 1954, **58,** 624
[16] HUNT, M. L., NEWMAN, S., SHERAGA, H. A. and FLORY, P. J. *J. phys. Chem.* 1956, **60,** 1278
[17] HUQUE, M. M., GORING, D. A. I. and MASON, S. G. *Canad. J. Chem.* 1958, **36,** 952
[18] SELLEN, D. B. and LEVI, M. P. *Polymer, Lond.* 1967, **8,** 633
[19] LINDSLEY, C. H. and FRANK, M. B. *Industr. Engng Chem. (Industr.),* 1953, **45,** 2491
[20] MOORE, J. C. *J. Polym. Sci. A,* 1964, **2,** 835
[21] MALEY, L. E. *J. Polym. Sci. C,* 1965, **8,** 253
[22] ZIMM, B. H. *J. chem. Phys.* 1958, **16,** 1099
[23] BENOIT, H. *J. Polym. Sci.* 1953, **11,** 507
[24] BENOIT, H., HOLTZER, A. M. and FLORY, P. J. *J. phys. Chem.* 1954, **58,** 635
[25] KRAHTOCHVIL, P. *J. Polym. Sci. C,* 1968, **23,** 143