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Specific Interaction of Aniline Blue with (1 → 3)-ß-D-Glucan*

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SUMMARY

Interaction between aniline blue and curdlan, a (1→3)-β-D-glucan, has been studied using absorption and fluorescence spectroscopy. The evidence suggests that a minor, weakly fluorescent component of commercial dyes forms a strongly fluorescent complex with curdlan, with an excitation maximum of 395 nm and an emission maximum of 495 nm. This component was partially purified by TLC on silica gel. Of many polysaccharides surveyed, a number showed weak interactions with the major component of aniline blue but only (1→3)-β-D-glucans such as pachyman, curdlan and laminaran induced fluorescence in the minor component. Fluorescence was less with laminaran than with curdlan and decreased with increasing alkali concentration suggesting conformational control of the dye-binding mechanism. As little as 5 μg/ml of curdlan induced easily detectable fluorescence increases in aniline blue, and this was used to demonstrate the presence of (1→3)-β-D-glucan in a fungal cell wall preparation. (1→3)-β-D-glucan in cereal grain sections was located as bright yellow-green fluorescent particles. In barley these stained particles, located in association with the sub-aleurone endosperm cell wall, showed a fluorescence excitation maximum at 395 nm and emission maximum of 495 nm.

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INTRODUCTION

The dye aniline blue has long been used as a stain for 'callose' (Currier, 1957, and refs. therein; Eschrich, 1961) and successful use of this reaction has continued to the present day (Fulcher *et al.*, 1977; Bacic & Stone, 1981*a*; Waterkyn, 1981) despite in most instances, lack of complete knowledge of the chemical nature of either reactant. Morphological specificity has been acceptable to, and exploited by, most investigators, but a chemical specificity for (1→3)-β-D-glucan (rather than 'callose', sometimes defined as that which stains with aniline blue) has been less readily accepted.

The concept of histochemical specificity remains controversial. Smith & McCully (1978) pointed out that only in one instance (Kessler, 1958) had a rigorous chemical identification of the staining bodies as (1→3)-β-D-glucan been made. Since that time, however, Vithanage *et al.* (1980) have isolated a substance from rye pollen, produced in response to incompatible pollination, that fluoresces in the presence of aniline blue and appears to contain both (1→3)- and (1→4)-β-linked D-glucose. Mixed linkage β-D-glucan was identified but the presence of homopolymers could not be ruled out. Hinch & Clarke (1982) using specific enzymes, suggested that aniline blue 'positive' deposits in fungal infected *Zea mays* contained a glucan with both (1→4)- and (1→3)-β-D-linked glucose units.

Circumstantial evidence for the identity of fluorescently stained bodies in barley as (1→3)-β-D-glucan has been obtained (e.g. Fulcher *et al.*, 1977) and, despite their criticism of claims for specificity, Smith & McCully (1978) accept that the morphological specificity observed does often seem to correspond to (1→3)-β-D-glucan. Faulkner *et al.* (1973) described briefly the staining of isolated polysaccharide samples and concluded that no particular structural specificity exists, although differential staining was observed. Unfortunately, absorption behaviour of isolated polysaccharide fractions (both specific and non-specific) may be erratic and comparison of intensities in such a system may be unreliable.

Recently, chemical investigations (Bacic & Stone, 1981*b*) have strongly supported the earlier histochemical observations of Fulcher *et al.* (1977) that there are aniline blue 'positive' (1→3)-β-D-glucan deposits associated with barley sub-aleurone endosperm cell walls. Isolation of the stained bodies was not, however, reported. Although

this exercise would be of value in a particular instance, and is the completely rigorous approach, the task may be difficult. We have attempted in this study a more general approach by looking for evidence of interaction in solution and determining the specificity of any interactions observed by surveying a number of polysaccharides. A similar approach has been applied to the study of interaction of congo red and calcofluor white with polysaccharides (Wood & Fulcher, 1978; Wood, 1980).

EXPERIMENTAL SECTION

General methods

Absorption spectra were recorded with a Beckman model 26 spectrophotometer, and fluorescence spectra (uncorrected) with a Hitachi-Perkin-Elmer MPF-2A spectrofluorimeter, normally at 30°C. Aniline blue or water soluble aniline blue (CI 42755) was supplied by Eastman Kodak Co., Rochester, NY 14650, USA (EAB), Aldrich Chemical Co. Inc., Milwaukee, Wisconsin 53233, USA (AAB), and Polysciences Inc., Warrington, PA 18976, USA (Lot 1532; PAB). The 'fluorochrome' component in PAB was isolated by thin layer chromatography using the method of Smith & McCully (1978), but the component of interest was located using a spray of curdlan (1 mg/ml) in 0·05 M NaOH. The sources, and main structural features of the polysaccharides used are listed in Table 1. The identity of curdlan was confirmed by ^{13}C-NMR spectroscopy using $DMSO_{d6}$ as solvent. Acid hydrolysis was with 1 M H_2SO_4 for 3 h at 100°C and component sugars were identified following neutralisation with $BaCO_3$, by paper chromatography using 8 : 2 : 1 (v/v) ethyl acetate–pyridine–water.

Absorption and fluorescence spectroscopy of dye–polysaccharide mixtures

Solutions of polysaccharide, first clarified by centrifugation, were tested for interaction with dye (~ 10 μg/ml) by studying changes in the absorption and fluorescence spectra of the dyes. Measurements were made, between 1 and 24 h after mixing, in 0·05 M NaOH and (in initial studies comparing the different commercial sources of aniline blue) in

TABLE 1
Source and Main Structural Features of Polysaccharides Investigated

Polysaccharide	*Source*	*Supplier*	*Main structural features*
Curdlan	*Alcaligenes faecalis*	T. Harada	(1→3)-β-D-Glc*p*
Laminaran	*Laminaria hyperborea*	Sigma Chemical Co.	(1→3)-β-D-Glc*p*
Carboxymethylpachyman (CMP) and pachyman	*Pachyman* (from *Poria cocos*)[a]	J. J. Marshall	(1→3)-β-D-Glc*p*
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	(1→4)- and (1→3)-β-D-Glc*p*
Hydroxyethylcellulose Natrosol 250 M (HEC)	Cellulose	Hercules Co.	(1→4)-β-D-Glc*p*
Potato starch	*Solanum tuberosum*	Fisher Scientific	(1→4)- and (1→6)-α-D-Glc*p*
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*
Pustulan	*P. papullosa*	Calbiochem	(1→6)-β-D-Glc*p*
Maize stem hemicellulose	*Zea mays*	A. J. Buchala	(1→4) and (1→3)-β-D-Glc*p*
Cotton fibre hemicellulose	Cotton	A. J. Buchala	(1→3)-β-D-Glc*p*
Xanthan gum	*Xanthomonas campestris*	Kelco Co.	(1→4)-β-D-Glc*p*, oligosaccharide sidechains
(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	*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*

Guar gum	*Cyanaposis tetragonolobus*	Tragacanth Trading Co. Ltd	(1→4)-β-D-Man*p*, D-Gal*p* substituents
Tamarind powder	*Tamarindus indica*	Tragacanth Trading Co. Ltd	(1→4)-β-D-Glc*p*, oligosaccharide side chains
W. pyramidata gum	*Watsonia pyramidata*	I. C. M. Dea	(1→4)-β-D-Xyl*p*, oligosaccharide substituents
ι-Carrageenan	Rhodophyceae	I. C. M. Dea	(1→3)-β-D-Gal*p* and (1→4)-α-L-3,6-anhydro-Gal*p* + sulphate ester
Agarose	Rhodophyceae	I. C. M. Dea	(1→3)-β-D-Gal*p* and (1→4)-α-L-3,6-anhydro-Gal*p*
Pectin (low methoxy)	Citrus	I. C. M. Dea	(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)-β-D-Glc*p*$NHSO_4$-6-SO_4
Hyaluronic acid	Connective tissue	M. B. Mathews	(1→4)-β-D-Glc*p*A and (1→3)-β-D-Glc*p*NAc
K18	*Klebsiella*	G. G. S. Dutton	[b]
K24	*Klebsiella*	G. G. S. Dutton	[c]
K32	*Klebsiella*	G. G. S. Dutton	[d]
Locust bean gum	*Ceratonia siliqua*	Stein, Hall and Co. Ltd	(1→4)-β-D-Man, D-Gal*p* substituents

[a] Prepared according to Clarke & Stone (1962).
[b] Dutton *et al.* (1978).
[c] Choy *et al.* (1973).
[d] Bebault *et al.* (1978).

0·05 M NaOH containing 0·5 M NaCl. Difficulties with dye solubility in these solutions were overcome by preparation of the initial stock solution in water. The blue colour of the dye fades rapidly on making alkaline and the final solution used was colourless. In higher alkali concentrations and in salt, precipitate may form on standing. Therefore, measurements were preferably made on the same day as preparation.

Because of solubility problems with some polysaccharides, the survey of polysaccharides was carried out without salt in the medium. Polysaccharides were dissolved with gentle heating if necessary, then centrifuged and the supernatant used for testing. Many of the polysaccharides showed some insoluble material which was washed, dried and weighed. Because of small starting sample size this procedure was inherently inaccurate but adequate to determine if sufficient polysaccharide had dissolved for testing. In most cases less than 25% of the starting material was recovered as insoluble residue with the exception of cotton fibre hemicellulose and maize stem hemicellulose which seemed to be mainly insoluble. (Very small amounts of these were available; results suggest some solubility for the former.) Polysaccharides were mostly dissolved at 1 mg/ml but with small samples this was reduced to 0·5 mg/ml. The concentration dependence of the interaction was tested with curdlan between 0 and 1 mg/ml and laminaran between 0 and 4 mg/ml. The effect of alkali concentration, and alkali plus salt, was tested with 250 μg/ml of curdlan.

For fluorescence studies, samples were compared generally at the same excitation and emission slit widths and amplifier sensitivity settings but the wide range of intensities examined sometimes required different settings. The numerical values of intensities reported are therefore arbitrary. Where useful, relative fluorescence intensity (RFI), the ratio of fluorescence intensity of sample plus dye to fluorescence intensity of dye alone at particular excitation and emission wavelengths, is quoted. However, very low fluorescence of dye alone tended to make this value unreliable. In all cases, control solutions were examined to determine intrinsic fluorescence of the polysaccharide sample, if any, and to avoid possible confusion with Raman scatter peaks.

Extraction of hyphal cell walls from *Fusarium sulphureum*

Hyphal cell walls (50 mg) from *Fusarium sulphureum* (Barran *et al.*, 1975) were extracted by stirring and gentle warming with 0·05 M NaOH

(10 ml), the mixture centrifuged and 1 ml of the supernatant mixed with PAB (0·2 ml, 100 μg/ml) then diluted to 2 ml with 0·05 M NaOH and the fluorescence spectra obtained.

Isolated extracts of hyphal cell walls were also tested. Hyphal cell wall, defatted with chloroform–methanol (2:1) and treated with refluxing 70% ethanol, was extracted twice with water at 100°C (430 mg; 2 × 100 ml H_2O). The hot water-soluble material was concentrated and lyophilised to give 34 mg of material which showed galactose, glucose, mannose and (tentatively) some uronic acid on acid hydrolysis and paper chromatography. The water-insoluble material was treated with 0·5% ammonium oxalate (90°C, 5 h) but this reagent failed to solubilise significant amounts of material. Further extraction with 5% KOH containing 0·1% $NaBH_4$ under nitrogen at room temperature for 24 h gave soluble material, isolated by adjustment of the pH to 4·5 and precipitating with 3 vol. of ethanol. This 5% KOH-soluble fraction (21 mg) showed a similar constituent sugar profile to the hot water-soluble fraction. The hot water-soluble and 5% KOH-soluble fractions were tested for interaction with aniline blue at concentrations of 450 and 500 μg/ml respectively.

Fluorescence microscopy and microspectrofluorometry

Mature barley kernels (*Hordeum vulgare* L. cv Himalaya) were glutaraldehyde-fixed, embedded in glycol methacrylate, and sectioned for microscopic examination as described by Fulcher *et al.* (1977). Sections 1–2 μm thick were stained with 10 μg/ml PAB in 0·05 M NaOH and examined after 1–2 min using a Zeiss Universal research microscope equipped with a III RS epi-illuminating fluorescence condenser and HBO 200 illuminator. Aniline blue-induced fluorescence was detected using exciter filter G365 (transmission λ_{max} 365 nm) and barrier filter LP 418 (transmission $\lambda_{max} > 418$ nm). Stained sections are shown in Fig. 9.

For microspectrofluorometry, sections were stained for 5 min, blotted dry and shipped to Carl Zeiss Ltd (Oberkochen, W. Germany) for analysis. There, the sections were rehydrated and aniline blue-stained sub-aleurone deposits scanned for excitation and emission maxima using a Zeiss Universal microscope equipped with epi-illuminating condenser II P UV and HBO 100 illuminator and scanning exciter and barrier monochromators. Excitation spectra were obtained using a fixed detection wavelength of 520 ± 28 nm; the emission spectra

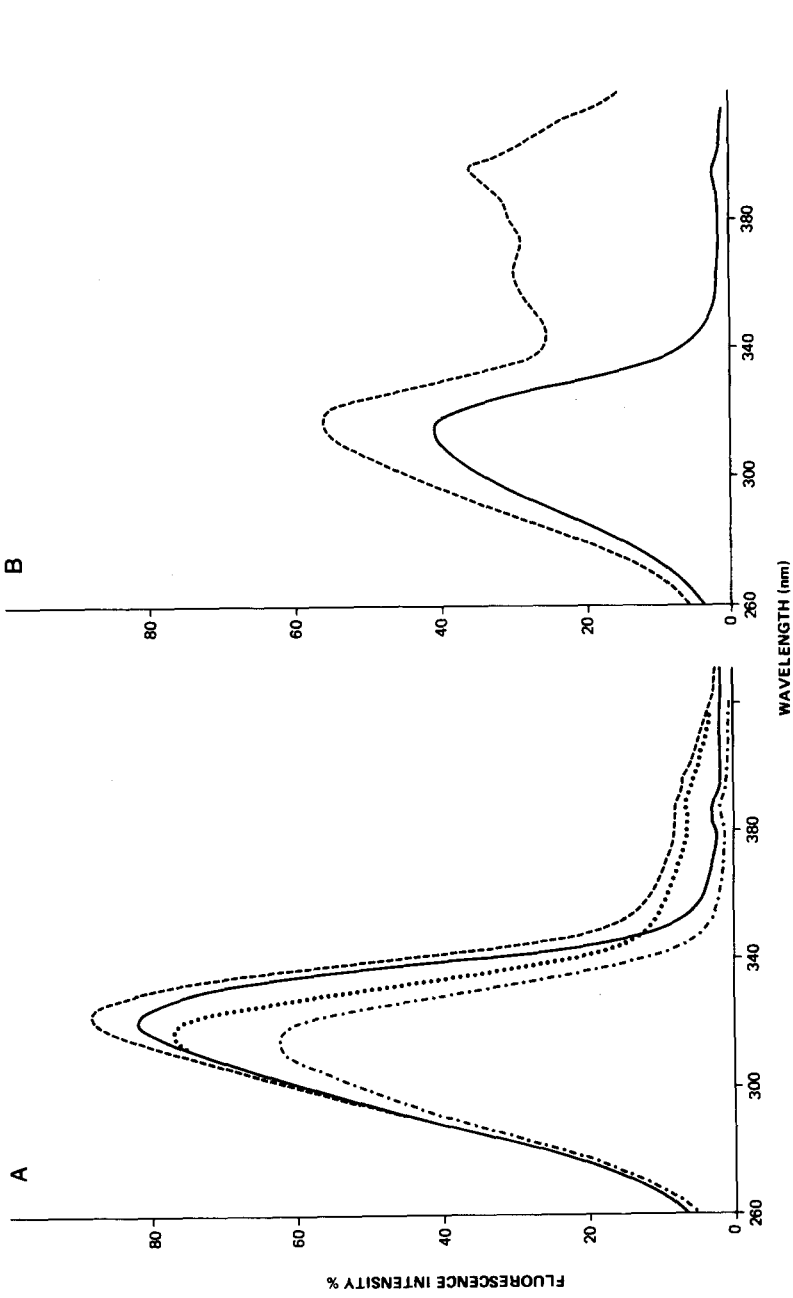

Fig. 1. Fluorescence excitation spectra (emission ~450 nm) of (A) AAB and EAB (10 μg/ml) and (B) PAB (10 μg/ml) in the presence and absence of curdlan (1 mg/ml) in 0·05 M NaOH–0·5 M NaCl. (A) —— AAB alone; ------- AAB plus curdlan; -·-·-·- EAB alone; ····· EAB plus curdlan. (B) —— PAB alone; ------- PAB + curdlan.

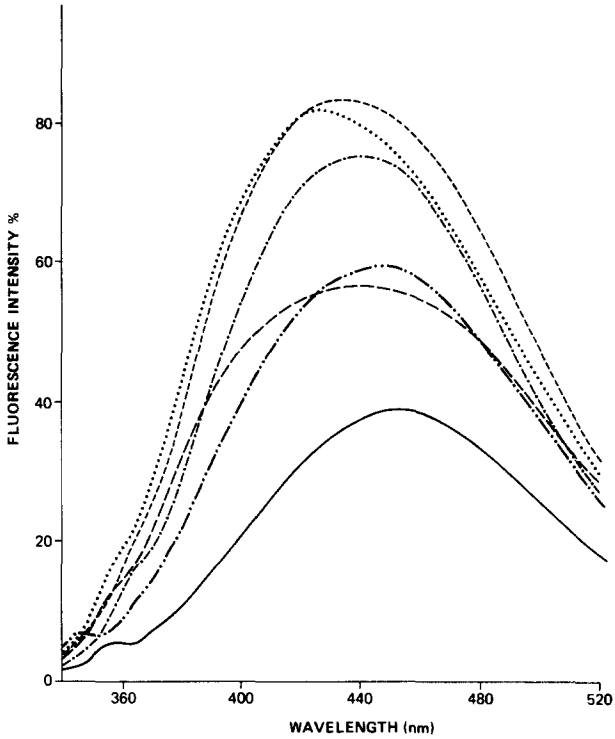

Fig. 2. Fluorescence emission spectra (excitation ~320 nm) of aniline blue (10 μg/ml) in the presence and absence of curdlan (1 mg/ml) in 0·05 M NaOH-0·5 M NaCl. —— PAB alone; ----- PAB + curdlan; -·-·-· AAB alone; ····· AAB + curdlan; -·-·-·-· EAB alone; ····· EAB + curdlan.

possessed an excitation maximum fixed at 365 ± 10 nm. The averages of 8 scans are illustrated in Fig. 10.

RESULTS

The major absorption peak of aniline blue in alkali occurs at ~310 nm. At this wavelength, the extinction coefficients of the three commercial dyes decreased in the order Aldrich > Eastman > Polysciences. The main fluorescence peaks showed the same order of intensities, presumably reflecting the purity of the dyes (Figs 1 and 2; Table 2).

TABLE 2

λ_{max} and Fluorescence Intensities of Emission Spectra of Aniline Blue (10 μg/ml) in the Presence and Absence of Curdlan (1 mg/ml) in 0·05 M NaOH–0·5 M NaCl

Supplier	*Band widths*[a]	*Dye alone*			*Dye + curdlan*		
		Excitation λ_{max} *(nm)*	*Emission* λ_{max} *(nm)*	*Fluorescence intensity (%)*	*Excitation* λ_{max} *(nm)*	*Emission* λ_{max} *(nm)*	*Fluorescence intensity (%)*
AAB	1	323	440	75·5	323	432	83·5
	2	395	495[b]	3·0	395	495	94·0
EAB	1	318	445	62·5	318	424	81·5
	2	395	495[b]	4·0	395	495	70·0
PAB	1	318	452	39·0	318	435	56·5
	1	395	493[b]	1·6	395	493	88·0

[a] (1) Excitation 3 nm, emission 8 nm; (2) excitation 5 nm, emission 10 nm.
[b] No distinct peak.

In the absence of curdlan both the excitation (λ_{max} ~ 315–320 nm) and the emission (λ_{max} ~ 450 nm) spectra of each dye were similar in appearance (Figs 1 and 2). In the presence of curdlan, with emission set at ~450 nm, the main excitation peak intensities increased slightly but the greatest effect was observed between 360 and 400 nm. EAB and AAB showed intensity increases in this region and PAB showed two new major overlapping peaks at 365 nm and 395 nm (Fig. 1). The emission spectra obtained for each dye from excitation at the curdlan-shifted λ_{max} values (318 nm for EAB and PAB and 323 nm for AAB) showed increased fluorescence intensity values of 1·1, 1·3 and 1·4 for AAB, EAB and PAB respectively and hypsochromic shifts (Fig. 2; Table 2). Curdlan alone showed slight background fluorescence but no significant peaks (other than small Raman scatter) both at these and the other excitation wavelengths of interest, namely 365 nm and 395 nm.

Excitation of all three dye samples at either of these latter wavelengths (peak shoulder values shown in Fig. 1(B)) produced fluorescence emission peaks at 495 nm in the presence of curdlan. Spectra obtained with excitation at 395 nm are shown in Fig. 3. Major fluorescence emission peaks occurred at 495 nm which were absent for dye or curdlan alone, with all three dye samples but with AAB and EAB the absolute intensity values were much less, requiring wider slit widths equivalent to a four- to five-fold sensitivity increase (Table 2). (In consequence, a Raman scatter peak could be observed now at ~456 nm with these samples of dye alone.) For each dye the increase in fluorescence intensity was essentially from background or close to 'zero' intensity, making RFI values inaccurate, but useful as a guide to the magnitude of the effect. RFI values observed were AAB 31·3, EAB 17·5 and PAB 55. These results showed that the most effective wavelengths for study were excitation 395 nm and emission 495 nm, and the most suitable source of dye was Polysciences. PAB was therefore used in subsequent experiments.

The absorbance spectra of aniline blue in the presence of curdlan and some other polysaccharides [guar gum, locust bean gum, starch, O-(hydroxyethyl)cellulose, tamarind powder, laminaran, O-(carboxymethyl)pachyman] in 0·05 M NaOH–0·5 M NaCl showed little if any changes although UV absorbing contaminant in some of the samples caused additive hypsochromic shifts in λ_{max}. Difference spectra of curdlan plus PAB against PAB alone, however, showed a small peak at ~395 nm. Fluorescence spectra of these polysaccharides and other

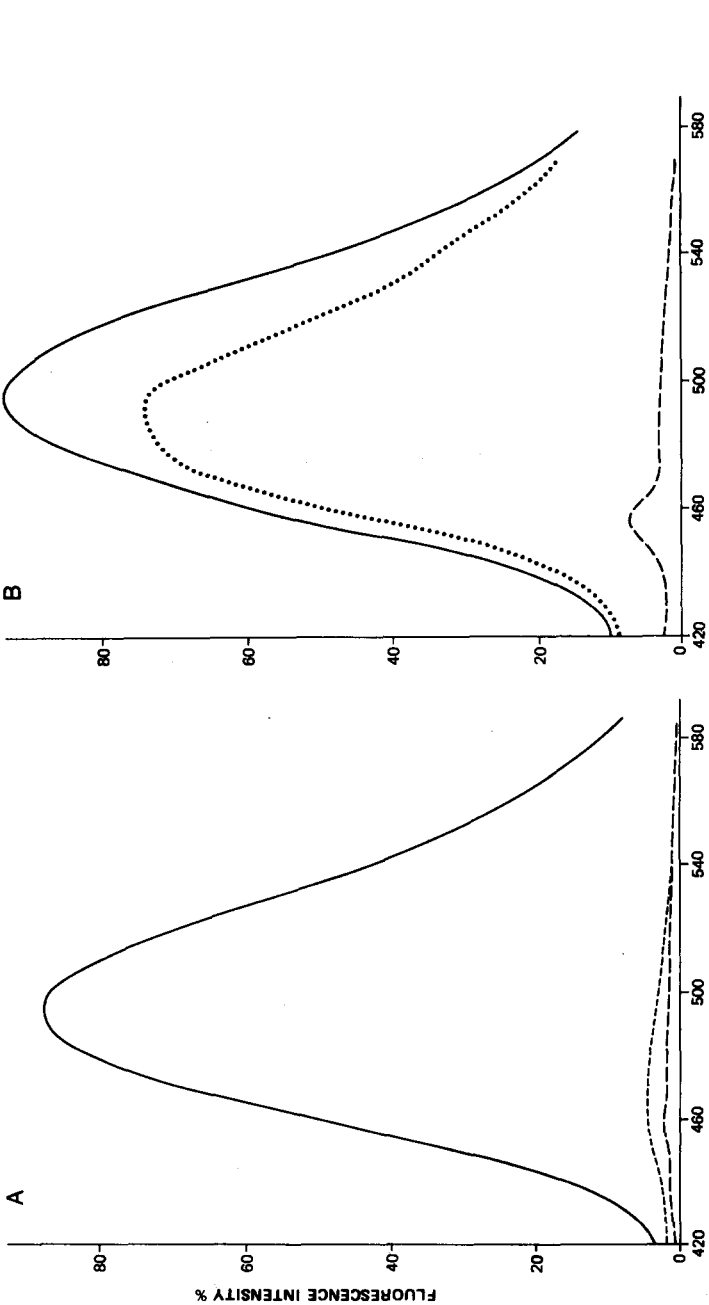

Fig. 3. Fluorescence emission spectra (excitation 395 nm) of aniline blue (10 μg/ml) in the presence and absence of curdlan (1 mg/ml) in 0·05 M NaOH–0·5 M NaCl. (A) ----- PAB alone; ..... curdlan alone; —— PAB + curdlan. (B) ----- AAB alone; —— AAB + curdlan, ... EAB + curdlan.

polysaccharides in 0·05 M NaOH were examined. A number of samples influenced the main fluorescence emission peak (~450 nm, excitation ~315 nm) usually by inducing a hypsochromic shift and increasing the intensity. In many instances this was caused, in part or wholly, by the intrinsic fluorescence of the sample alone. Similarly a number of the polysaccharides contained components contributing to the fluorescence emission at 495 nm from excitation at 395 nm. These were clearly distinguished from the samples which induced major increases in fluorescence intensity of the dye, namely curdlan, pachyman, O-(carboxymethyl)pachyman, laminaran and cotton fibre hemicellulose (Table 3).

The 'fluorochrome' fraction purified by TLC was not isolated in the dry state. Appropriate concentrations to use for fluorescence spectra were determined by examining the absorption spectra in 0·05 M NaOH in the presence and absence of curdlan. The results showed that 50–

TABLE 3

Fluorescence Emission Intensity at 495 nm (Excitation 395 nm) of Polysaccharide Samples Alone (~1 mg/ml) and in the Presence of PAB (10 μg/ml)

Sample	*Fluorescence intensity (495 nm) (curdlan + PAB = 100)*	
	Sample alone	*Sample + PAB*
0·05 M NaOH–0·5 M NaCl		
PAB	2	na
Curdlan	3	100
CMP	1	141
Laminaran	5	35
Potato starch	1	5
HEC	<1	3
Oat β-D-glucan	1	2
Guar gum	5	6
Locust bean gum	2	2
Tamarind powder	14	14
0·05 M NaOH		
PAB	2	na[a]

TABLE 3 continued

Sample	*Fluorescence intensity (495 nm) (curdlan + PAB = 100)*	
	Sample alone	*Sample + PAB*
Curdlan	2	100
Pachyman	1	71
CMP	nd	74
Cotton fibre hemicellulose	9	41
Pustulan	7	15
Potato starch	nd	2
HEC	nd	6
Oat β-D-glucan	nd	3
Barley β-D-glucan	4	4
Guar gum	2	1
Xanthan gum	nd	4
Tamarind powder	12	12
W. pyramidata gum	3	2
Xylan	2	2
Pullulan	3	1
Elsinan	3	2
(4-O-Methylglucurono)xylan	4	3
Arabino-(4-O-methylglucurono)xylan	1	3
D-gluco-D-mannan	4	5
D-galacto-D-gluco-D-mannan	5	6
β-(1→4)-D-galactan	12	12
Maize stem β-D-glucan[b]	2	3
Chondroitin 4-sulphate	1	2
Dermatan sulphate	1	3
Heparin	<1	2
Hyaluronic acid	<1	2
ι-Carrageenan	2	2
Agarose	3	3
Pectin	3	2
K18	6	7
K24	1	2
K32	2	3

[a] Abbreviations: na, not applicable; nd, not done.
[b] Tested at ~500 μg/ml.

100 μl of the isolated fraction of PAB diluted to 2 ml gave suitable absorbancies. λ_{max} was observed at 375 nm and in the presence of curdlan there was a bathochromic shift of 17 nm to 392 nm (Fig. 4) (i.e. $\sim\lambda_{max}$ of the fluorescence excitation peak observed with unfractionated PAB). Excitation of a solution of the 'fluorochrome' fraction alone at 310 nm produced no distinguishable fluorescence peak at ~450 nm (λ_{max} of the PAB emission spectra), nor were peaks evident at 495 nm (Fig. 5). However, in the presence of curdlan (1 mg/ml) excitation at any wavelength from ~300 to 395 nm produced a major fluorescence emission peak at 495 nm, with maximum intensity from irradiation at 395 nm, and the excitation spectra, with emission set at 495 nm, showed a λ_{max} of 395 nm with a shoulder at 365 nm. At the same sensitivity and slit widths the dye fraction alone was essentially non-fluorescent.

The relation between fluorescence intensity of 10 and 20 μg/ml PAB in the presence of increasing concentration of curdlan and laminaran is shown in Fig. 6. The fluorescence in the presence of curdlan approached saturation at ~200–250 μg/ml but continued to show a

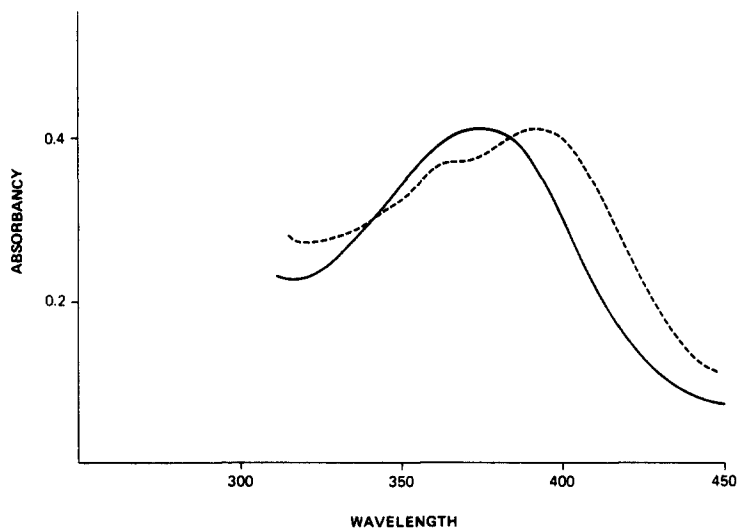

Fig. 4. Absorption spectra of partially purified component from PAB which interacts with curdlan in 0·05 M NaOH. —— In the absence of curdlan; -------- in the presence of curdlan (1 mg/ml).

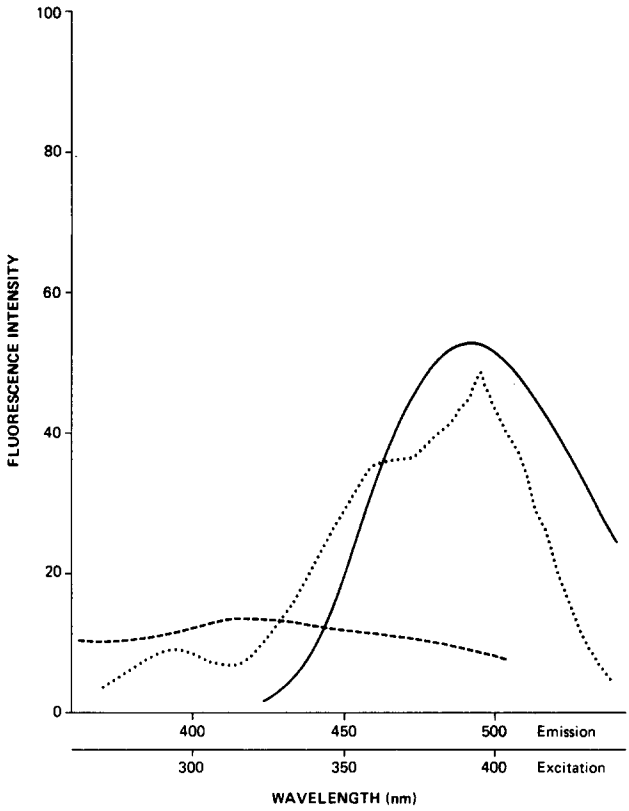

Fig. 5. Fluorescence emission and excitation spectra of partially purified component from PAB which interacts with curdlan (1 mg/ml) in 0·05 M NaOH. Emission spectra: ·------· component alone (excitation 310 nm); —— component + curdlan (excitation 395 nm). Excitation spectra (emission 495 nm): ····· component + curdlan.

steady increase with laminaran up to 4 mg/ml (the highest concentration tested).

The effect of alkali concentration is shown in Fig. 7. Both in the presence and absence of 0·5 M NaCl the fluorescence intensity at 495 nm showed a steady decrease until ~0·2–0·25 M NaOH then rapidly decreased to values essentially the same as in the absence of curdlan.

The fluorescence spectra (excitation 395 nm) of fungal cell wall extracts is shown in Fig. 8. The crude NaOH extract and water extract

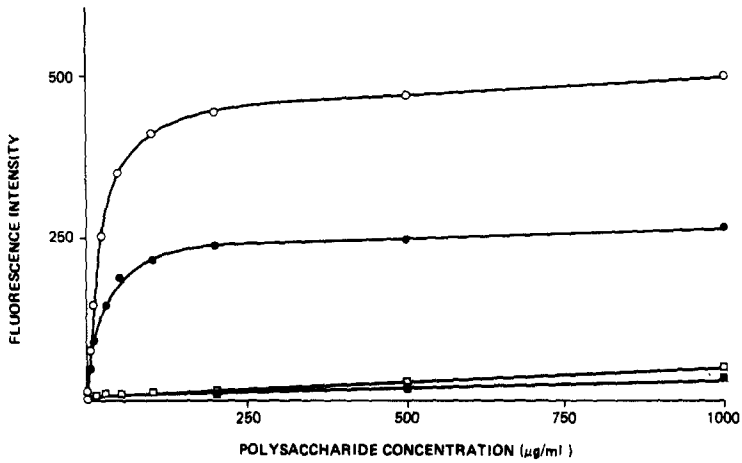

Fig. 6. Effect of curdlan (○, ●) and laminaran (□, ■) on fluorescence intensity (emission 495 nm, excitation 395 nm) of PAB in 0·05 M NaOH. Open symbols 20 μg/ml, closed symbols 10 μg/ml.

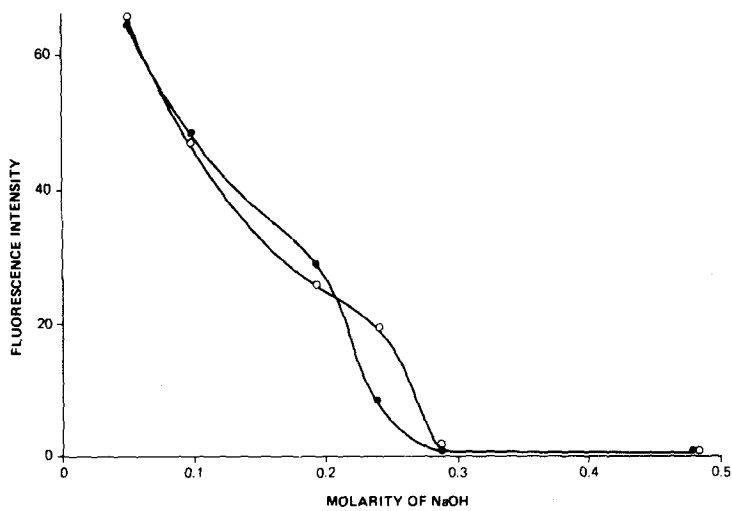

Fig. 7. Effect of concentration of NaOH on fluorescence intensity of PAB in the presence of curdlan (250 μg/ml) in 0·05 M NaOH (●) and 0·05 M NaOH–0·5 M NaCl (○). (In the latter solvent, the curdlan concentration was 1·2 mg/ml for 0·05 and 0·1 M NaOH.)

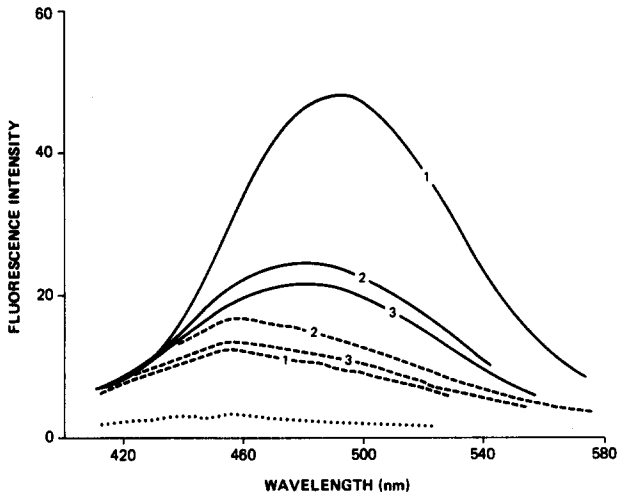

Fig. 8. Effect of fungal cell wall extracts on fluorescence spectra of PAB (10 μg/ml). 1, KOH extract (500 μg/ml); 2, crude 0·05 M NaOH extract; 3, H_2O extract (250 μg/ml). —— Sample + PAB; -------- sample alone; ····· PAB alone.

induced a small increase in fluorescence intensity of PAB with excitation at 395 nm but because of the fluorescence of the samples alone the emission peak was shifted to ~480. The KOH extracted fraction, however, induced a major emission peak at 495 nm, with an RFI of about 20 (after allowance for sample fluorescence).

Histochemically, aniline blue induced intense yellow-green fluorescence in distinct, cell wall-associated barley endosperm structures (Fig. 9). In the sub-aleurone region, the deposits were roughly spherical and aggregated, ranging from 5 to 30 μm in diameter (Fig. 9(a)). In the mid-endosperm (Fig. 9(b)) they were also associated with the cell walls but were much smaller (0·2–1 μm). Microspectrofluorometric analysis (Fig. 10) of the PAB-stained sub-aleurone structures demonstrated a well defined excitation peak at 395 nm (emission ~520 nm) and an emission maximum at 495 nm (excitation 365 nm).

DISCUSSION

Previous investigations of the affinity of aniline blue for cell wall structures, possibly (1→3)-β-D-glucan, have examined tissue sections mostly

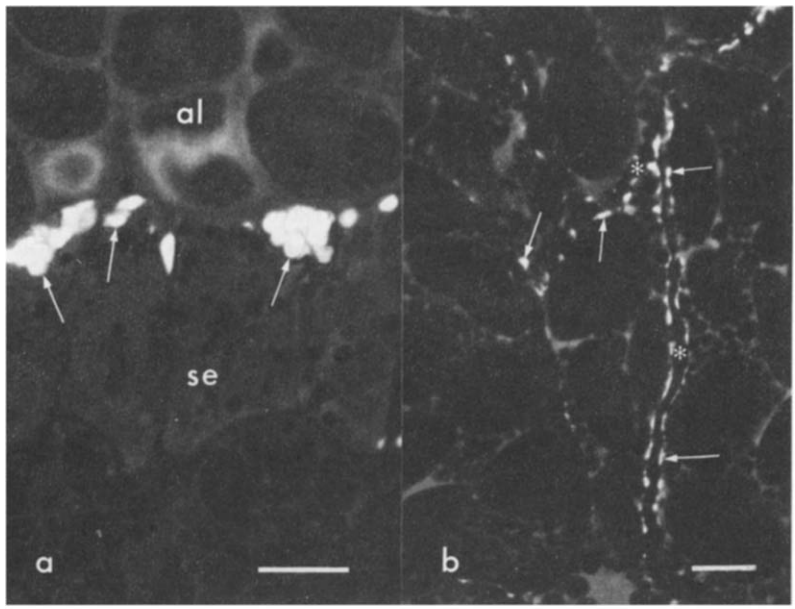

Fig. 9. Sections of barley endosperm after PAB staining showing large deposits of aniline blue fluorescent material (arrows) at the starchy endosperm (se) and aleurone layer (al) junction (a), and smaller structures (arrows) associated with cell walls (*) in the mid-endosperm (b). Scale bars equal 35 μm.

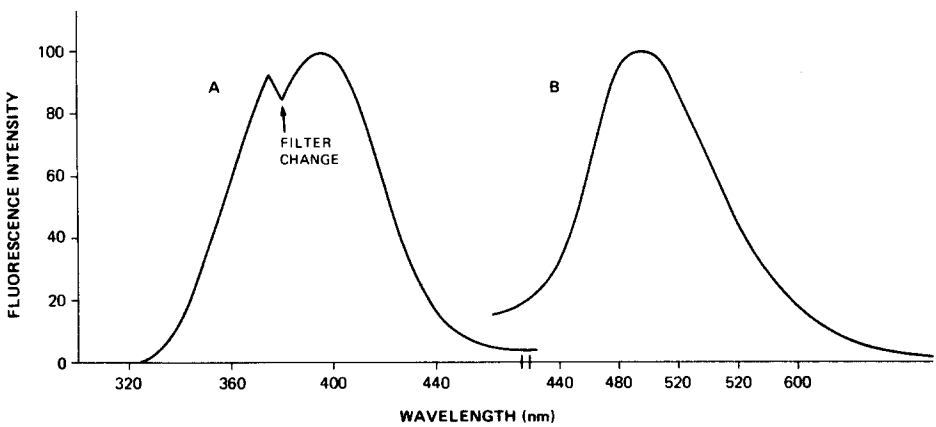

Fig. 10. Fluorescence spectra obtained by microspectrofluorometric measurements on aniline blue 'positive' deposits in barley. (A) excitation spectra (emission 520 ± 28 nm); (B) emission spectra (excitation 365 ± 10 nm).

or solid polysaccharide samples (Faulkner *et al.*, 1973; Fulcher *et al.*, 1977; Smith & McCully, 1978). In both these approaches quantitative measurement is not possible without sophisticated instruments, and adsorption characteristics of isolated fractions are difficult to predict. The present study relied heavily upon solution measurements to investigate the nature of aniline blue–polysaccharide interactions. Previously, attempts to study interaction in solution have been reported by Nakanishi *et al.* (1974) and Smith & McCully (1978). Are such solution studies relevant to the heterogeneous reactions taking place with fixed and embedded tissue? Smith & McCully (1978) pointed out the dangers inherent in extrapolating from such model systems to tissue sections, where a complex array of various hydrophilic/hydrophobic surfaces, component densities and embedding resin distributions might affect binding capacities.

However, contrary to these arguments, there are sound theoretical reasons to suppose behaviour in solution might be relevant to the solid state and hence tissue sections. An ordered triple helical conformation for (1→3)-β-D-glucans has been demonstrated for the solid state by X-ray diffraction and such conformational features may be required by the plant for biological functionality (Marchessault & Deslandes, 1979; Bluhm *et al.*, 1982). (1→3)-β-D-glucans may also possess ordered structure in solution (Bluhm *et al.*, 1982) and the helical conformation (and formation of triple helices) has been postulated as responsible for many of this polysaccharide's properties in solution, including dye-binding (Ogawa *et al.*, 1973). Conformational order in the polysaccharide may thus be the common factor connecting histochemical observations to solution properties.

Our results, like those of Smith & McCully (1978), demonstrated that commercial supplies of aniline blue have differing purities. Since the major fluorescence peak ($\lambda_{max} \sim 450$ nm) was not decreased in intensity by the addition of curdlan (Fig. 2) despite the appearance of a major new fluorescence peak (excitation 395 nm, emission 495 nm) (Figs 1 and 3), and since the absorption spectra of the dye in the presence of curdlan remained essentially unchanged (but with a detectable small increase in absorbancy at 395 nm) it seemed likely that the major changes observed in the fluorescence spectra on addition of curdlan were caused by a minor component of the dye, which is intensely fluorescent in the presence of curdlan. The difference between the three dyes supported this contention, and clearly demonstrated the

need for careful choice and description of dye. Our results agree with Smith & McCully (1978) who found that their Polysciences product was the best source of the reactive component.

It is important to note, however, that this component is not, by itself, highly fluorescent. Possibly this explains the use by Smith & McCully (1978) of rather high concentrations of their 'fluorochrome' fraction for measurement of the fluorescence spectra. Unfortunately, for solution studies with additives (glucose and cellobiose) at alkaline pH (8·5) these workers only did absorbancy measurements, whereas with added tannic acid they did fluorescence measurement, but at pH 7. Consequently the remarkable and unmistakable appearance of intense fluorescence (at 495 nm with 365–395 nm excitation) in the presence of (1→3)-β-D-glucan was not observed. Nevertheless, the absorption spectra and weak intrinsic fluorescence of the component partially purified in this study suggest it may be identical to the 'fluorochrome' isolated by Smith & McCully (1978).

To do these studies with curdlan it was necessary to use alkali to dissolve the polysaccharide. Fortunately this coincides with the common histochemical use of 'decolourised' aniline blue at alkaline pH. In 0·05 M NaOH, aniline blue is essentially colourless, and these studies are concerned solely with the component that fluoresces with curdlan in alkali, and with the fluorescent staining of (1→3)-β-D-glucan. Although it is reasonable to suppose some relationship between these observations and those of Nakanishi *et al.* (1974) on the behaviour of the blue dye at neutral pH, and the conventional blue staining of curdlan (Nakanishi *et al.*, 1976) and other (1→3)-β-D-glucans (Kessler, 1958) the nature of the connection is not clear at present.

Some interaction between the major component of the dye (emission ~450 nm) and curdlan was indicated by hypsochromic shifts and/or fluorescence intensity increase, in 0·05 M NaOH, with or without 0·5 M NaCl. Similar hypsochromic shifts and increases in fluorescence emission intensity, not accountable for in terms of sample alone, were observed for some other polysaccharides, most notably O-(hydroxyethyl)cellulose and starch. Indeed, in 0·05 M NaOH–0·5 M NaCl, starch induced the greatest increase (~2-fold) of any polysaccharide tested, including curdlan, at these wavelengths. However, no significant dye-binding by starch was observed histochemically. The fluorescence intensity of the major component of PAB in the presence of some polysaccharides was less than the sum of fluorescence intensities of

dye alone plus polysaccharide alone. The results with this area of the spectrum were therefore not easy to interpret other than that interaction was evident with (1→3)-β-D-glucan but also with some other polysaccharides. There was no clearly defined specificity of interaction.

In contrast the interaction of the minor component (excitation 395 nm, emission 495 nm) was highly specific for (1→3)-β-D-glucan (Table 3). Fluorescence was weak in the presence of the low molecular weight (1→3)-β-D-glucan, laminaran (Fig. 6), and decreased with increasing alkali concentration until, at 0·5 M NaOH there was little interaction evident (Fig. 7). The high specificity of the interaction and the requirement for high molecular weight and low alkali concentration all suggest that an ordered conformation of the polysaccharide is required for dye-binding. This agrees with published data for congo red interaction with the (1 → 3)-β-D-glucan, lentinan (Saito *et al.*, 1979), but the transition was not sharp, as reported for curdlan and congo red (Ogawa *et al.*, 1972). However, the *rate* of fluorescence decrease was more rapid in the region 0·2–0·25 M NaOH (Fig. 7). This inflexion point appeared to be delayed by the presence of 0·5 M NaCl, indicative of suppression of coulombic repulsions from alkali-induced ionisation. Interaction with the anionic CMP was increased by addition of salt (Table 3).

Having established a specificity of interaction in solution it was of interest to determine if interaction with aniline blue could be used to detect (1→3)-β-D-glucan in crude extracts from plants and fungi. Crude extracts from barley contained too much interfering fluorescent substances, but extracts from the hyphal cell walls of *Fusarium sulphureum* (known from Schneider and Barran's (unpublished) work to contain (1→3)-β-D-glucan) clearly indicated the presence of (1→3)-β-D-glucan.

Interaction of aniline blue with (1→3)-β-D-glucan is easily detected with as little as 5 μg/ml of curdlan (16-fold increase in fluorescence intensity, see Fig. 6) providing a potentially highly sensitive and specific assay system. Different (1→3)-β-D-glucans may be less easily detected, i.e. laminaran requires 50–100 μg/ml for reliable increases in intensity.

Recent studies from these laboratories (Wood *et al.*, 1983) have used isolated cereal cell wall fractions to establish the specificity, histochemically and in solution, of the interaction of congo red and calcofluor with (1→3),(1→4)-β-D-glucan in cereal grains. We have not similarly isolated the (1→3)-β-D-glucan deposits of barley (seen in Fig. 9), but by means of microspectrofluorometry, have obtained the

reflectance fluorescence spectra from the PAB stained bodies in tissue sections. The average excitation and emission spectra observed corresponded exactly to observations in solution.

In conclusion, changes in the fluorescence spectra of aniline blue may be used to detect dye-binding by (1→3)-β-D-glucan in solution. The interaction is specific for (1→3)-β-D-glucan, and corresponds to histochemical staining of endosperm cell wall associated particles in barley. These results further identify these particles as (1→3)-β-D-glucan, which is now known, from chemical studies, to be present in barley. The very high specificity of interaction in solution suggests that, with care (or by isolation and identification of the active dye component) aniline blue fluorescence may be used histochemically to locate (1→3)-β-D-glucan in a variety of tissues. (As has been done heuristically by biologists for many years.) Interpretation of observed staining should be confirmed by application of a secondary specific technique (such as enzyme digestion).

It is possible that confusion has arisen in the past from lack of distinction between bright field and fluorescence microscopy, from differences between dye sources and from difficulty in distinguishing between the major component's interaction with a variety of structures compared to the minor component's specific affinity for (1→3)-β-D-glucan. A rigorous assessment of the histochemical specificity of aniline blue, however, cannot be claimed here. Isolation of the active dye component in larger quantities, and application of solid state techniques, including microspectrofluorometry, would be worthwhile further studies.

(1→3)-β-D-glucans are currently of considerable interest from the point of view of both fundamental (conformational) aspects, and potential industrial and medical applications. Induced fluorescence of aniline blue may prove useful in further studies of this ubiquitous polysaccharide.

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