INTERACTION OF SOME DYES WITH CEREAL β-GLUCANS[1]

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ABSTRACT   Cereal Chem. 55(6): 952-966

Low concentration of the dyes Calcofluor White M2R New, Tinopal CBS-X, and Congo Red cause preferential precipitation of β-glucan from aqueous solutions of oat gum containing protein, pentosan, and starch. Tests with a number of polysaccharides indicate a specificity for β-1,4-linked glucopyranosyl units. The precipitation with Calcofluor occurs between pH 6 and 10 and is not appreciably affected by temperature (5 and 25° C), but the precipitate dissolves at elevated temperatures. Salt suppresses the precipitation. Viscosity measurements and absorption spectrophotometry afford other evidence for specific dye-polysaccharide complex formation. The dyes concerned are useful in microscopic studies of oat and barley endosperm cell walls, and can be used to observe the changes that occur during extraction of oat gum.

The optical brightener Calcofluor White M2R New, the major active ingredient of which is the disodium salt of 4,4'-bis[4-anilino-6-[bis(2-hydroxyethyl)amino] -s-triazin-2-yl]amino]-2,2'-stilbenedisulfonic acid (compound I), is a fluorescent dye used commercially as a whitening agent and found to be a useful fluorochrome for microscopic studies (1,2). Particularly intense fluorescence has been noted for barley endosperm cell walls (2,3) in which the major component (4) is a linear mixed linkage β-glucan composed of β-1,4- and β-1,3-glucopyranosyl units in the approximate ratio of 7:3 (5,6).

In general, there appears to be a specificity for β-linked glucans (7,8), but these studies have been directed mainly toward the histochemical value of the dye and reactivity toward substrates has been determined by visual estimation of adsorption from solution onto undissolved polysaccharides. To our knowledge, no studies have been done to date of the behavior of solutions of polysaccharides with this dye, although an effect on the viscosity of solutions of substituted celluloses is known (9).

In this article, we describe a relatively specific precipitation reaction between the mixed linkage β-glucan of oats and barley and Calcofluor White M2R New,

and some other cellulose substantive dyes, such as Congo Red, also known to be a useful stain for plant cell walls. The value of the interaction in microscopic studies of oat and barley cell walls is demonstrated.

MATERIALS AND METHODS

R. Hyldon, of The Quaker Oats Co., Barrington, IL, provided oat gum prepared by alkaline extraction from rolled oats. Acid hydrolysis and paper chromatography revealed the presence of major amounts of glucose, lesser amounts of arabinose and xylose, and traces of galactose. The minor polysaccharide constituents and most of the contaminating protein were removed in two successive precipitations with 20−25% w/v ammonium sulfate, to give oat β-glucan.

American Cyanamid Company, Bound Brook, NJ, provided Calcofluor White M2R New (Calcofluor); Ciba-Geigy Ltd., Dorval, Que., Tinopal CBS-X (Tinopal); and W. Przybylski, Health and Welfare Canada, water-soluble food dyes (permitted in Canada). Other dyes were obtained commercially. Carbohydrates were obtained commercially except for tamarind amyloid, which was a crude preparation precipitated by ethanol from an aqueous extract of tamarind seed—carboxymethyl-pachyman—prepared by the method of Clarke and Stone (10), and barley β-glucan, which V. M. Bendelow (Agriculture Canada, Winnipeg Research Station), provided. β-Glucan was estimated as previously described (11). In quantitative studies of the precipitation of oat β-glucan, the glucan concentrations of all solutions were determined by the modified cysteine-sulfuric acid reaction (11); otherwise, nominal solution concentrations are reported in terms of the weight of polysaccharide initially dissolved.

Precipitation of β-Glucan From Solutions of Oat Gum

Oat gum (2 g) was dissolved in water (200 ml), and Calcofluor (200 mg) in water (50 ml) was added with vigorous stirring. The mixture was centrifuged and the supernatant decanted from the gel-like dye-polysaccharide complex. Addition of more Calcofluor to the supernatant produced no further precipitation. To isolate polysaccharides not precipitated by Calcofluor, 3 vol of 2-propanol (IPA) were added to the supernatant and the precipitate was isolated by centrifugation, washed with IPA, and air dried with gentle warming to give the noncomplexing fraction S (216 mg). The dye-polysaccharide complex was suspended in water (200 ml), stirred, and heated. Some swelling and dispersion took place. The cooled mixture was dialyzed overnight against tap water, then heated again to 60°C with stirring. One volume of IPA was added and the mixture centrifuged. The supernatant, which contained Calcofluor, was discarded and the precipitate redispersed in water and precipitated by 1 vol of IPA. This was repeated once more and the precipitate washed with IPA and air dried with gentle warming to give fraction P (1.35 g).

Experiments with barley β-glucan, and with the dyes Tinopal and Congo Red, were performed similarly, but the dialysis step, which was not successful in removing Calcofluor, was omitted.

Removal of Calcofluor From β-Glucan Complex

Fraction P (50 mg) obtained from precipitation of oat gum was dissolved in water (10−15 ml) and stirred with cellulose powder, Dowex 1 × 8 (Cl⁻) or

dimethylaminoethyl-cellulose (Cl⁻) for 2 hr. Samples were stored overnight at 5° C, then centrifuged, or in the case of cellulose, filtered. The amount of bound Calcofluor was estimated relative to an untreated control by absorbance measurements.

Interaction of Carbohydrates With Calcofluor and Congo Red

When possible, carbohydrate was dissolved in water to 1% w/v solution. Starch was dissolved in 0.05N NaOH and the solution neutralized with acetic acid. Samples were also dissolved in phosphate buffer, pH 7, ionic strength (I) 0.05. Each solution was centrifuged (21,000 × g) for 1 hr. The supernatant carbohydrate solution (4 vol) was thoroughly mixed with dye (1 vol, 10 mg/ml) and examined for precipitate immediately and after standing overnight at room temperature. Samples with Calcofluor were protected from light.

Testing of Dyes for Precipitation Reaction With Oat β-Glucan

Solutions of dyes were added to oat gum (1% w/v) and the mixture examined for possible precipitate.

Light Sensitivity of Calcofluor in Water and Dimethylsulfoxide in Presence and Absence of β-Glucan

Solutions of Calcofluor (10 and 4 μg/ml) were prepared in dimethylsulfoxide (DMSO) and water with and without added β-glucan (250 and 100 μg/ml). One portion of each solution was protected from light until measurement of absorbance at the wavelength of maximum absorption and another portion was exposed to normal daylight for 3 hr or more before measurement of absorbance.

Effect of Calcofluor on Precipitation Reaction and Dye-Binding Measurements

Solutions of oat β-glucan (2 ml, 0.93% w/v) were mixed with solutions of Calcofluor (0.25 ml and 0.5 ml) and water to give a final volume of 2.5 ml and a range of Calcofluor concentrations. Samples, which were protected from the light, were kept at room temperature for at least 1 hr and then centrifuged; aliquots were removed and diluted as required for assay of glucan and Calcofluor (355 nm). Precipitates were washed once with water, dissolved in warm DMSO, and diluted with DMSO as required for spectrophotometric assay at 365 nm.

Effect of pH, Salt, and Temperature on Precipitation of β-Glucan by Calcofluor

Oat β-glucan (0.7–0.8% w/v, 1 ml) was mixed with Calcofluor (0.25 ml) to give final concentrations of Calcofluor from 100 to 2,000 μg/ml. Samples were left at 25° C for at least 1 hr before centrifugation at room temperature and determination of the glucan content of the supernatant. β-Glucan solutions were prepared in water at pH 6.5; phosphate buffer at pH 6.0, 7.0, and 8.0; carbonate buffer at pH 10; and M NaCl. The I of the buffers was 0.05. A precipitation reaction was also performed at 5° C using water as solvent and centrifuging at this temperature.

Effect of Added Dye (Congo Red and Calcofluor) on Viscosity of Oat β-Glucan Solutions

Viscosities were measured in Cannon Manning semimicro viscometers (Cannon Instrument Company, State College, PA) at 20° C using aliquots from mixtures of oat β-glucan (1 mg/ml) and dye (0.5 mg/ml). Viscosities were

redetermined after a suitable interval to determine if they were stable; if not, they were maintained for 18—24 hr at 20°C, and a further measurement was made. Any precipitate that formed was removed by centrifugation before viscosity measurements.

Fluorescence and Bright-Field Microscopy

Mature, unimbibed seeds of barley (*Hordeum vulgare* L. cv Vanier) and oats (*Avena sativa* cv Hinoat) were cut in half transversely and fixed in 6% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 6.8, for 24—48 hr at 0.4°C. Fixed specimens were dehydrated successively in Methyl Cellosolve®, ethanol, *n*-propanol, and *n*-butanol and embedded in glycol methacrylate. Sections were cut 0.5—2.0 μm thick, affixed to glass slides, and stained in one of the following solutions: 1) 0.01% (w/v) Calcofluor in distilled water, 60 sec, 2) 0.01% (w/v) Congo Red in distilled water, 5 min, 3) 0.01% (w/v) Trypan Blue in distilled water, 10 min. Excess stain was removed by washing in running water for 1—2 min; sections were allowed to air dry before mounting in immersion oil. Specimens stained with Calcofluor or Congo Red were routinely examined by fluorescence optics using a Zeiss Universal Research Microscope equipped with a III RS condenser for epiillumination by an HBO 200 w/2 mercury arc lamp. Calcofluor fluorescence was maximized using ultraviolet exciter filter G 365 in combination with barrier filter LP418; Congo Red was examined with filters BP546/10 (exciter) and LP590 (barrier). Trypan blue-stained material was slightly fluorescent, but provided maximum contrast with bright-field optics.

RESULTS

Mixture of solutions (about 1% w/v) of oat gum or barley β-glucan with one tenth part by weight of Calcofluor, Tinopal, or Congo Red produced precipitates. Qualitative analysis (Table I) demonstrated in each case a fractionation of glucan from polysaccharides containing pentose and galactose. Quantitative analysis of the precipitate and supernatant obtained from interaction of Calcofluor with oat gum showed, in addition, purification from ash, protein, and starch. (The method for starch analysis [11] gives a starch content for α-amylase—treated gum controls of 0.5—0.6%. Thus, the oat gum precipitate and all barley β-glucan fractions were essentially starch free.)

The interaction of Congo Red and Calcofluor with solutions of a number of carbohydrates was studied (Table II). Precipitation or gel formation both in water and buffer was observed for Calcofluor with oat β-glucan, lichenan, hydroxyethyl cellulose, and tamarind amyloid. Slight precipitation was observed with pectin. Congo Red gave precipitation with oat β-glucan, lichenan, hydroxyethyl cellulose, and tamarind amyloid, and partial precipitation with starch.

Of the dyes tested, only Calcofluor, Tinopal, Congo Red, Trypan Blue, and Brilliant Yellow (weak reaction) were capable of giving a precipitate with oat β-glucan (Fig. 1). The following dyes were not reactive in this respect: Alizarin Red S, Rosaniline Hydrochloride, Orange G, and the food dyes (permitted in Canada), namely, Brilliant Blue, Indigotine, Fast Green, Amaranth, Erythrosine, Ponceau SX, Allura Red, Tartrazine, and Sunset Yellow. Also ineffective were 4,4'-diamino-2,2'-stilbenedisulfonic acid, 4-acetamido-4'-iso-

TABLE I
Analysis of Fractions Obtained From Dye Interaction
With Oat Gum and Barley β-Glucan

Sample[a]		Yield (%)	Ash[b]	Nitrogen[b]	Starch[b]	Glucan[b]	Constituent Sugars[c]			
							Gal	Gluc	Arab	Xyl
OG			3.49	0.97	2.1	85[d]	Tr	+++	+	+
OG, Calcofluor	P	68	0.31	0.16	0.5	95	...	+++	...	...
OG, Calcofluor	S	11	5.23	1.85	7.0	39[d]	+	+++	++	++
OG, Congo Red	P	68					...	+++	...	...
OG, Congo Red	S	11					+	+++	++	++
OG, Tinopal	P	63					...	+++	...	...
OG, Tinopal	S	16					+	+++	++	++
BG			0	0.41	0.5	93	...	+++	Tr	Tr
BG, Calcofluor	P	68			0.3	94	...	+++	Tr	Tr
BG, Calcofluor	S	17			0.7	87	...	+++	+	+

[a]OG = oatgum, BG = barley β-glucan, P = precipitated by dye, S = not precipitated by dye, Tr = trace.
[b]Percentage, dry wt. basis, in sample.
[c]Gal = galactose, gluc = glucose, arab = arabinose, xyl = xylose.
[d]Note: galactose interferes in glucan assay.

thiocyanostilbene-2,2′-disulfonic acid, ferulic acid, and *p*-coumaric acid. The structures of a few of these are shown in Fig. 2.

Removal of final traces of Calcofluor (or Congo Red) from the dye−β-glucan complex was difficult, although repeated dispersion in hot water and precipitation by IPA removed sufficient dye to restore room temperature solubility. Treatments with Dowex-1, cellulose, or DEAE-cellulose removed some dye but were unsuccessful in removing last traces of fluorescence. A single treatment with DEAE-cellulose, however, which gave a recovery of β-glucan of about 85%, reduced the Calcofluor content as estimated by absorbance spectrophotometry about tenfold compared with a control. Absolute measurements were not possible because of unavoidable exposure to light that occurred during sample preparation. Calcofluor is light sensitive in dilute solution. This is shown in Table III, which also illustrates the effect of solvent (dimethylsulfoxide [DMSO] or H₂O) and the presence or absence of β-glucan on absorption spectra. In DMSO, a 20-nm red shift occurs in the absorption maxima. In water, the presence of β-glucan at concentrations lower than that at which precipitation occurs brings about a smaller red shift than in DMSO. Relatively little change occurred in the extinction coefficient. Thus, if samples are not exposed to light, the concentration of dye can be measured both in the aqueous solution and in the precipitated complex, which can be dissolved in DMSO. Furthermore, for the concentrations in question, aqueous solutions of glucan can be measured by the cysteine-sulfuric acid reaction (11) without interference from Calcofluor. Thus, the dye-polysaccharide interaction can be

TABLE II
Interaction of Congo Red and Calcofluor With Carbohydrates

Sample	Structural Units of Main Chain	Precipitation Reaction[a]	
		Congo Red	Calcofluor
Oat β-glucan	β-1,3 and β-1,4-Glc	+	+
Lichenan[b] (*Cetraria islandica*)	β-1,3 and β-1,4-Glc	+	+
Cellobiose[c]	β-1,4-Glc	−	−
Laminaran[b]	β-1,3-Glc	−	−
Carboxymethyl cellulose[d]	β-1,4-Glc	−	−
Hydroxyethyl cellulose[e]	β-1,4-Glc	+	+
Guar gum[f]	β-1,4-Man	−	−
Locust Bean Gum[g]	β-1,4-Man	−	−
Carboxymethyl-pachyman	β-1,3-Glc	−	−
Starch (potato)[h]	α-1,4-Glc	±	−
Pectin[b]	α-1,4-GalA	−	±
Tamarind amyloid	β-1,4-Glc	+	+

[a] + = strong gelation or precipitation, ± = slight precipitation.
[b] Sigma Chemical Co., St. Louis, MO.
[c] Eastman Kodak Co., Rochester, NY.
[d] Grade 7H3 SXF, Hercules Incorporated, Wilmington, DE.
[e] Natrosol 250 M, Hercules Incorporated, Wilmington, DE.
[f] Tragacanth Trading Co., New York, NY.
[g] Stein, Hall & Co., Inc., New York, NY.
[h] Fisher Scientific Co., Pittsburgh, PA.

monitored for percentage of β-glucan precipitated and for dye bound, which may be determined either directly by redissolving the precipitate in DMSO or indirectly from the dye remaining in solution. Precipitates obtained at high dye concentration redissolved with difficulty and problems with manipulation
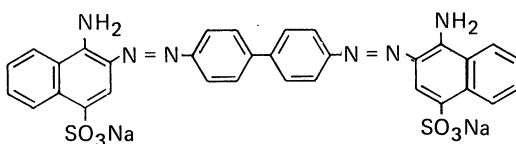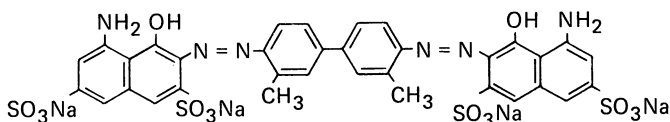
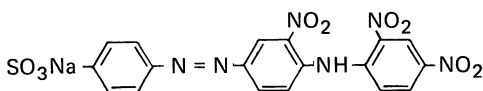CALCOFLUOR
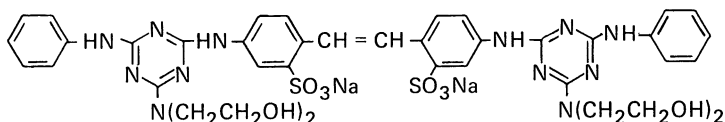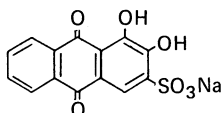
TINOPAL
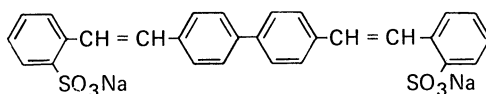
CONGO RED

TRYPAN BLUE

BRILLIANT YELLOW

Fig. 1. Molecular formula of compounds that give precipitate with oat β-glucan.

probably made these the less accurate measurements. For the most part, the two measurements were in agreement (Table IV). Once precipitation neared completion, the proportion of dye bound became approximately constant (93–95%).
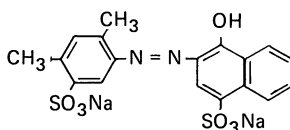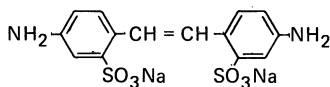
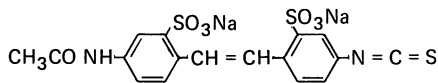•

Alizarin Red

Orange G
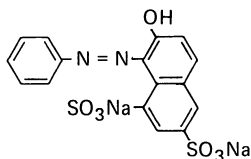
Ponceau SX

Di–sodium salt of 4,4'–diaminostilbene–2,2'–disulfonic acid

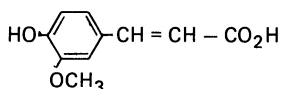Di–sodium salt of 4–Acetamido–4'–isothiocyanostilbene–2,2'–disulfonic acid

Ferulic acid

Fig. 2. Molecular formula of selection of compounds that do not give precipitate with oat β-glucan.

The course of the precipitation reaction differed little between 5 and 25° C (Fig. 3), but in M NaCl, the precipitation was considerably suppressed. The precipitate will redissolve at higher temperatures (90—100° C, lower if excess dye is removed). In buffered solutions (pH 6—10), the precipitations were as shown in Fig. 4. A slight suppression of precipitation was observed at pH 6 relative to 7, 8, and 10. Glucose calibrations in the cysteine-sulfuric acid reaction (11) were either unchanged or only slightly depressed in phosphate, carbonate, or M NaCl. Since measurements were made on diluted aliquots, interference from added salts was negligible.

This series of precipitation reactions was done with concentrations of β-glucan of 0.7 to 0.8% w/v. At lower concentrations of β-glucan ($\sim$ 0.4% w/v),

TABLE III
Effect of Solvent, Presence of β-Glucan, and Exposure to
Light on Optical Absorbance of Calcofluor

Solvent	Concentration of Calcofluor (μg/ml)	Concentration of β-Glucan (μg/ml)	Treatment	λmax[b]	Absorbance
H$_2$O	4	0	Exposed	350	0.041
H$_2$O	4	100	Exposed	355	0.044
H$_2$O	4	0	Unexposed	350	0.159
H$_2$O	4	100	Unexposed	355	0.165
DMSO[a]	10	0	Exposed	369	0.292
DMSO	10	250	Exposed	369	0.294
DMSO	10	0	Unexposed	369	0.413
DMSO	10	250	Unexposed	369	0.411

[a]DMSO = dimethylsulfoxide.
[b]λmax = Maximum absorption.

TABLE IV
Precipitation of Oat β-Glucan[a] by Calcofluor

Concentration of Calcofluor (μg/ml)	β-Glucan Precipitated (%)	Dye Bound (mg) Direct Measurement[b]	Dye Bound (mg) By Difference[c]	Added Dye Bound[d] (%)
100	3	...	...	...
200	70	0.39	0.40	81
400	94	0.93	0.91	91
600	98	1.37	1.37	91
800	98	1.84	1.86	93
1,000	99	2.24	2.33	93
2,000	99	4.20	4.69	94
4,000	99	8.35	9.47	95

[a]0.93% w/v in water.
[b]From absorbance of dimethylsulfoxide solution of precipitate.
[c]By difference from absorbance of supernatant from precipitation.
[d]Calculated from indirect (ie, supernatant) measurement.

precipitates tended to be more gel-like and the results less reproducible. At 0.1% w/v, the shape of the precipitation curve was considerably changed, as shown in Fig. 5. Also shown are the viscosity changes that occur when Calcofluor or Congo Red are added to β-glucan. Both dyes bring about an initial small decrease in viscosity followed by a steady increase until, in the case of Calcofluor, the onset of precipitation. At higher concentrations of dye, viscosities slowly increased with time. Dyes, which in solution alone do not greatly differ in
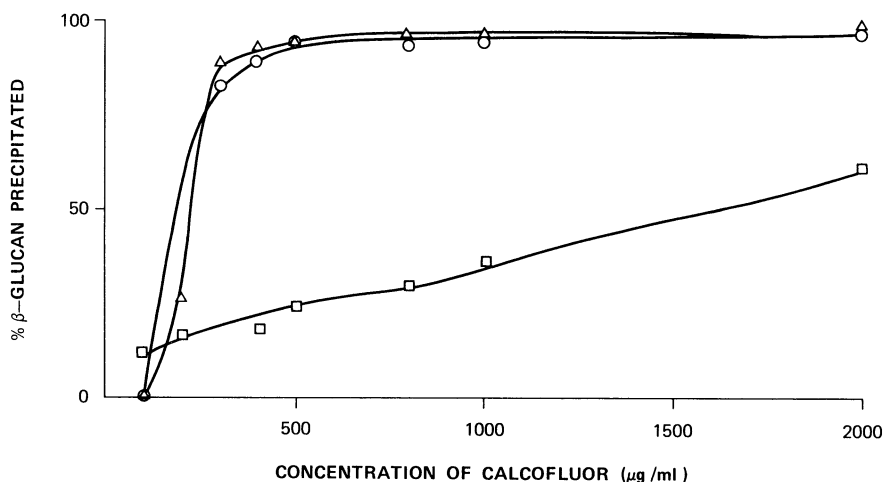

Fig. 3. Precipitation curves of oat β-glucan: o————o in water at 20° C; △———△ in water to 5° C; □———□ in 1M NaCl.
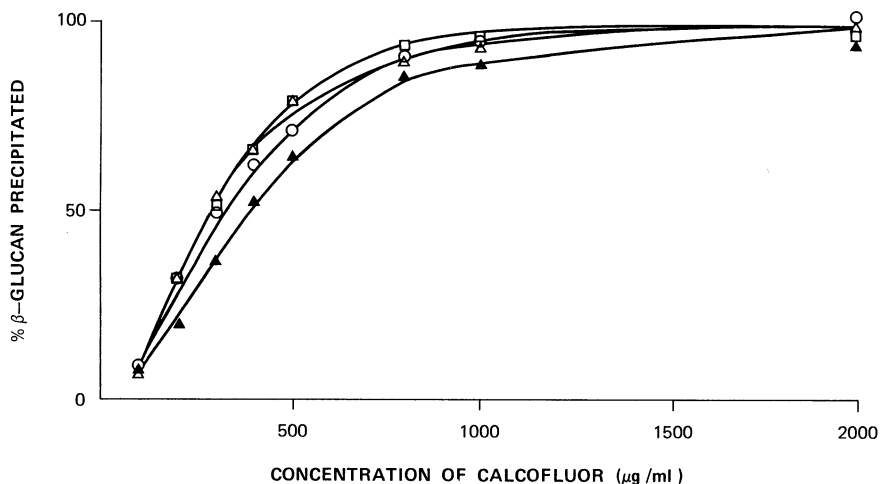
Fig. 4. Precipitation curves of oat β-glucan: ▲————▲ at pH 6; △———△ at pH 7; □———□ at pH 8; o————o at pH 10.

viscosity from water, can double the viscosity of dilute solutions of β-glucan. The viscosities of solutions of β-glucan alone in water, salt, or alkali (pH 10) were 1.7, 1.7, and 1.8 centistokes (cSt), respectively.

DISCUSSION

The precipitation of oat β-glucan by Calcofluor and other dyes is a somewhat unusual reaction for neutral polysaccharides. Precipitation of neutral polysaccharides as their metal complexes is well known (12), and polyanions form insoluble coacervates with certain cationic detergents and dyes (13). To our knowledge, however, no reports are available of polysaccharide precipitation from interaction with comparitively low molecular weight organic molecules at low concentration. The precipitation of amylose as the butanol complex (14) is superficially similar.

Clearly β-glucan is specifically precipitated from solutions of oat gum (Table I). Starch, pentosan, and protein did not precipitate. Of the carbohydrates tested, only neutral polymers containing β-1,4-linked glucopyranosyl units gave a strong precipitation or gel reaction with Calcofluor and Congo Red (Table II). Considerably more testing of defined polysaccharides will be required, however, before the significance of this apparent structural specificity can be assessed. The results with starch, locust bean gum, and guar gum nevertheless indicate some
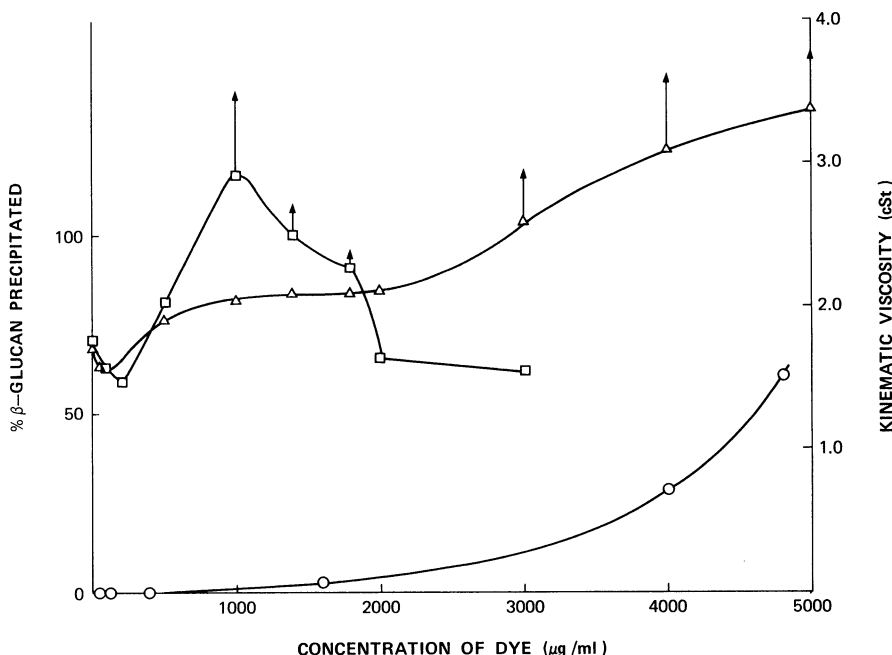

Fig. 5. Effect of dye addition to low concentrations of oat β-glucan: o———o precipitation curve, 0.09% w/v; □---□ viscosity changes, Calcofluor; △---△ viscosity changes, Congo Red. Arrows indicate viscosity changes with time.

specific steric requirements for interactions either in the monomer units and linkages or in the polysaccharide conformation in solution.

Dye binding to neutral polysaccharides in solution without precipitation is a more frequently encountered phenomenon (15,16). Changes in the absorption spectra of Congo Red occur in the presence of amylose (17) and certain β-1,3-glucans (18). We have also observed changes in the wavelength of maximum absorption of Congo Red in the presence of oat β-glucan, lichenan, laminaran, hydroxyethyl cellulose, and tamarind amyloid. Similar changes were observed with Calcofluor. More detailed studies of dye binding by absorption and fluorescence spectrophotometry are in progress.

Data as yet are insufficient to draw conclusions as to structural requirements for the dye molecules. All reactive dyes (Fig. 1) belonged to that class known as cellulose substantive, and are extended, conjugated molecules containing sulfonic acid groups and either an azo or stilbene group. As seen in Fig. 2, however, the presence of these features does not necessarily confer reactivity. Ferulic and p-coumaric acids were tested because of the known ability of phenolic acids to cause gelation when esterified to certain polysaccharides (19), because they occur naturally in cereals and because they are somewhat similar structurally to the stilbene-type dyes.

Calcofluor, and other stilbene-type dyes, can exist as the cis- and trans-isomers. Evidently the trans-isomer is the active component in the dye's role as a whitening agent, but under the influence of light, particularly in dilute solution, trans-cis isomerization might occur (20). The effect of binding on this transformation is complex, but in systems studied here, β-glucan (at low concentration) has no effect on the trans-cis conversion (Table III). The red shift in the wavelength of maximum absorption of the dye in aqueous solutions in the presence of added β-glucan is good evidence that complex formation is taking place, even below concentrations at which precipitation occurs.

No attempt was made to protect Calcofluor solutions from light in the earlier fractionation experiments. Subsequent repeats of the experiments, with appropriate precautions taken, indicated no difference in ability to precipitate β-glucan. In dilute solutions in which dye concentrations were to be determined by spectrophotometry, however, protection from light was necessary.

Calcofluor as supplied is not a pure compound, but according to the manufacturer, about 70% is "active."[4] If the dye supplied is 100% compound I, molecular weight 960, however, the precipitation curves indicated that in water, precipitation was virtually complete with one dye molecule per 60 anhydroglucose units. At this dye/β-glucan ratio, precipitation was almost quantitative, but in the presence of M NaCl, precipitation reached only 70% completion even in the presence of 2.5 times that amount of dye.

Since 93−95% of added Calcofluor was bound by oat β-glucan, the endosperm cell walls of oat and barley show a strong fluorescence when stained with Calcofluor (Fig. 6 and 7). The subaleurone endosperm cell walls, particularly in the oat section, are considerably thicker than the inner endosperm walls. This is shown at higher magnification in Fig. 8. The technique is capable of showing considerable detail. No intracellular staining occurred, and lamellar structure can be observed in the walls. Otherwise, no distinction is apparent between different regions across the wall. Similar observations can be made using

[4]F. G. Villaume, American Cyanimid Company, Bound Brook, NJ. Private communication.

fluorescence optics with Congo Red, or bright-field optics with Trypan Blue.

We have been using three successive alkaline extractions routinely to obtain oat gum from dehulled oat flour (11,21). The course of this extraction can be observed histochemically. Figure 9 shows a typical coarse flour particle. The tendency for the thick-walled subaleurone endosperm cell walls to remain with the aleurone cells accounts for the known concentration of gum in coarse, or bran, fractions of the flour. After the first alkaline extraction (Fig. 10),
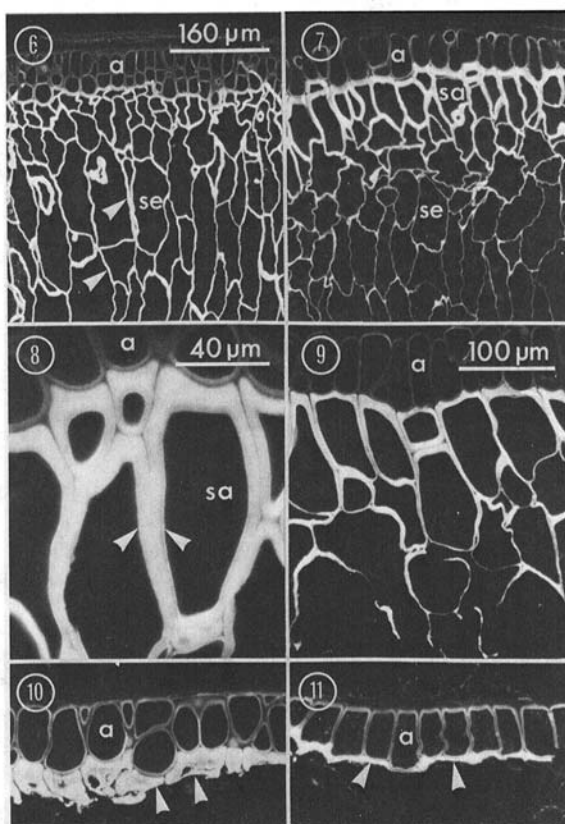
Fig. 6. Transverse section of barley endosperm showing intense Calcofluor fluorescence (arrows) in starchy endosperm cell walls. a, Aleurone layer; se, starchy endosperm. **Fig. 7.** Transverse section of oat endosperm also showing Calcofluor staining throughout the endosperm cell walls. Note greater thickness of walls in subaleurone region (sa). Scale as in Fig. 6. **Fig. 8.** Portion of sa of oat endosperm showing lamellar organization of thickened subaleurone cell walls (arrows). **Fig. 9.** Portion of coarse oat flour particle before alkaline extraction. **Fig. 10.** As in Fig. 9, but after first alkaline extraction. Most of inner endosperm cell walls have been removed during treatment, leaving only those materials (arrows)adhering directly to a. Scale as in Fig. 9. **Fig. 11.** As in Fig. 9, but after third alkaline extraction. Only thin layer of Calcofluor-positive material (arrows) adheres to a. Scale as in Fig. 9.

fluorescence has been removed largely from the inner, thinner endosperm cell walls. The thicker subaleurone walls are still prominent, but show signs of attack. After further alkaline extraction (Fig. 11), most of the fluorescence has been removed. Unextracted mixed linkage β-glucan or possibly cellulose might be responsible for the residual fluorescence. The remaining wall structures show considerable pitting. Interestingly, the gum obtained from the first extraction shows higher protein and lower viscosity than do those from the second and third extractions (21).

Histochemical observations involve surface adsorption phenomena, and dye-substrate interactions may be quite different from those observed in solution. A detailed understanding of the mechanism of dye-β-glucan precipitation is desirable, both from the point of view of developing practical applications in cereal chemistry and more generally since exploitable differences may exist in behavior of different dyes with different polysaccharides. In the first instance, the dye appears to interact with polysaccharide in solution before precipitation occurs. This can be detected by changes in the physical properties of both dye (absorption spectra) and polysaccharide (viscosity). The viscosity increase before precipitation may be a result of coil expansion in the dye/polysaccharide complex (which will be polyanionic in nature) and thus indicates a role for polymer-polymer interaction in the precipitation reaction. The precipitation may also involve dye, or dye-aggregate, cross-linking. The nature of the interaction may be similar to the known gelation of polyvinyl alcohol by Congo Red and similar dyes (22).

In conclusion, a strong interaction between oat β-glucan and certain dyes, such as Calcofluor, has been demonstrated. The potential for exploitation of this interaction in analytic, structural, and histochemical applications is evident.

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