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FACTORS AFFECTING PRECIPITATION AND SPECTRAL CHANGES ASSOCIATED WITH COMPLEX-FORMATION BETWEEN DYES AND β-D-GLUCANS*†

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ABSTRACT

The formation of complex between β-D-glucans, in particular oat β-D-glucan, and the dyes Calcofluor White M2R New (Calcofluor) and Congo Red was investigated. Oat β-D-glucan could be quantitatively precipitated by Calcofluor. The degree of precipitation was influenced by initial concentration of oat β-D-glucan, amount of Calcofluor added, and ionic strength of solution. Complex formation with as little as 0.5 μg/mL of β-D-glucan could also be detected by changes in the absorption and fluorescence spectra of the dyes. The effect of concentration of oat β-D-glucan on dye binding, as indicated by bathochromic shifts in the absorption spectra and increase in fluorescence intensity, was studied. Precipitation interfered with quantitative measurements, but there was a linear relationship between absorbance at λ_{max} of the difference spectra and concentration of oat β-D-glucan between 0 and 5 μg/mL in carbonate buffer pH 10.0, ionic strength 0.2. Precipitates formed more readily with Congo Red than Calcofluor, and occurred with as little as 1–2.5 μg/mL of oat β-D-glucan in the presence of salts. The interaction of anionic polysaccharides, and starch and β-(1→3)-D-glucan dissolved in dilute alkali, was considerably enhanced in the presence of salt. The interaction of oat β-D-glucan was also increased by increasing the ionic strength of the solution, but was inhibited by high concentrations of sucrose and was not detectable in 7M urea.

INTRODUCTION

Such direct or cellulose substantive dyes as Calcofluor and Congo Red are useful, in microscopic studies, as stains for plant and fungal cell-walls[1,2]. Uptake of these dyes would of course be expected for cellulose-containing cell-walls, although a more general histochemical affinity for β-linked polysaccharides has been claimed[3,4]. Such claims were based on visual estimation of dye adsorption from solution onto undis-

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solved polysaccharides. Reports that barley endosperm cell-walls stained intensely with Calcofluor[5] and contained considerable amounts of the mixed linkage (1→3 and 1→4) β-D-glucan, but very little cellulose[6], suggested that the mixed linkage β-D-glucan might interact with Calcofluor. Investigation of this possibility led to the observation that oat and barley β-D-glucan were precipitated from solution by Calcofluor and Congo Red[7], and this has allowed new and specific methods of analysis of these polysaccharides[11,18]. Below concentrations at which precipitation occurred, the cereal endospermic β-D-glucans induced major bathochromic (red) shifts in the absorption maxima of both dyes, changed the fluorescence spectra of Calcofluor from a single to a double peak, and increased the fluorescence intensity from four- to seven-fold[8]. Investigation of a variety of polysaccharides showed that major interactions of this nature were only shown by polysaccharides known to contain contiguous β-(1→4)-linked D-glucopyranosyl residues[8], although other polysaccharides showed some aspects of this behavior, for example a β-(1→3)-D-glucan (curdlan) produced a major red shift in the spectra of Congo Red. Alkali was required to dissolve this latter polysaccharide and we have since observed interaction with Calcofluor if sodium chloride (M) is added to the solution.

I now report further on aspects of the complex formation between β-D-glucans, in particular oat endospermic β-D-glucan, and Congo Red and Calcofluor. These two dyes are representative, respectively, of the azo-type and stilbene-type of dye with affinity for cellulose.

EXPERIMENTAL

General methods. — Oat β-D-glucan (98% glucan, 0.13% nitrogen, 0.24% ash) was prepared from crude oat gum (provided by R. Hyldon, Quaker Oats Co., Barrington, Illinois) by two successive precipitations with ammonium sulfate and precipitation with 2-propanol. In one experiment, a similarly prepared oat β-D-glucan (98% glucan, 0.83% nitrogen, 0.39% ash) was also used, but this was from an oat gum of considerably higher viscosity, made in this laboratory from the cultivar Hinoat. Congo Red (C.I. 221120) was purchased from Canadian Laboratory Supplies Ltd. and Calcofluor White M2R New {{C.I. 40622; C.I. Fluorescent Brightener 28; major active ingredient, the disodium 4,4'-bis{4-anilino-6-[bis(2-hydroxyethyl)-amino]-1,3,5-triazin-2-yl}amino-2,-2'stilbenedisulfonate}} was a gift from the American Cyanamid Co., Bound Brook, N.J., U.S.A. and was quoted as containing 70% active component (F. G. Villaume, personal communication). Calcofluor undergoes *trans–cis* isomerization when exposed to light, and solutions were therefore protected from light.

Other polysaccharides studied were gifts or were from commercial sources[8]. Rapeseed hot-water extract was prepared from dehulled, defatted and 80% ethanol extracted *β campestris* (Echo) rapeseed, essentially as described by Siddiqui and Wood[9]. The hot-water-soluble material was isolated by lyophilization.

Absorption spectra were obtained with a Unicam SP-1800 or Beckman Model

26 spectrophotometer, and fluorescence spectra (uncorrected) on a Hitachi Perkin Elmer MPF-2A spectrofluorimeter.

Precipitation reaction. — Solutions (~0.1–1%, w/v) of oat β-D-glucan were prepared by "wetting" with ethanol and heating and stirring in water, phosphate buffer pH 7.0 ionic strength (I) 0.05 or 0.2, or carbonate buffer pH 10, I 0.2. Solutions were clarified by centrifugation and the supernatant solutions (4 vol) thoroughly and rapidly mixed with solutions of Calcofluor (1 vol) in the appropriate buffer to give final Calcofluor concentrations of 0.01–0.2%. After 1 h at 25°, the mixture were centrifuged and the supernatant solutions analyzed for β-D-glucan by a modified cysteine–sulfuric acid reaction[10]. Neither dye nor buffers significantly interfered with the assay at the concentrations used. The amount of β-D-glucan precipitated was calculated by difference. The concentrations of β-D-glucan quoted will be those present initially (before mixing). The concentrations of Calcofluor quoted will be those which would have been finally obtained if precipitation had not occurred. (The precipitated complex strongly adsorbs Calcofluor from solution).

Absorption and fluorescence spectroscopy. — Oat β-D-glucan solutions were clarified by centrifugation and appropriate aliquots rapidly mixed with buffer and dye to give final concentrations of 0.5–1000 μg/mL of polysaccharide and 10 μg/mL of dye. Portions of the solutions were used to record absorption and fluorescence spectra, using the appropriate buffer or, in the case of difference spectra, the dye alone, as a blank.

Other polysaccharides [*O*-(carboxymethyl)pachyman, *O*-(carboxymethyl)cellulose, curdlan, and potato starch] were tested using solutions (0.1% w/v), clarified by centrifugation, and containing dye (10 μg/mL) in the presence and absence of M sodium chloride. Rapeseed hot-water extract (0.1% w/v) was tested in phosphate buffer pH 7.0, I 0.05.

Fluorescence spectra were recorded with similarly prepared solutions. It should be noted that Calcofluor's light sensitivity resulted in a decreasing fluorescence intensity during scans of emission or excitation spectra, despite the use of narrow excitation bandwidths. The emission spectra shown were recorded with excitation at 350 nm.

RESULTS AND DISCUSSION

It was previously reported that oat β-D-glucan can be quantitatively precipitated by Calcofluor from solutions (0.7–0.9% w/v) in water or dilute buffers (pH 6–10)[7]. However at lower initial concentrations of oat β-D-glucan (0.09% w/v) the shape of the plot of (percent β-D-glucan precipitated) against (concentration of Calcofluor) was changed (Fig. 2 and ref. 7) and quantitative precipitation was not achieved. This was not promising from the point of view of developing methods for isolation of oat β-D-glucan as the concentration in crude extracts of oat flour is normally <0.7% w/v.

Results from further studies of the effect of initial β-D-glucan concentration and ionic strength are summarized in Figs. 1 and 2. At both ionic strength 0.05 and

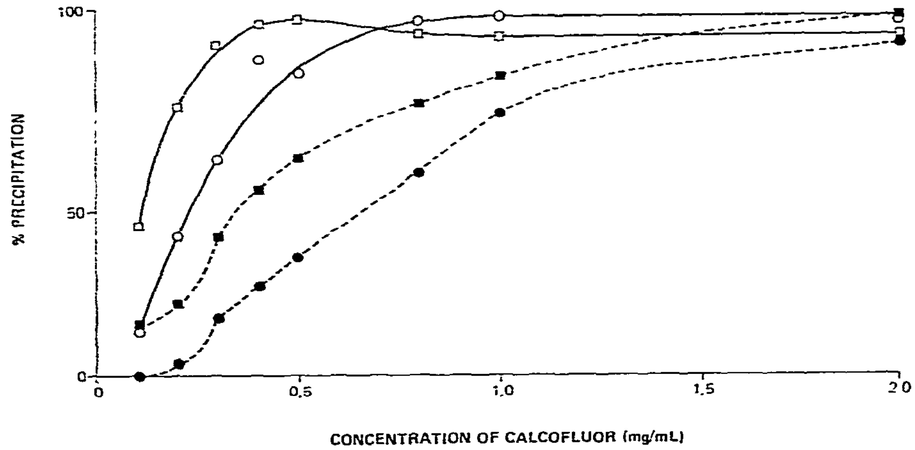

Fig. 1. Precipitation of oat β-D-glucan by Calcofluor in phosphate buffer pH 7.0, I 0.05 (0.3% □—□; 0.6% ○—○) and phosphate buffer pH 7.0, I 0.2 (0.3% ■—■; 0.6% ●—●).

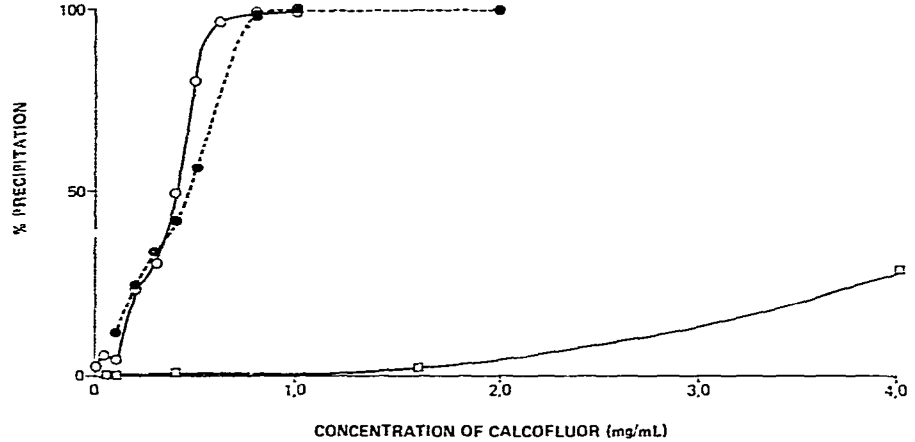

Fig. 2. Precipitation of oat β-D-glucan by Calcofluor in phosphate buffer pH 7.0, I 0.2 (0.8% ○—○), carbonate buffer pH 10.0, I 0.2 (0.12% ●—●), and water (0.09%, □—□).

0.2, the more concentrated β-D-glucan solution (0.6% w/v) required more Calcofluor to produce the same degree of precipitation, and precipitation occurred less readily in the buffer of higher ionic strength, in agreement with the previous observation that M sodium chloride inhibited precipitation[7] (Fig. 1). Despite this, as shown in Fig. 2, quantitative precipitation of low concentrations of oat β-D-glucan was achieved by use of the higher ionic-strength buffers, at both pH 7.0 and 10.0. The enhancement of precipitation of dilute solutions of oat β-D-glucan in the presence of these buffers, as compared to water, is in apparent contradiction to the inhibitory effect of salt at higher concentrations. Studies with some intermediate concentrations and ionic strengths gave irreproducible results.

Although the present report deals with the precipitation of oat β-D-glucan by Calcofluor, it is clear that other dyes, such as Congo Red, and other β-D-glucans important from the nutritional or industrial point of view, may be studied in this fashion[7,8]. As anticipated by the results in Fig. 2, it was possible to use precipitation by Calcofluor to isolate oat β-D-glucan directly from an alkaline extract of oat flour[11]. Somewhat laborious procedures had previously been required to obtain material similarly free from pentoglycosan and protein.

Complex formation between β-D-glucan and dye may be detected by changes in the absorption and fluorescence spectra of the dyes[8,11]. In general, increasing the ratio of β-D-glucan: dye induced red shifts and increased the extinction coefficient at λ_{max} (Figs. 3 and 4), and this was conveniently monitored by plotting concentration of oat β-D-glucan against absorbancy at λ_{max} of the difference spectra (~380 nm for Calcofluor, as shown in Fig. 5). In buffer, saturation values were obtained at lower concentrations of β-D-glucan than in water. Unfortunately, as demonstrated in Fig. 4, spectrophotometric measurements were subject to interference from formation of precipitates, and at higher β-D-glucan concentrations, absorbancy curves were frequently displaced from isosbestic points, even when, as with Calcofluor, no precipitate was evident. Precipitate and adsorbed dye could be centrifuged and the degree of precipitation then judged by measuring loss of dye from the supernatant solution. The amount of precipitate, which was generally much less with Calcofluor than with Congo Red, tended to pass through a maximum at intermediate β-D-glucan concentration. Thus, for example, with Congo Red in phosphate buffer pH 7.0, ionic strength 0.05, there was very little precipitate at 1000 μg/mL, and 0–5 μg/mL, but considerable precipitate at 50–250 μg/mL. In phosphate buffer pH 7.0, ionic strength 0.05, containing M sodium chloride or M sodium sulfate, precipitation was observed with Congo Red at 1 μg/mL of β-D-glucan, and was maximum at 2.5–5.0 μg/mL. (Congo Red is unstable in high salt concentrations, but this precipitate is readily distinguishable from the complex precipitate). Since precipitation is dependent upon glucan and dye concentration, it is important to mix solutions rapidly for reproducible results. The spectra of samples in which dye was layered onto polysaccharide solution before mixing differed from the spectra of rapidly mixed solutions. The formation of precipitates, the differences observed with different modes of mixing, and the departure of spectra from isosbestic points, suggest that the interaction may not be reversible, and that more than one form of the bound dye is present. Thus previous attempts[11] to estimate free and bound dye are probably in error. Despite this, some qualitative observations were possible. Dye binding, as shown by spectral shifts, was observed in phosphate pH 7.0, carbonate pH 10.0, maleate pH 6.7, 2-(*N*-morpholino)ethanesulfonic acid (MES) pH 6.5, borate pH 10.0, and water. Salts (sodium chloride or sodium sulfate, used in phosphate buffer, pH 7.0, ionic strength 0.05) enhanced dye binding, but sucrose (20% w/v) inhibited binding. Binding was less in borate than the other buffers, and no binding was observed in 7M urea.

In carbonate buffer, pH 10.0, ionic strength 0.2, Calcofluor showed little or no precipitate and an isobestic point was observed at 350–355 nm for concentrations of

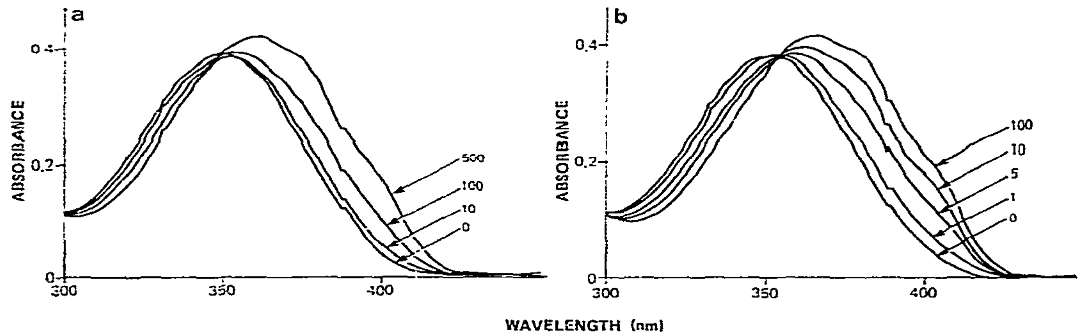

Fig. 3. Absorption spectra of Calcofluor (10 μg/mL) alone (0) and in the presence of oat β-D-glucan in a, water; b, carbonate buffer pH 10.0, I 0.05. Concentrations (μg/mL) of oat β-D-glucan are as marked.

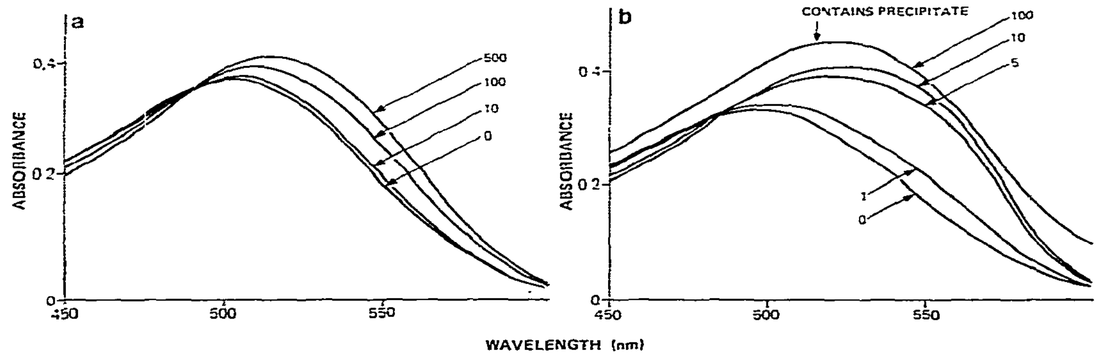

Fig. 4. Absorption spectra of Congo Red (10 μg/mL) alone (0) and in the presence of oat β-D-glucan in a, water; b, carbonate buffer pH 10.0, I 0.05. Concentrations (μg/mL) of oat β-D-glucan are as marked.

β-D-glucan up to 10 μg/mL. An isosbestic point (~490 nm) was also observed over the same concentration range, in the Congo Red spectra, although some precipitation was evident at 10 μg/mL. Plots of absorbancy (at least duplicate determinations) at λ_{max} of the difference spectra against β-D-glucan concentration are shown for Congo Red and Calcofluor in Fig. 6. Two different preparations of oat β-D-glucan were used and the responses of the two preparations were similar, suggesting that the system might be useful for assay of oat β-D-glucan, although the narrow linear range (0.1 absorbancy units and 0–5 μg/mL of β-D-glucan) makes the method subject to error. Carbonate buffer pH 10, ionic strength 0.2 is, however, a useful medium for extraction of oat β-D-glucan, and we have used this system to demonstrate that there was no detectable β-D-glucan in the feces of animals fed diets high in oat β-D-glucan, which confirmed histochemical observations (R. G. Fulcher and P. J. Wood, unpublished observations). In the case of cereal extracts, only the mixed linkage β-D-glucan interacts with the dyes (P. J. Wood, R. G. Fulcher, and B. A. Stone, unpublished results, and ref. 8), and so the procedure is specific and, although difficult to mani-

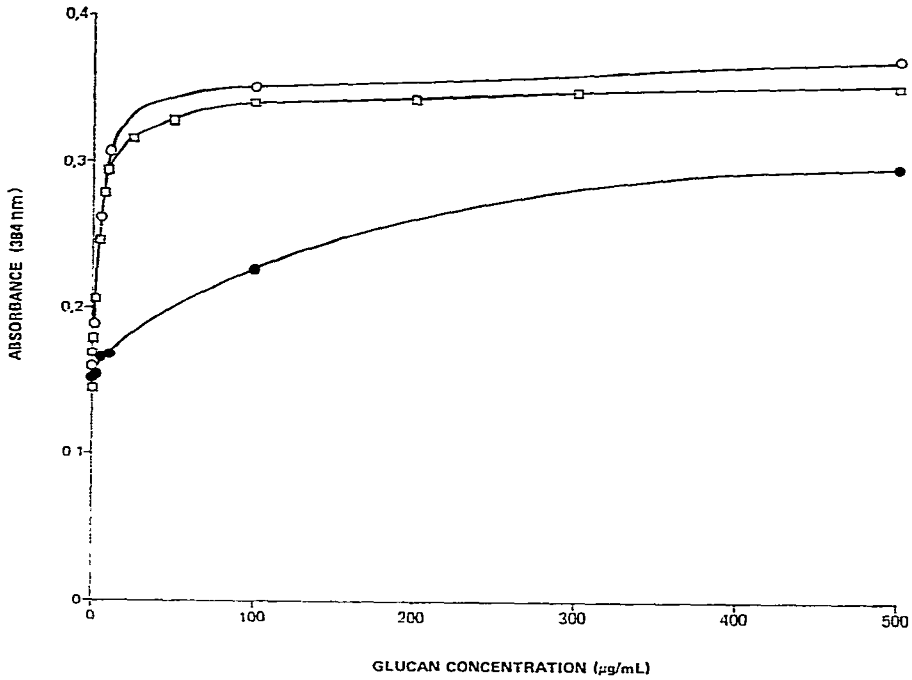

Fig. 5. The effect of concentration of oat β-D-glucan on absorbance at 384 nm of Calcofluor (10 μg/mL) in water (●—●), carbonate pH 10.0, I 0.05 (○—○), and phosphate pH 7.0, I 0.05 (□—□).

pulate quantitatively, is a useful and sensitive "spot" test, with detectable wavelength shifts induced by as little as 0.5 μg/mL oat β-D-glucan. Other β-D-glucans not present in cereals, such as xyloglucans and substituted celluloses, similarly interact[8] and thus may be detected in food or plant extracts. As an example, Fig. 7 shows the effect of a hot-water extract from rapeseed flour on the absorption spectra of Congo Red. Characteristic spectral shifts, and changes in fluorescence spectra were also observed with Calcofluor. The spectral changes presumably arise from rapeseed "amyloid", known to be present in hot-water extracts from rapeseed flour[9]. The presence of this component in the cell walls of the seed can be demonstrated histochemically.

The fluorescence intensity of both Calcoflour and Congo Red was markedly increased in the presence of oat β-D-glucan. The single rather broad, asymmetric emission peak of Calcofluor alone (λ_{max} 430 nm) characteristically split into two peaks at about 420 and 440 nm in the presence of β-D-glucan, and the response to β-D-glucan showed an enhancement, relative to water, in the presence of buffer (Fig. 8). Congo Red is a diazo dye and thus normally exhibits only a weak fluorescence, and so the enhancement of fluorescence in solution is an interesting confirmation of histochemical results, which demonstrate intense red fluorescence in oat endosperm cell-walls and lesser staining in the aleurone cell-walls. Development of assay techniques based on fluorescence measurements are in progress.

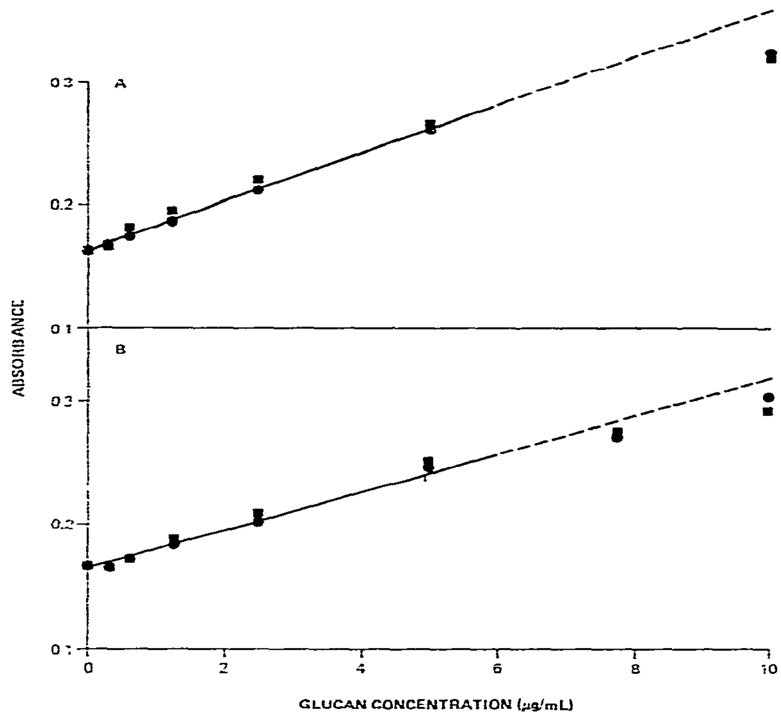

Fig. 6. Effect of oat β-D-glucan concentration on absorbance (A), at 384 nm (Calcofluor, 10 μg/mL) and (B) at 540 nm (Congo Red, 10 μg/mL) in carbonate buffer, pH 10, I 0.2. (●, Hinoat oat β-glucan; ■ Quaker Oat β-glucan).

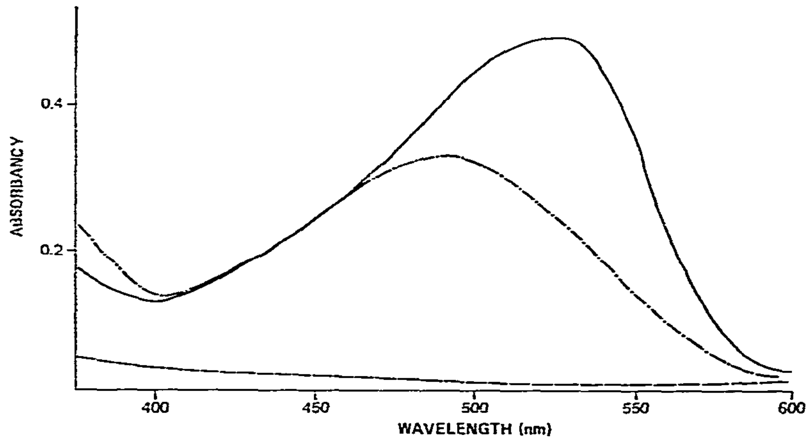

Fig. 7. Effect of rapeseed hot-water extract (1 mg/mL) on the absorption spectra of Congo Red (10 μg/mL) in phosphate buffer pH 7.0: Congo Red alone, –·–·–·–; rapeseed extract alone, ———; Congo Red plus rapeseed extract, ———.

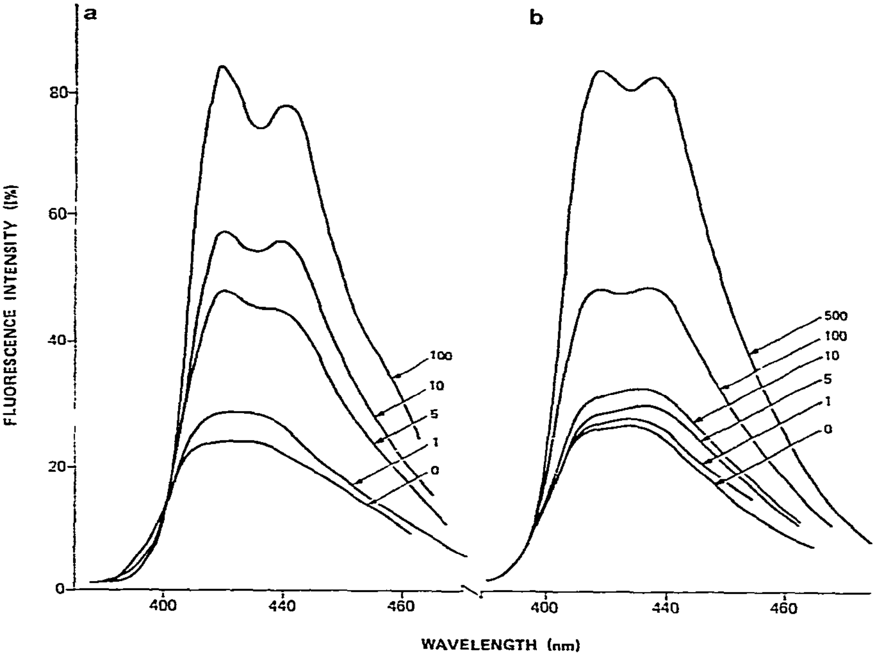

Fig. 8. Fluorescence emission spectra of Calcofluor (10 μg/mL) alone (0) and in the presence of oat β-D-glucan in a, carbonate buffer pH 10.0, I 0.05; b, water. Concentration (μg/mL) of oat β-D-glucan are as marked.

The results described here are mainly of a qualitative nature and do not allow definite conclusions regarding the nature of the dye–polysaccharide complex, or explanations for some of the phenomena observed. However, some tentative interpretations are possible. As reported previously[8] the specificity of the interaction may be dependent upon conformational factors, and the wavelength shifts and changes in fluorescence spectra on binding are similar to those which occur in decreasing polarity of solvent. It is known that ordered conformations of polysaccharides may possess defined regions of differing polarity[13], and ligand binding may occur in regions of decreased polarity. This is known for amylose inclusion complexes, and may also be true for Congo Red interaction with (1→3)-β-D-glucan, where an ordered conformation in the polysaccharide was necessary for binding[14] (although the nature of the binding forces involved was not clear). In the case of the more flexible polymer, poly(vinyl alcohol), it was suggested that the interaction with Congo Red (similar to that for β-D-glucans) was dependent upon a dye-induced order in the polymer chain[15,16].

The prime requisite for interaction would appear to be an ordered conformation in the polymer, either indigenous or induced. For polyanions, the more compact, ordered form is favored when electrostatic repulsions are reduced by charge screening from the presence of salt. Thus O-(carboxymethyl)pachyman and O-(carboxymethyl)-cellulose both showed increased interaction with Congo Red and Calcofluor in the

TABLE I

THE EFFECT OF M SODIUM CHLORIDE ON DYE–POLYSACCHARIDE INTERACTIONS

Polysaccharide	*Medium*	$\Delta(\lambda_{max})$ *C.R.*[a]	*Cal.*[a]	*R.F.I.*[a,b]
CMC[a]	H_2O	0	0	0.9
CMC	M NaCl	35	14, 27[c]	4.4
CMP[a]	H_2O	0	1	1.0
CMP	M NaCl	39	12	3.5
Curdlan	0.05M NaOH	26	0	0.8
Curdlan	0.05M NaOH + M NaCl	39	9	2.9
Potato starch	0.05M NaOH	10	1	0.7
Potato starch	0.05M NaOH + M NaCl	20	0	1.0
Oat β-D-glucan	0.05M NaOH	10	4	1.6
Oat β-D-glucan	0.05M NaOH — M NaCl	6[d]	13	3.4

[a] Abbreviations: C.R., Congo Red; Cal., Calcofluor; R.F.I., relative fluorescence intensity; CMC, *O*-(carboxymethyl)cellulose 7H3 SXF; CMP, *O*-(carboxymethyl)pachyman. [b] The relative fluorescence intensity is the ratio of fluorescence intensity of Calcofluor plus sample/fluorescence intensity of Calcofluor alone. [c] Two peaks. [d] Precipitate formation.

presence of salt (Table I). Similary oat β-D-glucan, starch, and curdlan [a (1→3)-β-D-glucan] showed increased interaction in dilute sodium hydroxide in the presence of salt, presumably because hydroxyl groups in the polysaccharides were ionized by the alkali. Starch, however, did not induce spectral shifts in Calcofluor under any of the conditions tested, suggesting other (probably steric) requirements for interaction.

If binding is a consequence, in part at least, of partition of dye into regions of decreased polarity (such as the hydrophobic interior of the polysaccharide helix) then addition of salt would favor dye adsorption, whereas sucrose would decrease adsorption. The effect of salt on dye binding with neutral polysaccharides at neutral pH (oat β-D-glucan, barley β-D-glucan, *O*-(hydroxyethyl)cellulose, and xyloglucan[8,10]) may therefore be an effect of solvent polarity or, possibly, charge screening of initially bound dye. These factors all require enhancement of dye binding with increased salt concentration, and yet precipitation of oat β-D-glucan at 0.3–1% concentration was inhibited by salt. This result suggests that precipitation is a secondary reaction taking place following dye binding and that more than one precipitation mechanism may operate. In the absence of dye, solutions of oat β-D-glucan were stable, showing only small amounts of precipitate on being kept.

This, and previous reports[7,8,11], indicate some of the factors influencing the interaction of Congo Red and Calcofluor with polysaccharides, and place previous histochemical reports in a new perspective. Development of new methods for assay of certain β-D-glucans and β-D-glucanases[17] are in progress.

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