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SPECIFICITY IN THE INTERACTION OF DIRECT DYES WITH POLYSACCHARIDES*

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ABSTRACT

A number of polysaccharides have been shown to interact in solution with the direct dyes Calcofluor White M2R New (Calcofluor) and Congo Red. Complex formation was detected by changes in solubility and in the fluorescence and absorption spectra of the dyes. Strongest interaction, as evidenced by precipitation, bathochromic shifts >20 nm with Congo Red and >10 nm with Calcofluor, and a major (4–7 fold) increase in fluorescence intensity, was shown by polysaccharides known to contain contiguous (1→4)-β-linked D-glucopyranosyl units, such as cereal β-D-glucans, xyloglucan, and substituted celluloses. Evidence of complex formation was also shown by some other polysaccharides, such as (1→3)-β-D-glucan and hemicellulosic galactoglucomannans, but most polysaccharides showed little or no interaction. The two dyes differed in their affinities for the different polysaccharides tested. Cellopentaose and higher, but not lower, oligomers showed interaction.

INTRODUCTION

The interaction of direct dyes with cellulose[1], of significance in the textile industry, and the interaction of cationic dyes with anionic polysaccharides[2–4] important, for example, in studies of connective tissue glycosaminoglycans, are well known. The former phenomenon is a heterogeneous interaction. The latter has been extensively studied in solution, although a major practical benefit of such interactions has been in histochemistry[5] and, therefore, in a heterogeneous system. We reported[6] that direct dyes, as exemplified by Congo Red and Calcofluor, form complexes in solution with cereal β-D-glucans and some other polysaccharides, and showed how this interaction was of value histochemically. The interaction between stilbene-type, fluorescent "whitening" agents and β-D-glucans, used in histochemistry, is reported to show some specificity[7,8], but these suggestions have been based on subjective estimates of a heterogeneous adsorption phenomenon. The present study forms part of a program to give a firmer chemical basis, if possible, to such histo-

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chemical observations. In view of the importance of specificity for biology in general and for applications of the interaction with cereal β-D-glucans in particular, we undertook a survey of the interaction of a number of polysaccharides with Congo Red and Calcofluor, examples respectively of the diazo and stilbene type of direct dye. The interactions described all take place, initially at least, in solution, and as such are distinct from the binding of direct dyes by cellulose but, surprisingly, the interactions show a high degree of specificity for the cellulosic-type structure, namely contiguous (1→4)-β-D-glucopyranosyl units.

The interactions described here are between anionic dyes and neutral or anionic polysaccharides, and the driving force is not, therefore, electrostatic in origin. Dye, or ligand, interactions of this nature with polysaccharides have been described previously. Congo Red is known to form complexes with starch and amylose[9,10], and with (1→3)-β-D-glucan[11,12], as shown by changes in the absorption and c.d. spectra. Circular dichroism studies have also indicated an interaction between direct dyes and mannans[10]. A variety of low-molecular-weight, organic molecules, including some fluorescent dyes, form complexes with amylose and cyclodextrin[13–15].

Interaction between substituted celluloses and Congo Red and other direct dyes, as manifested by changes in viscosity and absorption spectra of the dyes, has been previously reported[16,17], and a precipitation of tamarind amyloid by certain dyes has been noted[18].

EXPERIMENTAL

General. — Absorption spectra were recorded with a Unicam SP-1800 or Beckman Model 26 Spectrophotometer, and fluorescence spectra with a Hitachi–Perkin–Elmer MPF-2A spectrofluorimeter. Congo Red (C.I. 22120) was purchased from Canadian Laboratory Supplies Ltd., and Calcofluor White M2R New {{C.I. 40622; CI Fluorescent Brightener 28; major component, the disodium 4,4′-bis{4-anilino-6-[bis(2-hydroxyethyl)amino]-1,3,5-triazin-2-yl}amino-2,2′-stilbenedisulphonate}} was a gift from the American Cyanamid Co., Bound Brook, N.J. 08805. The source and main structural features of the polysaccharides are listed in Table I. In some cases, the identity of the monosaccharide constituents of the polysaccharides was verified by acid hydrolysis (M sulphuric acid, 100°, 3 h) and paper chromatography in 8:2:1 (v/v) ethyl acetate–pyridine–water. A highly purified sample of *Bacillus subtilis* β-D-glucan endohydrolase (EC 3.2.1.73) was kindly provided by B. A. Stone.

Preparation of cello-oligosaccharides. — Cellodextrin acetates were prepared by acetolysis[27] of ashless cellulose powder (Whatman), and the crude mixture fractionated on plates (20 × 20 cm) of Whatman PLK 5 Linear K Silica Gel (1000 μ) by use of two developments with 23:2 (v/v) benzene–methanol. The oligosaccharide acetates were detected by spraying with water, and the fractions obtained were refractionated on silica gel and crystallized from 95% ethanol. Melting points and optical rotations were in good agreement with literature values[27]. The acetates were

TABLE I

SOURCE AND MAIN STRUCTURAL FEATURES OF POLYSACCHARIDES INVESTIGATED

Polysaccharide	*Source*	*Supplied by*	*Main structural features*
Oat β-D-glucan	*Avena sativa*	R. Hyldon	(1→4)- and (1→3)-β-D-Glc*p*
Barley β-D-glucan	*Hordeum vulgare*	V. Bendelow D. T. Bourne	(1→4)- and (1→3)-β-D-Glc*p*
Lichenan	*Cetraria islandica*	Sigma Chemical Co.	(1→4)- and (1→3)-β-D-Glc*p*
Hydroxyethylcellulose Natrosol 250 M	Cellulose	Hercules Co.	(1→4)-β-D-Glc*p*
Amyloid (xyloglucan)	*Tamarindus indica*	I. R. Siddiqui	(1→4)-β-D-Glc*p*, oligosaccharide side-chains
Carboxymethylcellulose 7H3 SXF	Cellulose	Hercules Co.	(1→4)-β-D-Glc*p*
Xanthan gum	*Xanthomonas campestris*	Kelco Co.	(1→4)-β-D-Glc*p*, oligosaccharide side-chains
Reduced pneumococcus polysaccharide Type III	*Pneumococcus*	B. A. Stone	Alternating (1→3)- and (1→4)-β-D-Glc*p*
Curdlan	*Alcaligenes faecalis*	T. Harada	(1→3)-β-D-Glc*p*
Laminaran	*Laminaria hyperborea*	Sigma Chemical Co.	(1→3)-β-D-Glc*p*
Carboxymethylpachyman	*Pachyman* (from *Poria cocos*)[a]	J. J. Marshall	(1→3)-β-D-Glc*p*
Barley pentosan	*Hordeum vulgare*	G. H. Palmer	(1→4)-β-D-Xyl*p*, L-Ara*f* substituents
Wheat pentosan	*Triticum aestivum*	G. O. Aspinall	(1→4)-β-D-Xyl*p*, L-Ara*f* substituents
(4-*O*-Methylglucurono)xylan	*Populus tremuloides*	T. E. Timell	(1→4)-β-D-Xyl*p*, 4-*O*-methyl-Glc*p*A substituents
Arabino-(4-*O*-methylglucurono)xylan	*Picea abies*	T. E. Timell	(1→4)-β-D-Xyl*p*, 4-*O*-methyl-Glc*p*A and Ara*f* substituents
D-Gluco-D-mannan	*Populus tremuloides*	T. E. Timell	(1→4)-β-D-Man*p* and D-Glc*p*
D-Galacto-D-gluco-D-mannan A	*Tsuga canadensis*	T. E. Timell	(1→4)-β-D-Man*p* and D-Glc*p*, D-Gal*p* substituents
Galacto-D-gluco-D-mannan B	*Tsuga canadensis*	T. E. Timell	(1→4)-β-D-Man*p* and D-Glc*p*, D-Gal*p* substituents
β-D-(1→4)-galactan	*Larix laricina*	T. E. Timell	(1→4)-β-D-Gal*p*
Arabino-D-galactan	*Larix occidentalis*	T. E. Timell	(1→3)-β-D-Gal*p*, D-Gal*p* and Ara*f* substituents
Elsinan	*Elsinoe leucospila*	A. Misaki	(1→4)- and (1→3)-α-D-Glc*p*
Pullulan	*Pullularia pullulans*	H. Jennings	(1→4)- and (1→6)-α-D-Glc*p*
Algal D-xylan	*Chaetangium erinaceum*	H. Parolis	(1→4)- and (1→3)-β-D-Xyl*p*
W. pyramidata gum	*Watsonia pyramidata*	I. C. M. Dea	(1→4)-β-D-Xyl*p*, oligosaccharide substituents
Guar gum	*Cyamopsis tetragonolobus*	Tragacanth Trading Co. Ltd.	(1→4)-β-D-Man*p*, D-Gal*p* substituents

TABLE I (*continued*)

Polysaccharide	*Source*	*Supplied by*	*Main structural features*
Locust-bean gum A	*Ceratonia siliqua*	Stein, Hall and Co. Ltd.	(1→4)-β-D-Man*p*, D-Gal*p* substituents
Locust-bean gum B	*Ceratonia siliqua*	G. O. Aspinall	(1→4)-β-D-Man*p*, D-Gal*p* substituents
Pectin	Citrus	Sigma Chemical Co.	(1→4)-α-D-Gal*p*A
Chondroitin 4-sulphate	Connective tissue	M. B. Mathews	(1→4)-β-D-Glc*p*A and (1→3)-β-D-Gal*p*NAc-4-SO_4
Dermatan sulphate	Connective tissue	M. B. Mathews	(1→4)-α-L-Ido*p*A and (1→3)-β-D-Gal*p*NAc-4-SO_4
Heparin	Connective tissue	M. B. Mathews	(1→4)-α-L-Ido*p*A-2-SO_4, (1→4)-β-D-Glc*p*A, and (1→4)-α-D-Glc*p*NSO_4-6-SO_4
Heparan sulphate	Connective tissue	M. B. Mathews	(1→4)-α-L-Ido*p*A-2-SO_4, (1→4)-β-D-Glc*p*A, and (1→4)-α-D-Glc*p*NAc-6-SO_4
Rh. Trifolii	*Rhizobium trifolii*	P. E. Jansson	Ref. 20
K5	*Klebsiella*	G. G. S. Dutton	Ref. 21
K18	*Klebsiella*	G. G. S. Dutton	Ref. 22
K24	*Klebsiella*	G. G. S. Dutton	Ref. 23
K32	*Klebsiella*	G. G. S. Dutton	Ref. 24
K36	*Klebsiella*	G. G. S. Dutton	Ref. 25
K55	*Klebsiella*	G. G. S. Dutton	Ref. 26

[a]Prepared according to ref. 19.

O-deacetylated by catalytic amounts of barium methoxide in dry methanol[28]. Cello-oligosaccharides were also prepared by fractionation of the *O*-deacetylated crude acetolysate on columns of Bio-Gel P-2. The effluent was monitored by means of a Waters R403 differential refractometer. Four fractions, corresponding to Glc_4–Glc_7, were collected, concentrated, and rechromatographed to give four symmetrical peaks. These fractions were collected, concentrated, and used for testing with dyes. Concentrations of the oligosaccharides used for testing dye-interactions were determined by a modified cysteine–sulphuric acid method[29].

Enzyme treatment of wheat and barley pentosans. — Wheat and barley pentosans were treated with *B. subtilis* β-D-glucanase in 0.05M Mes (2-morpholinoethanesulphonic acid) buffer, pH 6.5, for 65 h at 37° in the presence of toluene. Barley pentosan was given a second treatment (48 h). The digestion was terminated by heating the solution in a boiling-water bath for 5–10 min, and the mixture then dialysed in the presence of chloroform, and freeze-dried.

Removal of pyruvate residues by acid hydrolysis. — Pyruvate residues were hydrolysed from K32 polysaccharide by treatment of its aqueous solution with Bio-Rad AG 50W X-8, (H^+) ion-exchange resin[24], and from *Rh. trifolii*, K36, K5, and xanthan polysaccharides by 0.1M trifluoroacetic acid for 1 h at 95–100°. The hydrolysate was dialysed against tap and distilled water, and freeze-dried. Analysis[30] showed that the pyruvate content after acid hydrolysis was <0.1% in each case.

Precipitation of polysaccharide by dye. — Polysaccharide solutions (1%, w/v) were prepared in phosphate buffer, pH 7.0 [ionic strength (I) 0.05], carbonate buffer (pH 10, I 0.05), or 0.05M sodium hydroxide. In some instances, the latter solution was neutralised with phosphate buffer before testing. Resort to alkaline solutions was necessary for polysaccharides poorly soluble in neutral phosphate buffer. The solutions of polysaccharide were clarified by centrifugation, before testing the supernatant (4 vol.), by addition with rapid mixing of 1 vol. of a solution (1%, w/v) of the dye in the appropriate solvent. The samples were examined for the presence of a precipitate immediately and after 24 h. Calcofluor solutions were protected from light.

Absorption and fluorescence spectroscopy of dye–polysaccharide mixtures. — Solutions (0.1%, w/v) of polysaccharide, first clarified by centrifugation, were tested for interaction with dye (10 μg/mL) by studying the changes in the absorption and fluorescence spectra of the dyes. Assuming 100% purity for Congo Red and 70% for Calcofluor[31], this corresponds to 14.3μM Congo Red and 7.29μM Calcofluor. Calcofluor solutions were protected from light, and fluorescence spectra obtained were uncorrected. The spectral changes appeared to occur immediately upon mixing and were stable. However, Calcofluor solutions are light-sensitive, and decreased intensity of fluorescence emission was observed during repeated scans despite the use of narrow, excitation-slit widths. As for precipitation testing, phosphate buffer (pH 7.0, I 0.05) was the preferred medium, but solubility problems sometimes necessitated the use of alkali. Some polysaccharide samples containing acidic groups and oligosaccharides were tested in solutions of higher ionic strength to enhance interactions. Spectra of controls without added dye were also obtained. The effects

of detergents, solvents, and ionic strength on the spectra of the dyes were studied, at concentrations as described in the text.

RESULTS

Precipitation. — Ideally, each polysaccharide should be tested under the same conditions. We have shown[6] that four parts by weight of oat β-D-glucan are quantitatively precipitated from 0.7–1.0% solutions by one part by weight of Calcofluor in phosphate buffer (pH 7.0, I 0.05) or in carbonate buffer (pH 10.0, I 0.05). Unfortunately, not all polysaccharide samples showed complete solubility in these buffers. Amylose, pustulan, and soybean xylan, for example, gave reliably stable solutions only in 0.5M sodium hydroxide which, as shown for oat β-D-glucan (Table II), was not a suitable medium for testing. Where solubility allowed, however, testing was carried out in phosphate buffer (pH 7.0, I 0.05). Of polysaccharides soluble in this buffer, immediate precipitation (which tended to be stringy and gelatinous in nature) was observed with both the dyes for oat β-D-glucan (2 samples), barley β-D-glucan (3 samples), hydroxyethylcellulose Natrosol 250M, and tamarind amyloid. No other polysaccharide samples produced definite major precipitation of this nature. Lesser precipitation was observed, after 24 h, with both dyes with K32, *Rh. trifolii*, barley pentosan, and guar gum. Some precipitation, after 24 h, with Calcofluor only was noted with arabino-(4-*O*-methylglucurono)xylan, wheat pentosan, and pectin. The significance of this lesser precipitation-reaction is doubtful. No precipitate was observed with cellobiose, laminaran, carboxymethylpachyman, locust-bean gums A and B, tara gum, *W. pyramidata* gum, elsinan, carboxymethylcellulose 7H3 SXF, arabinogalactan, (1→4)-β-D-galactan, pullulan, and *C. erinaceum* xylan.

Of samples poorly soluble in phosphate buffer and thus tested in carbonate buffer (pH 10.0, I 0.05), galactoglucomannan A showed some precipitation with Congo Red. Galactoglucomannan B, glucomannan, and (4-*O*-methylglucurono)xylan showed no precipitation with either dye. Because of poor solubility, the concentrations of glucomannan and galactomannan B used to test precipitation were 0.2 and 0.7%, respectively. Curdlan and K55, tested in 0.05M sodium hydroxide, showed no precipitation with either dye. The sample of lichenan tested was not very soluble in the buffers used, but precipitation with both dyes was observed in 0.05M and neutralised 0.05M sodium hydroxide.

Oat β-D-glucan was precipitated by both dyes from phosphate buffer (pH 7.0, I 0.05 and I 0.2), carbonate buffer (pH 10.0, I 0.05), and 0.05M sodium hydroxide. Precipitation was not observed from 0.5M sodium hydroxide with Congo Red. A small amount of precipitate that formed with Calcofluor in 0.5M sodium hydroxide appeared to arise from difficulties in mixing. Certainly, precipitation with Calcofluor was inhibited by 0.5M sodium hydroxide.

Absorption spectrophotometry. — In most cases, absorption spectra of control solutions without dye showed low absorption values at wavelengths of interest, and no defined peaks. None of the major spectral changes noted could be attributed to addi-

tive effects, but the wood hemicelluloses [galactoglucomannans, glucomannan, and (1→4)-β-D-galactan] contained u.v. chromophores that interfered in the λ_{max} region of Calcofluor, and, with (1→4)-β-D-galactan, the Calcofluor peak was masked. However, the λ_{max} area of Congo Red was free from interference.

Oat and barley β-glucan, lichenan, hydroxymethylcellulose Natrosol 250M, and tamarind amyloid induced major changes in the absorption spectra of both dyes (Figs. 1 and 2, and Table II). [To avoid crowding, oat β-D-glucan and lichenan spectra are not shown, but they were similar to that of barley β-D-glucan. Lichenan however, showed a somewhat larger shift (35 nm) with Congo Red than oat and barley β-D-glucan]. Bathochromic (red) shifts in λ_{max} for Congo Red were 20 nm or more, and, for Calcofluor, greater than 10 nm. No other polysaccharide tested showed effects as great as this with both dyes. Carboxymethylcellulose 7H3 SXF (in I 0.2 phosphate), laminaran, and crude wheat-pentosan, which showed the presence of D-glucose on acid hydrolysis, induced >10-nm red shifts in the λ_{max} of the Congo Red and >5-nm red shifts in the λ_{max} of the Calcofluor spectrum. Another group of polysaccharides (galactoglucomannan A, glucomannan, curdlan, carboxymethylpachyman, and crude barley-pentosan, which showed the presence of D-glucan contaminant) induced major (11–33-nm) red-shifts in the λ_{max} of the Congo Red spectrum, but showed little or no interaction with Calcofluor. Minor interactions (4–8-nm red shifts), mainly with Congo Red, were induced by de-*O*-pyruvylated xanthan gum, reduced pneumococcus polysaccharide Type III, elsinan, pullulan, arabino-(4-*O*-methylglucurono)xylan, galactoglucomannan B, (4-*O*-methylglucurono)xylan, heparin, and dialysed *Rh. trifolii*. A group of polysaccharides (K32, K36, and *Rh. trifolii*) showed major hypsosochromic (blue) shifts in the λ_{max}

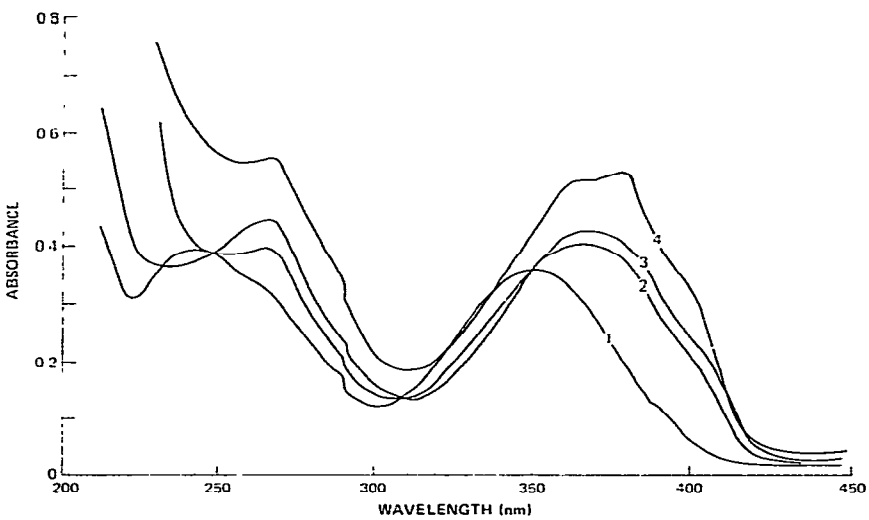

Fig. 1. Absorption spectra of solutions in phosphate buffer (pH 7.0, I 0.05) of: (1) Calcofluor (10 μg/mL) alone, (2) in the presence of hydroxyethylcellulose Natrosol 250M, (3) barley β-D-glucan, and (4) tamarind amyloid.

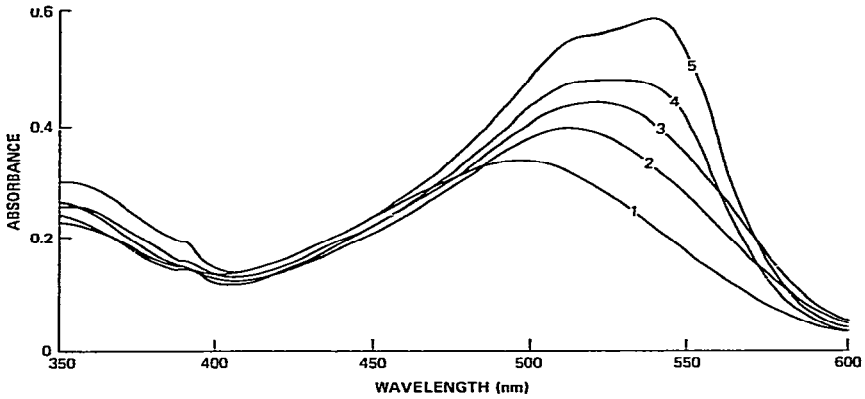

Fig. 2. Absorption spectra of solutions in phosphate buffer (pH 7.0, I 0.05) of: (1) Congo Red (10 μg/mL) alone, (2) in the presence of laminaran, (3) barley β-D-glucan, (4) hydroxyethylcellulose Natrosol 250M, and (5) tamarind amyloid.

TABLE II

CHANGES IN λ_{max} OF CONGO RED AND CALCOFLUOR SPECTRA, AND IN FLUORESCENT INTENSITY OF CALCOFLUOR IN THE PRESENCE OF POLYSACCHARIDES

Polysaccharide	*Medium*[a]	*Δ(λmax)*		*R.F.I.*[b,c]
		C.R.[b]	*Cal.*[b]	
Oat β-D-glucan	1	21	14	4.3
	2	29	14	7.3
	3	16	10	2.9
	4	10	4	[d]
	6	13	11	3.4
	7	30	17	4.7
	8	0	2	[d]
Barley β-D-glucan 1	1	25	15	4.3
Barley β-D-glucan 2	1	19	12	4.1
Barley β-D-glucan 3	1	23	12	4.3
Lichenan	1	35	16	4.4
	5	31	16	[d]
Hydroxyethylcellulose Natrosol 250M	1	23–38	14	4.2
Tamarind amyloid	1	41	25	6.7
Carboxymethylcellulose 7H3 SFX	1	3	3	1.3
	2	15	6	2.1
Xanthan gum	4	0	−1	[d]
O-Depyruvylated xanthan gum	2	8	0	[d]
Reduced pneumococcus polysaccharide	1[e]	8	6	[d]
Type III	4[f]	4	0	1.8
Barley pentosan	1	32	2	3.1
Wheat pentosan	1	17	10	2.0
Enzyme-treated barley pentosan	1	31	1	[d]
Enzyme-treated wheat pentosan	1	1	[d]	[d]

TABLE II *(continued)*

CHANGES IN λ_{max} OF CONGO RED AND CALCOFLUOR SPECTRA AND IN FLUORESCENT INTENSITY OF CALCOFLUOR IN THE PRESENCE OF POLYSACCHARIDES

Polysaccharide	*Medium*[a]	$\Delta(\lambda_{max})$		*R.F.I.*[b,c]
		C.R.[b]	*Cal.*[b]	
Galactoglucomannan A	3	14	−2	1.1
Glucomannan	3	18	−1	0.9
Curdlan	4	26	0	0.8
Laminaran	1	15	5	1.5
Carboxymethylpachyman	1	11	3	1.1
Elsinan	1	6	−1	0.6
Pullulan	1	4	1	1.0
Arabino-(4-*O*-methylglucurono)xylan	1	5	3	1.6
Galactoglucomannan B	3	7	−1	1.0
(4-O-Methylglucurono)xylan	3	7	1	1.0
Guar gum	1	0	0	1.1
Locust-bean gum A	1	0	0	0.9
Locust-bean gum B	1	−3	0	0.7
Pectin	1	1	1	1.0
Algal xylan	1	−2	0	0.9
β-D-(1→4)-Galactan	1	1	[g]	0.9
W. pyramidata gum	1	1	−3	0.8
Tara gum	1	2	0	0.8
Arabinogalactan	1	1	1	1.1
Chondroitin 4-sulfate	2	0	−3	0.3
Hyaluronic acid	2	1	2	0.8
Dermatan sulfate	9	0	0	0.7
Heparin	9	4	0	0.8
Heparan sulfate	9	0	0	0.8
K5	1	0	−1	0.8
K18	1	0	0	0.3
K24	1	0	0	0.6
K55	1	−1	−1	0.5
	4	1	−2	0.3
K32	1	−13	−1	0.3
O-Depyruvylated K32	1	0	0	[d]
Dialysed K32	1	0	−1	[d]
K36	1	−40	−10	0.6
O-Depyruvylated K36	1	0	0	[d]
Dialysed K36	1	0	0	[d]
Rh. trifolii	1	−30	−15	1.1
O-Depyruvylated *Rh. trifolii*	1	−8	−7	[d]
Dialysed *Rh. trifolii*	1	4	−2	[d]

[a](1) Phosphate buffer (pH 7.0, I 0.05); (2) phosphate buffer (pH 7.0, I 0.2); (3) carbonate buffer (pH 10.0, I 0.05); (4) 0.05M sodium hydroxide; (5) neutralised 0.05M sodium hydroxide; (6) water; (7) M sodium chloride; (8) 0.5M sodium hydroxide; and (9) phosphate buffer (pH 7.0, I 0.05) and 0.5M sodium chloride. [b]Abbreviations: C.R., Congo Red; Cal., Calcofluor; and R.F.I., relative fluorescence intensity. [c]The relative fluorescence intensity is the ratio of fluorescence intensity of Calcofluor plus sample/fluorescence to intensity of Calcofluor alone. [d]Not determined. [e]Supernatant solution. [f]Concentration of 300 μg/mL. [g]No peak.

of the spectra of both dyes, and changes in the spectra as shown in Fig. 3. Since removal of pyruvate groups removed or considerably diminished this interaction, it was initially suspected that the effect was related to pyruvate content. Subsequently, it was found that dialysis, without hydrolysis, largely removed the interaction, which indicated that a low-molecular-weight impurity might be responsible for the effect. Cetyltrimethylammonium bromide, which had been used in the purification of these polysaccharides, was probably the component responsible, as indicated by the similarity of the spectra (Fig. 3). The presence of cetyltrimethylammonium bromide (10 μg/mL) also completely masked the interaction of oat β-D-glucan (1 mg/mL) with Congo Red in phosphate buffer (pH 7.0, I 0.05).

None of the other polysaccharide samples tested induced significant changes in the absorption spectra of Congo Red or Calcofluor.

High concentrations (20 mg/mL) of cellobiose, and a mixture of cellotriose and cellotetraose, showed no interaction with either dye. Of samples prepared from the crystalline acetates, only cellopentaose induced spectral shifts. This result was confirmed with samples prepared by Bio-Gel P-2 chromatography, which showed with Congo Red progressively greater red-shifts from Glc_5 to Glc_7 (Table III).

Addition of small amounts of solvent, such as ethanol and 1,4-dioxane, did not affect the absorption spectra of the dyes, but larger concentrations produced small red-shifts in the λ_{max} of the spectra. Increasing ionic-strength of the medium produced a blue-shift in the absorption maxima.

Fluorescence spectroscopy. — Congo Red did not show a strong fluorescence in solution alone or in the presence of oat β-D-glucan. Calcofluor, however, was strongly fluorescent with an asymmetric emission peak at ~430 nm from excitation

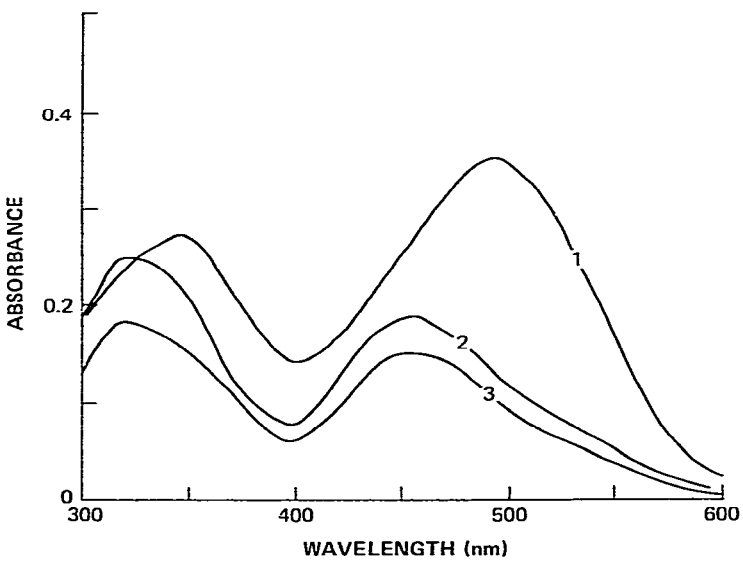

Fig. 3. Absorption spectra of solutions in phosphate buffer (pH 7.0, I 0.05) of: (1) Congo Red (10 μg/mL) alone, (2) in the presence of K36, and (3) cetyltrimethylammonium bromide (10 μg/mL).

TABLE III

CHANGES IN λ_{max} OF CONGO RED AND CALCOFLUOR SPECTRA, AND IN THE FLUORESCENT INTENSITY OF CALCOFLUOR IN THE PRESENCE OF CELLO-OLIGOSACCHARIDES[a]

Oligosaccharides	*Conc. (mg/mL)*	*Δ(λmax)*		*R.F.I.*[b]
		C.R.[b]	*Cal.*[b]	
Cellobiose	20	0	0	0.9
Cellotriose + cellotetraose	10	0	[c]	[c]
Cellotriose[d]	1	0	0	1.0
Cellotetraose[d]	1	0	0	1.3
Cellopentaose[d]	1	3	1	1.7
Cellotetraose[e]	2	0	0	0.7
Cellopentaose[e]	2	5	0	1.0
Cellohexaose[e]	2	9	0	1.9
Celloheptaose[e]	2	12	0	2.1

[a]For solutions in phosphate buffer (pH 7.0, I 0.2). [b]Abbreviations: C.R., Congo Red; Cal., Calcofluor; and R.F.I., relative fluorescence intensity. [c]Not done, because of sample–chromophore interference. [d]Prepared by *O*-deacetylation of crystalline acetates. [e]Prepared by Bio-Gel P-2 chromatography.

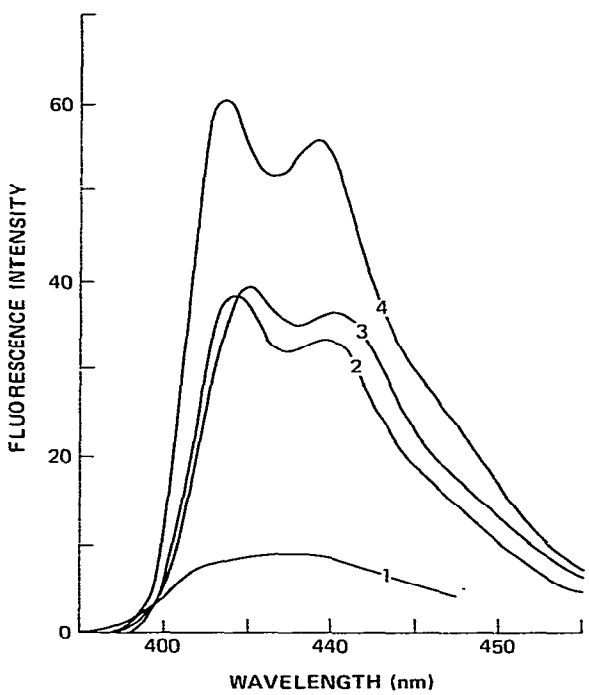

Fig. 4. Fluorescence spectra of solutions in phosphate buffer (pH 7.0, I 0.05) of: (1) Calcofluor (10 μg/mL) alone, (2) in the presence of hydroxyethylcellulose Natrosol 250M, (3) barley and oat β-D-glucan, and (4) tamarind amyloid.

at the absorption maximum (~350 nm). A considerable (4–7 fold) increase in fluorescence intensity (Table II) and a characteristic split of the emission spectra into two peaks at ~420 and 440 nm was observed in the presence of oat and barley β-D-glucan, lichenan, hydroxyethylcellulose Natrosol 250M, and tamarind amyloid (Fig. 4). In the presence of the (1→4)-β-D-glucans, hydroxyethylcellulose Natrosol 250 M, and tamarind amyloid, the peak wavelengths of emission were at a slightly shorter wavelength than with the mixed-linkage β-D-glucans. In the presence of phosphate buffer (I 0.2), carboxymethylcellulose 7H3 SXF induced a similar, but smaller, change in the fluorescence spectra. Unpurified wheat- and barley-pentosan also showed a similar effect. Slight changes in the fluorescence spectra with increased emission intensity were also noted for laminaran and arabino-(4-*O*-methylglucurono)-xylan, but with all other polysaccharides no significant changes were observed other than, in many instances, reduced intensity of emission. The significance of these small changes is uncertain. Although fluorescence is more subject to interferences (from contaminating chromophore groups, scattering, *etc.*) than absorption spectrophotometry, these results clearly delineate the group of most strongly-interacting polysaccharides, namely the (1→4)-β-D-glucans.

Although the cello-oligosaccharides induced little or no changes in the absorption spectra of Calcofluor, changes in the fluorescence spectra were observed (Table III). The results obtained are somewhat different depending on the method

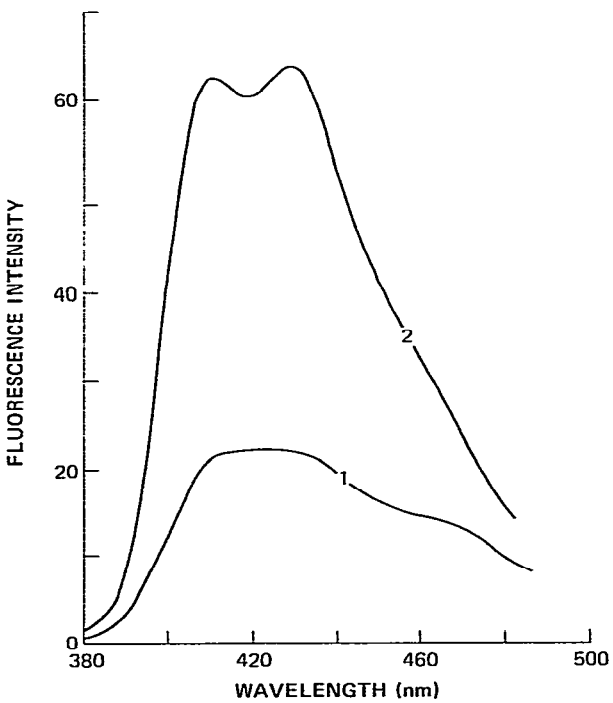

Fig. 5. Fluorescence spectra of solutions of Calcofluor (10 μg/mL) in: (1) water and (2) 1:1 (v/v) 1,4-dioxane–water.

of preparation of the oligosaccharides, possibly reflecting the presence of impurities in the sample prepared by gel chromatography. Overall, however, the results seem to confirm that interaction begins with Glc_5, and increases through Glc_6 and Glc_7.

High concentrations of 1,4-dioxane or ethanol produced changes in the fluorescence emission spectra similar to those caused by (1→4)-β-D-glucans (Fig. 5).

DISCUSSION

We have previously described[6] a precipitation reaction between cereal β-D-glucans and direct dyes. In this report, using solubility, and absorption and fluorescence spectroscopy as criteria of complex formation, we have surveyed a number of polysaccharides for their interaction with Congo Red and Calcofluor in order to determine whether this phenomenon is a general property of polysaccharides or depends on a structural specificity. Such a survey is inevitably incomplete but has led to some useful observations. Further studies, particularly into the physical nature of the interaction, will be required fully to establish the specificities indicated by the present study, in which polysaccharide samples were chosen for testing either on the basis of availability or because of structural features of interest. Thus, samples containing (1→4)- and (1→3)-β-linked pyranosyl units were of particular interest.

Isolation, purification, and characterisation of most polysaccharides is a difficult task and, for a study of this nature, there is thus a problem of sample availability, and sample purity and identity. Where appropriate, the latter has been verified by identification of the constituent sugars, but insistence on rigorous purity of samples would be unrealistic. It should be recognized, however, that spectroscopic studies are subject to interference from very small amounts of contaminant. For example, as little as 1 μg/mL of oat β-D-glucan produced a detectable red-shift in the absorption maxima of the dyes, and 10 μg/mL produced the maximum shift. Similarly, low concentrations of cetyltrimethylammonium bromide produced major blue-shifts in the absorption maxima. Consideration of the data as a whole can help avoid misinterpretation. Except where purified samples were used, the significance of small spectral changes is doubtful.

For spectroscopic studies, a polysaccharide concentration (1 mg/mL) known to produce saturation dye-binding with cereal β-D-glucan[32] was chosen. A reduced spectral-shift or fluorescence-intensity decrease, which will be referred to as decreased interaction, may reflect decreased dye-binding or a different mode of binding and environment for the dye. Quantitative, dye-binding studies would be required to make this distinction. This report indicates those polysaccharides that merit such further attention.

The naturally occurring mixed-linkage, (1→3)- and (1→4)-β-D-glucans had similar effects on the absorption and fluorescence spectra of the dyes, and all showed a definite precipitation-reaction. Testing of reduced pneumococcus polysaccharide Type III was hindered by low solubility and the small amount of sample available. However, this polysaccharide containing alternating (1→3)- and (1→4)-β-D-gluco-

pyranosyl units showed considerably reduced interaction with both dyes. It seems likely that this difference is related to sequence rather than ratio of linkages. The (1→3)-β-D-glucans (curdlan, laminaran) showed a small interaction with Calcofluor but strong interaction with Congo Red. The neutral (1→4)-β-D-glucans (hydroxyethylcellulose Natrosol 250 M and tamarind amyloid) induced major red-shifts in both dyes. Tamarind amyloid produced the greatest shifts and changes in fluorescence intensity. The interaction, as shown by spectral changes, therefore seems dependent both upon the proportion and distribution of (1→3)- and (1→4)-β-D-glucopyranosyl units.

The difference in interaction shown by tamarind amyloid and hydroxyethylcellulose Natrosol 250 M may depend on the different degrees of substitution of the cellulosic backbone. Hydroxyethylcellulose Natrosol 250 M (degree subst. 2.5) has on average more than one substituted hydroxyl group per D-glucose unit, whereas tamarind amyloid contains somewhat less than one substituent per D-glucose unit[33]. By use of hydroxyethylcelluloses of different degrees of substitution, it can be shown that the samples with the higher degree of substitution show less interaction[32].

The results with carboxymethylcellulose 7H3 SFX demonstrate an inhibition of interaction, probably as a result of Coulombic repulsion of the anionic dyes by the polyanion. Use of higher ionic-strength buffer partially overcame this effect. Dye binding by oat β-D-glucan was also increased by increasing the ionic-strength of the medium[32]. This may reflect anticooperative binding as a result of Coulombic repulsion effects, but other factors, such as state of aggregation of the dye, could play a part. In these studies, a higher ionic-strength medium was sometimes used in order to enhance possible interactions, and to counteract charge-effects from the polysaccharide. Unfortunately, in the case of xanthan gum, the salt necessary to overcome Coulombic repulsion favors an ordered secondary structure in the molecule[34], which may not be favorable for dye interaction. Turbidity of solution prevented absorption spectroscopy measurements, but no interaction, as judged by fluorescence spectroscopy, was observed in water or 0.12M sodium chloride at 25 or 50°. Xanthan gum was, thus, the only polymer known to contain contiguous (1→4)-β-D-glucopyranosyl units that did not show a strong interaction with Calcofluor. Following removal of the pyruvate residues, slight interaction with Congo Red was observed.

Of the wood hemicelluloses tested, galactoglucomannan A and glucomannan both showed definite interaction with Congo Red, and galactoglucomannan B showed slight interaction. Structural studies of these polymers have shown the presence of contiguous (1→4)-β-D-mannopyranosyl but not of contiguous (1→4)-β-D-glucopyranosyl units[35]. However, the (1→4)-β-D-mannans tested (guar gum, locust-bean gum, tara gum) did not show significant interaction with either dye. The significance of the small interaction shown by the two glucuronoxylans is not certain. Acid hydrolysis and paper chromatography did not reveal the presence of contaminating glucan.

The slight interactions shown by pullulan and elsinan are probably similar

to the known[9] interactions with starch. Starch did not detectably interact with Calcofluor.

The apparent interaction of wheat and barley pentosans was of interest because of the potential importance of analytical and histochemical applications of the dye interactions in studies of cereal β-D-glucans. Both pentosans were contaminated by a glucan. This and the dye interaction were removed from the wheat pentosan by digestion with β-D-glucanase. However, traces of glucan remained in the sample of barley pentosan, even after two successive treatments with enzyme. The major red-shift with Congo Red and a very small shift with Calcofluor that persisted with the enzyme-digested barley pentosan are not typical of the cereal β-D-glucans, but are more typical of (1→3)-β-D-glucans. The presence of (1→3)-β-D-glucan in barley has been suggested by histochemical studies[3,6]. The interaction could, of course, depend on the pentosan itself. The D-glucanase enzyme, which alone did not interact with the dyes, was active against oat β-D-glucan, and the degraded polymer showed very little dye-interaction.

Cellobiose, and a mixture of cellotriose and cellotetraose showed no interaction, even at a concentration of 20 mg/mL. Tests of pure fractions of trisaccharide, tetrasaccharide, and pentasaccharide indicated a slight interaction with the pentasaccharide. Since purified samples were used, the small spectral-shifts and increase of fluorescence intensity are probably significant. The results with fractions from Bio-Gel P-2 chromatography appear to confirm that a minimum d.p. of 5 is necessary for interaction. This indicates that it is the extended configuration of the polysaccharide chain rather than the configuration of the unit which is important for dye binding.

Nevertheless, it is the configuration of the units that controls conformation, and some of the structural features required for dye interaction are suggested by the present study. The results with elsinan, similar to cereal β-D-glucans but containing (1→3)- and (1→4)-α-D-glucopyranosyl units, show the importance of the anomeric configuration. The lack of interaction of *W. pyramidata* and *C. erinaceum* xylan shows the importance of CH_2OH-6. The structure of the latter xylan is so similar to that of cereal β-D-glucans that its lack of interaction was somewhat surprising. Analysis revealed the presence of a contaminating galactose component, but xylose was the major constituent sugar.

The importance of the configuration of both OH-4 and -2 is indicated by the lack of interaction of the D-galactan, arabino-D-galactan [(1→4)- and (1→3)-β-D-linked galactose units], and D-galacto-D-mannans [(1→4)-β-D-mannan].

The existence of favored conformation in polysaccharides suggests the possibility of specific, or conformation-related, ligand-binding. The required conformation could be that present naturally, or ligand-induced. The interaction of curdlan with Congo Red[11,12] appears to be dependent on the presence, or induction, of an ordered state, and similarly with amylose complexes. It is certainly clear that conformational changes take place on dye-binding, resulting in changes of physical properties such as viscosity[6,17] and solubility. It is interesting to note that decreasing the polarity of

the medium by addition of ethanol or 1,4-dioxane produced spectral changes in the dyes similar to those shown by the β-D-glucans. Dye-binding may therefore occur in regions of decreased polarity resulting from the particular conformation of the polysaccharide[37].

The strongest-interacting (1→4)-β-D-glucans belong to the A-type, an extended, ribbon-like, conformational class described by Rees and Scott[38], but so do the non-interacting mannans and xylans. Amylose and (1→3)-β-D-glucans which show interaction with Congo Red are of the B-type, a flexible helix.

Polyvinyl alcohol forms gels in the presence of diazo and stilbene-type, direct dyes, and the evidence suggests that conformational changes in the polymer result in polymer–polymer interactions which are responsible, at least in part, for the gelation[39,40]. Polyvinyl alcohol possesses considerable conformational flexibility compared to polysaccharides, and it is possible that both conformationally flexible and selected, conformationally more restricted, polyhydroxy polymers can interact with direct dyes. The present data, however, do not allow any conclusions other than that there is toward certain types of polysaccharide, a high degree of specificity, which is related in some fashion to the configuration of the monomeric units and the linkages joining them.

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REFERENCES

1 T. Vickerstaff, *The Physical Chemistry of Dyeing*, 2nd edn., Interscience, New York, 1954, pp. 167–190.
2 J. E. Scott, G. Quintarelli, and M. C. Dellovo, *Histochemie*, 4 (1964) 73–85.
3 A. L. Stone and D. F. Bradley, *Biochim. Biophys. Acta*, 148 (1967) 172–192.
4 M. D. Young, G. O. Phillips, and E. A. Balazs, *Biochim. Biophys. Acta*, 141 (1967) 374–381.
5 J. E. Scott and J. Dorling, *Histochemie*, 5 (1965) 221–233.
6 P. J. Wood and R. G. Fulcher, *Cereal Chem.*, 5 (1978) 952–966.
7 H. Maeda and N. Ishida, *J. Biochem. (Tokyo)*, 62 (1967) 276–278.
8 B. J. Harrington and K. B. Raper, *Appl. Microbiol.*, 16 (1968) 106–113.
9 B. Carroll and H. C. Cheung, *J. Phys. Chem.*, 66 (1962) 2585–2591.
10 J. A. Trisnadi, H. M. Boessler, and R. C. Schulz, *Colloid Polym. Sci.*, 252 (1974) 222–233.
11 K. Ogawa, J. Tsurugi, and T. Watanabe, *Carbohydr. Res.*, 29 (1973) 397–403.
12 K. Ogawa and M. Hatano, *Carbohydr. Res.*, 67 (1978) 527–535.
13 H. Kondo, H. Nakatani, and K. Hiromi, *Carbohydr. Res.*, 52 (1976) 1–10.
14 H. Nakatani, K.-I. Shibata, H. Kondo, and K. Hiromi, *Biopolymers*, 16 (1977) 2363–2370.
15 H. B. Edwards and J. K. Thomas, *Carbohydr. Res.*, 65 (1978) 173–182.
16 G. Centola, *Ric. Sci.*, 11 (1940) 905–906.
17 I. Jullander, *Ind. Eng. Chem.*, 49 (1957) 364–368.
18 G. R. Savur, *Indian Text. J.*, 66 (1956) 309–311.
19 A. E. Clarke and B. A. Stone, *Phytochemistry*, 1 (1962) 175–188.
20 P. E. Jansson, L. Kenne, B. Lindberg, and H. Ljunggren, *Int. Symp. Carbohydr. Chem., 9th*, (1978) G-21.
21 G. G. S. Dutton and M.-T. Yang, *Can. J. Chem.*, 51 (1973) 1826–1832.

22 G. G. S. DUTTON, K. L. MACKIE, AND M.-T. YANG, *Carbohydr. Res.*, 65 (1978) 251–263.
23 Y.-M. CHOY, G. G. S. DUTTON, AND A. M. ZANLUNGO, *Can. J. Chem.*, 51 (1973) 1819–1825.
24 G. M. BEBAULT, G. G. S. DUTTON, N. A. FUNNELL, AND K. L. MACKIE, *Carbohydr. Res.*, 63 (1978) 183–192.
25 G. G. S. DUTTON AND K. L. MACKIE, *Carbohydr. Res.*, 55 (1977) 49–63.
26 G. M. BEBAULT AND G. G. S. DUTTON, *Carbohydr. Res.*, 64 (1978) 199–213.
27 M. L. WOLFROM AND A. THOMPSON, *Methods Carbohydr. Chem.*, 3 (1963) 146–147.
28 T. S. STEWART, P. B. MENDERSHAUSEN, AND C. E. BALLOU, *Biochemistry*, 7 (1968) 1843–1854.
29 J. F. KENNEDY AND W. R. BUTT, *Biochem. J.*, 115 (1969) 225–229.
30 M. DUCKWORTH AND W. YAPHE, *Chem. Ind. (London)*, (1970) 747–748.
31 F. G. VILLAUME, American Cyanamid Company Ltd., personal communication.
32 P. J. WOOD, *Ind. Eng. Chem., Prod. Res. Der.*, 19 (1980) 19–23.
33 H. C. SRIVASTAVA AND P. P. SINGH, *Carbohydr. Res.*, 4 (1967) 326–342.
34 G. HOLZWARTH, *Biochemistry*, 51 (1976) 4333–4339.
35 T. E. TIMELL, *Adv. Carbohydr. Chem.*, 20 (1965) 408–483.
36 R. G. FULCHER, G. SETTERFIELD, M. E. MCCULLY, AND P. J. WOOD, *Aust. J. Plant Physiol.*, 4 (1977) 917–928.
37 D. A. REES, *M.T.P. Int. Rev. Sci., Biochem. Ser. One, Biochem. Carbohydr.*, 5 (1975) 1–42.
38 D. A. REES AND W. E. SCOTT, *J. Chem. Soc., B*, (1971) 469–479.
39 C. DITTMAR AND W. J. PRIEST, *J. Polym. Sci.*, 18 (1955) 275–298.
40 H. BELTMAN AND J. LYKLEMA, *Faraday Discuss. Chem. Soc.*, 57 (1974) 92–100.