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Binding of Methylene Blue to Alginate: Effect of Acid Treatment on Metachromasy

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SUMMARY

The spectral properties of methylene blue (MB) in solutions of Na alginate depend on the severity of prior acid treatment of the polysaccharide. The spectral properties affected are the fraction of MB in monomeric form, the relative amounts of metachromatic dye absorbing near 570 and near 595 nm, and the intensity and sign of circular dichroism (CD) activity associated with the 570 nm bands, at various ratios of polymer equivalents to dye (P/D) *from 1300 to 4. Acid treatment consisted of reaction of dry, alcohol-precipitated and presumably native alginate with 0·3 M HCl at room temperature for 5 min to 8 h. Acid-induced changes showed immediate (5 min) and slow (4–8 h) stages. In both stages the fractions of MB in monomeric form and in the 595 nm metachromatic form increased. CD activity was little affected by brief acid treatment (except in range* P/D = *165 to 55), but diminished at all* P/D *values on prolonged acid treatment. Minor changes were observed in the infrared spectra of alginate films. Fresh alcohol-precipitated alginate, untreated with acid, did not precipitate when dye was in excess, nor did it form gel beads in* $CaCl_2$ *solution. It is concluded that dilute acid treatment alters the stereospecific properties of native alginate, perhaps by inducing conformational changes in the constituent copolymer segments.*

INTRODUCTION

In a recent paper, absorption and CD spectra of methylene blue (MB) bound to five commercially prepared samples of Na alginate were described (Seely & Hart, 1979). In the range $P/D = 1$ to 1000, the

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position and intensity of metachromatic dye bands varied, and some samples showed strong CD bands while others showed weak bands or none. It was soon discovered that mild acid treatment (0·3 M HCl at room temperature for a few hours) of an alginate sample altered absorption bands in solutions subsequently prepared with dye, and weakened or obliterated CD phenomena. Since virtually all commercial preparations of alginate include an acid treatment step, we surmized that our commercial samples were already altered to some extent by acid, and that the differences observed in dye spectral properties owed as much to the severity of acid treatment during preparation as to the copolymer composition of the alginate.

To test this inference, it was necessary to examine a sample of Na alginate that had not been treated with acid at any stage of its preparation. We report here the absorption and CD spectral properties of MB bound to such a sample at *P/D* ratios from 1310 to 0·19, and the effects of prior treatment with acid on these properties. The results, and some observations on infrared spectra and gel-forming properties, lead to the conclusion that in some respects alginate prepared without the use of acid has very different binding properties from alginate prepared in the usual commercial way.

MATERIALS AND METHODS

The sample of Na alginate, prepared without the use of acid, was made available to us by Dr P. A. Sandford of the Kelco Company, San Diego, California. It had been prepared by extraction of seaweed (*Macrocystis pyrifera*) with Na_2CO_3 solution, filtration through Perlite, and precipitation with isopropanol. As preliminary handling of this material indicated an assay of less than 60% alginate, and furthermore its solutions were unstable, purification procedures were worked out.

The sample used in the survey of spectral properties of bound MB was purified by precipitating twice more from isopropanol–water, the first time in the presence of Na_2EDTA to sequester ions that might promote crosslinking or air oxidation. Analysis of the dried material (30·18% C, 4·10% H, 0·00% N) compared favorably with that calculated for $NaC_6H_7O_6 . 2H_2O$ (30·77% C, 4·73% H). Recovery was about 60%.

A somewhat more elaborate procedure, designed to eliminate neutral polysaccharide impurities, was used in the preparation of samples for

testing the effect of acid on dye-binding properties. Alginate (5 g) with Na_2EDTA (3·8 g) in 150 ml of water was precipitated by addition of 200 ml of isopropanol. The gel, after filtration and washing with methanol, was dissolved in water (150 ml) and precipitated with 200 ml of 0·1 M $CaCl_2$. The Ca alginate gel was treated with Na_2CO_3 (2·22 g) and filtered to remove the $CaCO_3$ residue. Alginate was precipitated twice by addition of isopropanol and dried. Recovery 2·88 g (58%); analysis: 30·54% C, 4·23% H, 0·02% N.

Alginate solutions were analyzed gravimetrically by precipitation with hexadecylpyridinium bromide (Scott, 1965). Typical analysis: 62·90% C, 9·60% H, 2·58% N; calculated for $C_{16}H_{33}{}^{+}NC_5H_5 . C_6H_7O_6^{-} . 2H_2O$, 62·88% C, 9·58% H, 2·72% N.

The copolymer block composition of the alginate sample was determined by the partial hydrolysis procedure of Haug *et al.* (1974) with the result: 44·9% poly(man), 17·0% poly(gul) and 38·1% poly(gul-man), where man = mannuronate residues, gul = guluronate, and poly(gul-man) is the alternating copolymer.

Methylene blue was purified by chromatography on neutral alumina with ethyl acetate–n-propanol mixtures as eluent. The molar absorptivity of the monomeric form of the dye in water was taken to be 82 000 M^{-1} cm^{-1} (Bergmann & O'Konski, 1963).

Solutions for the survey of spectral properties of MB bound to alcohol-precipitated alginate were prepared by adding an aliquot of MB stock solution ($0{\cdot}94 \times 10^{-3}$ M), diluted to 5 ml, to an aliquot of Na alginate stock solution ($1{\cdot}805 \times 10^{-2}$ N) also diluted to 5 ml. Solutions to test the effect of acid treatment on these properties were prepared similarly from stock solutions of similar concentrations. The dye-alginate solutions and their properties were quite stable for days.

Absorption spectra for the former series were recorded on a Cary 14R spectrophotometer. Absorption spectra and their second derivatives for the latter series were recorded on a Perkin-Elmer Hitachi 557 spectrophotometer. CD spectra were recorded on a modified Durrum-JASCO Model J-10 Spectropolarimeter at Battelle Memorial Institute, Columbus, Ohio. Infrared spectra of alginate films were recorded on a Beckman IR20A spectrophotometer.

The spectra were analyzed in a manner similar to that used previously (Seely & Hart, 1979). The concentration of dye in a monomeric state (whether bound or unbound to the polymer) was estimated from absorbances at 640, 665 and 685 nm. The estimated absorbance of

monomer MB was then subtracted from the total absorbance at wavelengths of interest (in particular 560, 570 and 600 nm) to leave the absorbance of the metachromatic forms of the dye. The wavelength scale in second derivative spectroscopy was calibrated with solutions of $NdCl_3$, with an estimated accuracy of about ± 1 nm.

RESULTS

Spectral data for mixtures of MB and alginate in the range $P/D > 3$ are collected in Table 1. In order to put these data in context, it is necessary to summarize previous observations with commercial samples of alginate that showed strong CD phenomena.

For these it was noted that the spectrum of dye in metachromatic form, the fraction (f_1) of dye in monomeric form and the CD spectrum varied in a complicated, but characteristic and reproducible, way as P/D varied from >1000 to 1. Comparison of CD and absorption spectra under a variety of conditions led to the characterization of three distinct types of optically active metachromatic bands, observed over different P/D ranges: Type A ($200 < P/D < 1500$, abs. max. 571 nm, CD extrema 540 (+), 575 (−)), Type B ($70 < P/D < 200$, abs. max. 558 nm, CD extrema 555 (−), 610 (+)) and Type C ($10 < P/D < 70$, abs. max. 565 nm, CD extrema 563 (+), 610 (−)). There were also CD-inactive forms, with absorption maxima at 595 nm ($10 < P/D < 50$) and 570 nm ($1 < P/D < 10$).

The commercial samples with weak CD activity had metachromatic band maxima generally in the range 580–600 nm, depending on P/D, because of a strong 595 nm absorption band component. It was found that treatment of commercial samples that showed strong CD activity with cold dilute HCl led to near obliteration of CD phenomena and to increase in dye absorption around 595 nm. For these reasons the metachromatic band around 595 nm was assigned to MB bound acid-altered polymer segments. Types A, B and C spectra were provisionally assigned to MB dimers bound to copolymer sequences poly(gul), poly(man) and poly(gul-man).

The data of Table 1 in general demonstrate that MB is more bound in optically active metachromatic forms to the alcohol-precipitated alginate than to commercial alginates. The fraction of dye in monomeric form is less than 0·10 over the range $P/D < 576$ and reaches a

TABLE 1

Absorption and CD Spectral Properties of Methylene Blue Bound to Purified, Alcohol-Precipitated Alginate at Various P/D Ratios[a]

$10^3\,[Alg]/N$	$10^5\,[MB]_t/M$	P/D	f_1	λ^{ag}_{max} (nm)	$10^{-4}\,\epsilon^{ag}_{570}$ $M^{-1}cm^{-1}$	$\epsilon^{ag}_{560}/\epsilon^{ag}_{570}$	$\epsilon^{ag}_{600}/\epsilon^{ag}_{570}$	$(\Delta\epsilon_{555}-\Delta\epsilon_{600})$ $M^{-1}cm^{-1}$
5·41	0·47	1 151	0·251	584	2·26	0·855	0·987	+0·31
5·41	0·94	576	0·102	572	2·52	0·901	0·817	0·62
5·41	1·88	288	0·049	570	2·66	0·926	0·728	0·75
5·41	2·82	192	0·027	570	2·65	0·924	0·707	0·72
5·41	3·76	144	0·020	570	2·65	0·928	0·700	0·55
1·80	1·41	128	0·023	571	2·60	0·912	0·720	0·64
1·80	1·88	96	0·024	571	2·70	0·914	0·732	+0·62
1·80	2·82	64	0·016	568	2·52	0·960	0·697	−0·014
1·80	3·76	48	0·012	565	2·40	0·991	0·666	+0·62
0·90	2·82	32	0·019	564	2·37	1·002	0·683	2·87
0·90	3·76	24	0·025	566	2·27	0·981	0·707	6·13
0·45	2·82	16·0	0·063	571	2·39	0·947	0·810	6·95
0·18	1·88	9·6	0·089	576	2·34	0·922	0·883	6·38
0·18	2·82	6·4	0·076	584	2·45	0·872	0·980	5·58
0·18	5·64	3·2	0·037	574	2·59	0·892	0·804	2·82

[a] The first four column headings are: total concentration of alginate, dye, the ratio of alginate carboxylate to dye, and fraction of dye in monomeric form. The next five columns refer to the spectrum of metachromatic dye, after subtraction of the absorption of monomeric dye as described in Seely & Hart (1979): the position of the metachromatic band peak, the molar absorptivity at 570 nm, the ratios of absorptivities at 560 and 600 nm to that at 570 nm, and the difference between the circular dichroism at 555 nm and that at 600 nm.

minimum of 0·012 at $P/D = 48$. With commercial samples of similar plant origin, f_1 was never less than 0·10 except at the lowest P/D values. The metachromatic dye spectrum itself (after subtraction of the monomer spectrum) is characterized in Table 1 by four quantities: the wavelength λ^{ag}_{max} of maximum absorbance, the absorptivity ϵ^{ag}_{570} at 570 nm of dye in non-monomeric form, and the ratios of absorptivities ϵ^{ag}_{560} and ϵ^{ag}_{600} at 560 and 600 nm to that at 570 nm, which describe the shape of the band. Variation in the band position with P/D features a wavelength minimum at $P/D = 32$ and a maximum at $P/D = 6{\cdot}4$. Similar extrema have been observed with the commercial samples, but at much higher values of P/D. The ratio $\epsilon^{ag}_{560}/\epsilon^{ag}_{570}$ exceeds 1 only at $P/D = 32$; more significantly, the ratio $\epsilon^{ag}_{600}/\epsilon^{ag}_{570}$ never exceeds 1.

The CD spectrum is characterized by the difference in dichroism at two wavelengths, 555 and 600 nm. This quantity varies from a small, more or less constant value when $P/D > 128$ to a rather larger more or less constant value in the range $24 > P/D > 6{\cdot}4$, through what appears to be a cusp at $P/D = 64$. The nature of the changes in the region $96 > P/D > 32$ is illustrated in Fig. 1. CD spectra in the range $P/D > 96$

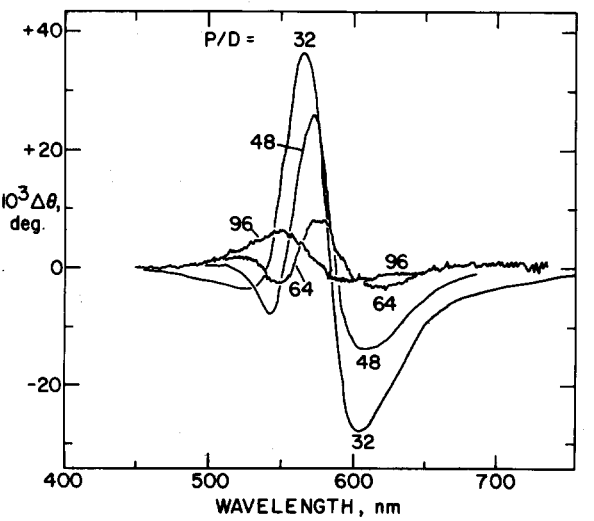


Fig. 1. CD spectra of MB with Na alginate purified by repeated alcohol-precipitation, in the region of transition from a Type A spectrum at $P/D = 96$ to a Type C spectrum at $P/D = 32$. Concentrations of MB are $1{\cdot}88 \times 10^{-5}$ M at $P/D = 96$, $2{\cdot}82 \times 10^{-5}$ M at $P/D = 64$ and 32, and $3{\cdot}76 \times 10^{-5}$ M at $P/D = 48$ (see Table 1). The ordinate is rotation in millidegrees in a 2 cm path length cell.

are similar to that at $P/D = 96$ and are characteristic of what has been called a Type A spectrum. The stronger spectra in the range $P/D < 32$ resemble the spectrum at $P/D = 32$ and are characteristic of Type C spectra. Between $P/D = 32$ and 96, there is a transition marked by incipient development of a trough near 550 nm as if to indicate superposition of a Type B spectrum on the Type A.

The alcohol-precipitated sample differed in a number of ways from even the best of the commercial samples examined. A larger fraction of dye was bound in metachromatic form, the proportion of metachromatic dye absorbing near 595 nm was much smaller, the P/D range over which the Type A spectrum was observed was increased, that over which the Type C spectrum appeared was displaced to smaller values, and that of the Type B spectrum was vestigial. All except the near absence of the Type B spectrum might have been anticipated by the assumption that native alginate contains stereospecific, strongly dye-binding sequences which become disordered on treatment with acid during commercial preparation.

TABLE 2
Absorption Spectral Properties of Solutions Prepared by Adding Purified, Alcohol-Precipitated Na Alginate to Methylene Blue, $1{\cdot}88 \times 10^{-5}$ M[a]

$10^5 \frac{[Alg]}{N}$	P/D	f_1	$\frac{[MB]_{ag}}{[Alg]}$	$10^{-4}\epsilon_{570}^{ag}$ $M^{-1}cm^{-1}$
0	0	1·00	–	–
0·36	0·19	0·83	0·89	2·38
0·72	0·38	0·73	0·71	2·58
1·08	0·58	0·62	0·67	2·71
1·44	0·77	0·52	0·62	2·71
1·84	0·98	0·44	0·57	2·70
2·34	1·25	0·37	0·51	2·69
3·61	1·92	0·24	0·40	2·69
5·41	2·88	0·16	0·29	2·66

[a] The columns list total alginate concentration, ratio of alginate carboxylate to dye, fraction of dye in monomeric form, ratio of metachromatic dye concentration to alginate, and molar absorptivity of metachromatic dye band which is at 570 nm.

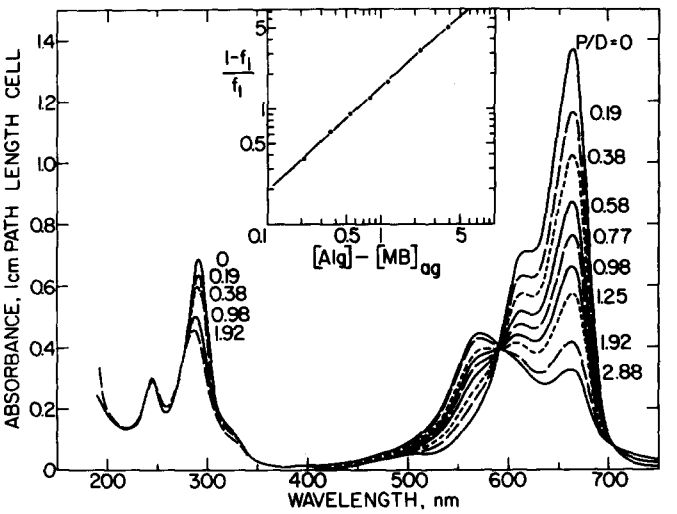


Fig. 2. Addition of purified, alcohol-precipitated Na alginate to MB solution, $1{\cdot}88 \times 10^{-5}$ M. Inset: plot of fraction of dye in metachromatic form according to empirical eqn. (1) of text.

We had previously observed that MB and alginate precipitated together when the ratio P/D approximated 1. Quite surprisingly, this did not happen with the alcohol-precipitated polymer even at $2{\cdot}8 \times 10^{-4}$ M concentration of each, and no difficulty was encountered in preparing stable solutions of MB and alginate at any concentration ratio. As an illustration of this, alginate was added in portions to a solution of MB, with the results shown in Fig. 2 and Table 2. The spectra show the large hypochromicity in the 665 and 296 nm bands during the change from monomeric to metachromatic dye. The peak of the metachromatic form is at 570 nm where its absorptivity is constant at $2{\cdot}70 \pm 0{\cdot}01 \times 10^4$ M^{-1} cm^{-1}.

In the Fig. 2 inset titration data are plotted according to an empirical equation of Yamaoka & Takatsuki (1978), which in our notation may be written

$$(1 - f_1)/f_1 = K([\text{Alg}] - [\text{MB}]_{ag})^{\alpha} \qquad (1)$$

where $[\text{MB}]_{ag}$ is the concentration of aggregated (metachromatic) dye and K and α are constants. Data for $P/D > 0{\cdot}38$ are correlated by eqn. (1) with $K = 1{\cdot}55 \times 10^5$ M$^{-\alpha}$ and $\alpha = 0{\cdot}882$.

Effect of acid treatment

In order to examine the effects of mild acid treatment, identical samples (0·45 g) of solid, purified Na alginate were treated for 0, 1, 2, 4 and 8 h with 50 ml of 0·3 M HCl at room temperature. The resulting alginic acid gels were recovered by filtration, washed well, dissolved by titration with NaOH (0·09 M) to pH 7·0 and diluted to a stock concentration of about $2{\cdot}06 \times 10^{-2}$ N. The '0 hour' sample was treated with acid only long enough (about 5 min) to allow penetration of the solid and conversion to alginic acid gel. Mixtures of MB with each of the alginate samples were prepared as before for a series of P/D ratios. A control solution (C) of untreated alginate was also prepared. Absorption and second derivative absorption spectra were recorded on all mixtures. In addition, absorption and CD spectra were recorded for the control and the 0, 2, 4 and 8 h acid treated samples at $P/D = 260$, 87 and 22, ratios chosen to represent intervals in which Types A, B and C appeared in commercial alginate (Seely & Hart, 1979).

The changes induced in the spectral properties of MB bound to alginate treated with acid for increasing lengths of time are more usefully expressed by the fraction f_1, or percent of dye which is monomeric (Table 3), and the ratio $\epsilon^{ag}_{600}/\epsilon^{ag}_{560}$ of absorptivities of metachromatic bound dye (Table 4). The former indicates the ability of the polymer to bind dye in stacked aggregates, and the latter indicates the relative amounts of dye species absorbing near 595 and 570 nm.

Inspection of Tables 3 and 4 shows that the acid-induced changes occur in two stages. The brief acid exposure involved in the '0-hour' sample was enough to increase f_1 markedly at all values of P/D except the lowest. The increase is greatest, percentagewise, in the range $110 > P/D > 18{\cdot}3$. On longer acid treatment there is a continued, gradual increase in f_1 at all P/D except the lowest. The actual values of f_1 are somewhat influenced by the total dye concentration, an effect which increases with time of acid treatment (compare values for $P/D = 165$ and 110), but the overall trend is clearly toward decreased capacity for metachromatic dye-binding as the duration of acid treatment increases.

Brief ('0-hour') acid treatment has little effect on $\epsilon^{ag}_{600}/\epsilon^{ag}_{560}$ in the ranges $1310 > P/D > 220$ and $P/D = 7{\cdot}3$–$3{\cdot}7$. In the range $165 > P/D > 55$, there is a distinct decrease in the absorptivity ratio, and in the range $37 > P/D > 11$, a distinct increase. On prolonged acid treatment the

TABLE 3

Fraction of Methylene Blue in Monomeric Form at Various *P/D* Values Before and After Treatment of Alginate with 0·3 M HCl for Increasing Lengths of Time, Expressed as Percent of Total Dye $[MB]_t$. C: Untreated Control Sample

P/D	*C*	*Duration of acid treatment (h)*					$10^5\,[MB]_t$ (*M*)
		0	1	2	4	8	
1310	21·8	31·4	26·3	29·8	32·8	42·1	0·47
660	10·4	13·2	12·1	14·7	18·2	22·4	0·94
330	4·6	7·7	7·2	7·2	10·7	16·8	1·88
220	2·5	4·5	5·6	7·7	10·0	13·6	2·82
165	2·0	3·8	5·1	5·1	8·0	11·4	3·76
110	1·1	4·1	5·0	5·7	11·0	16·3	1·88
73	0·8	3·6	3·4	6·4	12·2	15·8	2·82
55	0·7	4·2	4·0	6·9	10·8	15·4	3·76
37	0·8	6·4	6·8	10·6	13·1	17·3	2·82
27·5	1·1	9·6	8·5	11·6	13·8	13·3	3·76
18·3	3·3	11·5	11·5	11·8	13·0	14·2	2·82
11·0	6·7	13·1	11·5	13·8	15·3	13·2	1·88
7·3	6·7	8·5	8·7	8·8	9·1	8·4	2·82
3·7	4·5	4·8	5·2	4·3	3·4	3·8	5·64

absorptivity ratio increases at almost all *P/D* values as the metachromatic dye band is displaced to the red. The red shift is greatest in the range $55 > P/D > 11$.

It should be stressed that the purification procedure has little effect on the spectra of subsequently prepared solutions with MB, as long as acid is avoided. Spectral characteristics of the untreated control C, purified metathesis of Ca alginate, closely resembled those for the sample described in Table 1, which was purified by repeated precipitation with isopropanol, and those for the sample received, when its actual alginate assay was taken into account. There is therefore a basis for believing that the control samples consist of alginate essentially as it exists in nature.

Absorption (Figs 3a and 5a) and CD (Figs 3b and 5b) spectra of MB on alginate samples treated with acid for varying lengths of time illustrate more graphically the changes in these *P/D* ranges. Note that Figs 3b and 5b require different ordinate scales.

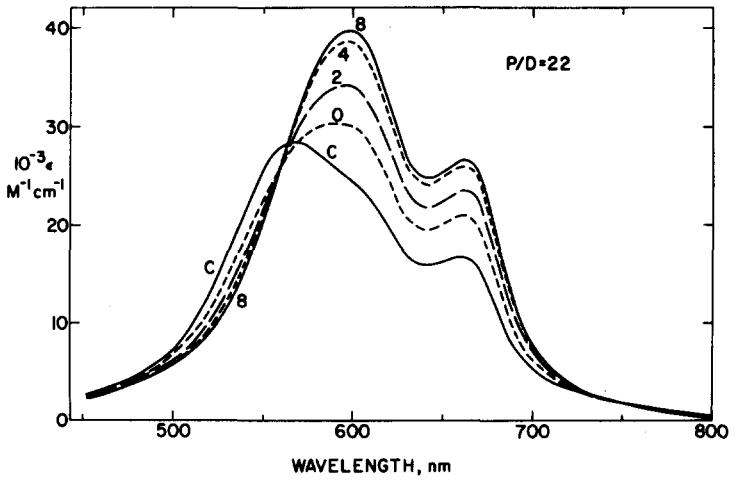
TABLE 4

Ratio $\epsilon^{ag}_{600}/\epsilon^{ag}_{560}$ of Metachromatic MB Absorptivities at 600 and 560 nm, at Various *P/D* Values Before and After Treatment of Alginate with 0·3 M HCl for Increasing Lengths of Time. C: Untreated Sample

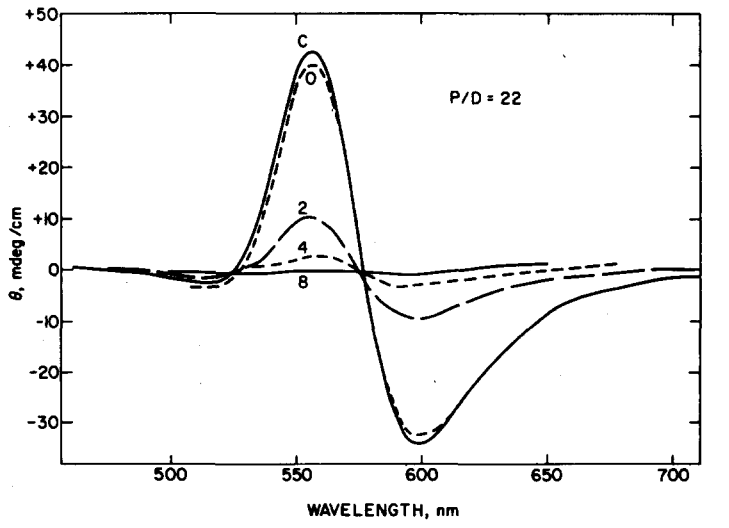
P/D	*C*	*Duration of acid treatment (h)*				
		0	1	2	4	8
1310	1·32	1·20	1·28	1·20	1·03	1·02
660	0·98	0·94	0·87	0·89	0·98	1·01
330	0·83	0·82	0·78	0·82	0·84	0·92
220	0·80	0·82	0·77	0·81	0·79	0·86
165	0·80	0·75	0·76	0·73	0·78	0·86
110	0·79	0·68	0·74	0·73	0·87	0·96
73	0·77	0·66	0·68	0·75	0·90	0·99
55	0·73	0·67	0·70	0·76	0·92	1·13
37	0·64	0·76	0·76	0·89	1·10	1·35
27·5	0·64	0·88	0·84	1·03	1·28	1·38
18·3	0·68	1·09	1·00	1·24	1·40	1·53
11·0	0·93	1·23	1·22	1·40	1·33	1·38
7·3	0·93	0·93	1·18	1·20	1·15	1·07
3·7	0·75	0·77	0·74	0·90	0·86	0·87

Changes at $P/D = 22$ are perhaps the simplest to interpret (Fig. 3a, b). Transition between the control and the '0-hour' sample is marked by decrease of absorption below 570 nm and increase around 595 nm. This shift intensifies on continued acid treatment, now with an isosbestic point at 560 nm. The CD spectrum of the control is typical of what has been called a Type C spectrum (Fig. 3b). There is little change after brief treatment with acid but on longer treatment the optical activity almost disappears. There is no CD band corresponding to the increasing absorption band at 595 nm.

At $P/D = 87$ brief acid treatment produced a small blue shift of the metachromatic dye band, and decreased absorbance (Fig. 4a). Continued acid treatment induces loss of absorbance below 570 nm, and increase above, with marked increase in the monomer dye absorption region around 665 nm. The CD spectrum of the control sample is what has been called Type B, and is *intensified* in the 0-hour sample (Fig. 4b). On continued acid treatment, the intensity of the Type B spectrum



a



b

Fig. 3. (a) Absorption spectra of MB in solutions with untreated Na alginate (C) and with alginate treated for 0, 2, 4 and 8 h with 0·3 M HCl. $[MB]_t = 2{\cdot}69 \times 10^{-5}$ M, $[Alg] = 5{\cdot}84 \times 10^{-4}$ N, $P/D = 22$. (b) Circular dichroism spectra of solutions of Fig. 3a.

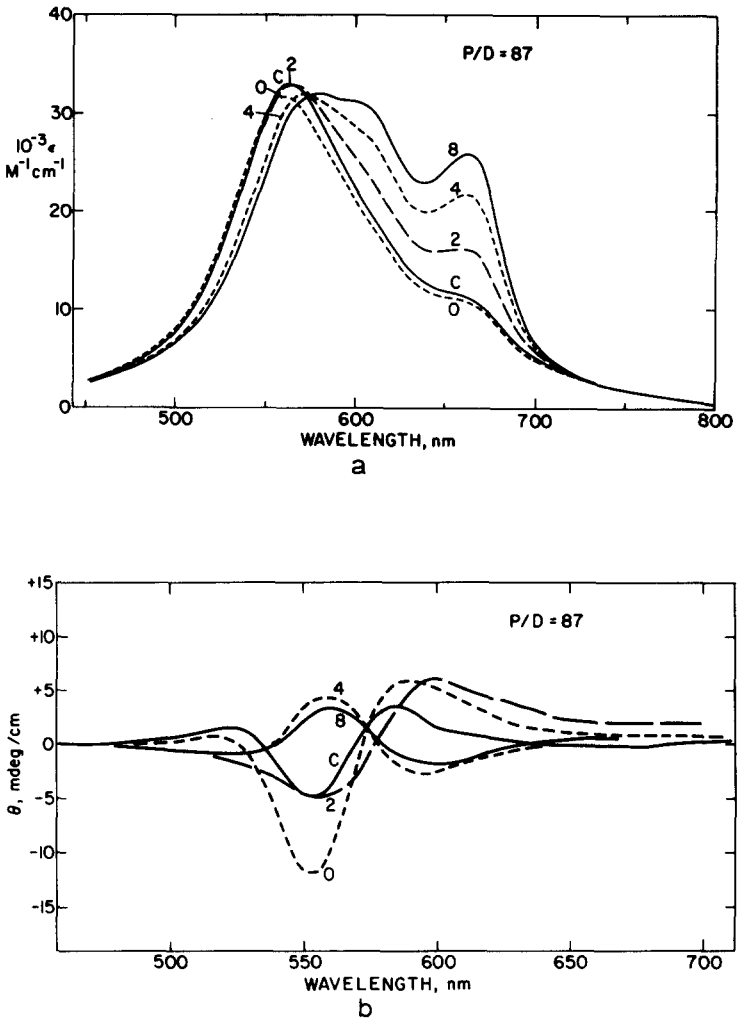


Fig. 4. (a) Absorption spectra of MB in solutions with untreated Na alginate (C) and with alginate treated for 0, 2, 4 and 8 h with 0·3 M HCl. $[MB]_t = 2{\cdot}69 \times 10^{-5}$ M, $[Alg] = 2{\cdot}33 \times 10^{-3}$ N, $P/D = 87$. (b) CD spectra of solutions of Fig. 4a.

decreases (2 h) and it is replaced by a weak Type C spectrum (4–8 h). Changes in this P/D range are clearly complex.

At the highest P/D value (260) the metachromatic dye spectrum is least affected by acid treatment, although the increase in monomer

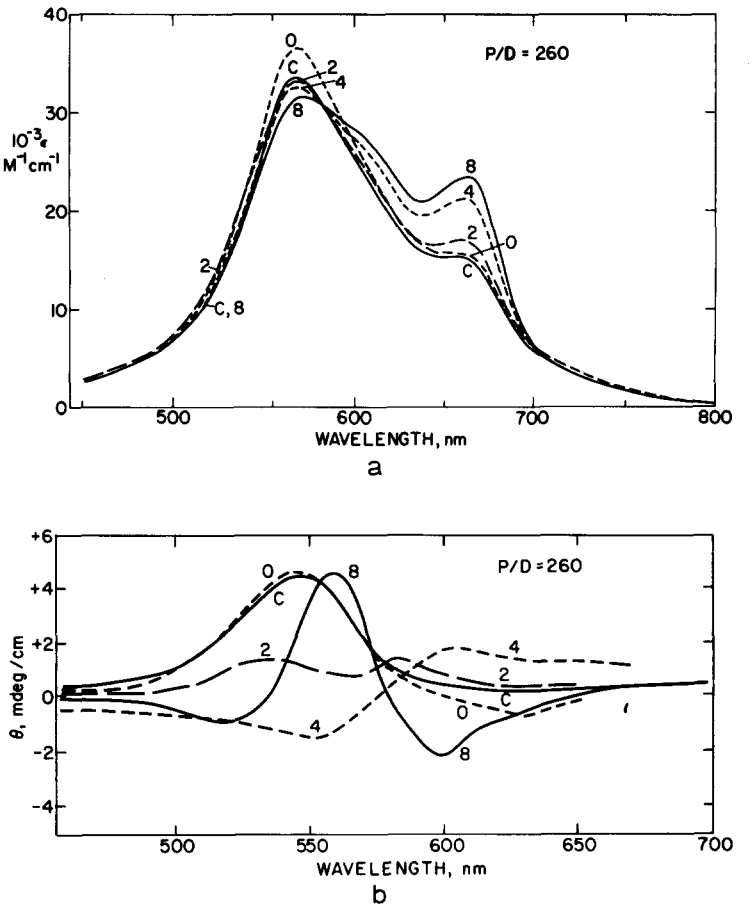


Fig. 5. (a) Absorption spectra of MB in solutions with untreated Na alginate (C) and with alginate treated for 0, 2, 4 and 8 h with 0·3 M HCl. $[MB]_t = 2{\cdot}69 \times 10^{-5}$ M, [Alg] = $7{\cdot}0 \times 10^{-3}$ N, $P/D = 260$. (b) CD spectra of solutions of Fig. 5a.

band intensity at 665 nm is evident (Fig. 5a). The CD spectrum of untreated alginate is what has been called a Type A spectrum, and is little affected by brief acid treatment (Fig. 5b). On prolonged acid treatment, the Type A spectrum fades and is replaced by a weak Type B spectrum, and then by a somewhat stronger Type C spectrum.

It would appear that polymer sequences that give rise to Type C spectra are somewhat more resistant to modification by acid than those that give rise to Types A and B spectra.

Second derivative absorption spectroscopy is an aid to determining the number of components in a complex absorption band and their location. Its application to the metachromatic spectra of MB is illustrated in two cases, for $P/D = 18$ and 73 (Fig. 6). On brief treatment with acid, the relatively smooth looking trough at 557 nm of metachromatic dye at $P/D = 18$ is converted into a partially resolved group of troughs at longer wavelengths. On prolonged treatment the component at 588 nm increases relative to the one at 572 nm. At $P/D = 73$, the blue shift of the metachromatic band on brief treatment and the red shift on prolonged treatment (as in Fig. 4a) are represented, but there is no resolution into distinguishable detail. In general, second derivative spectroscopy confirmed and extended that which could be inferred from absorption and CD spectroscopy but was not sensitive enough to resolve the metachromatic bands into their presumed components.

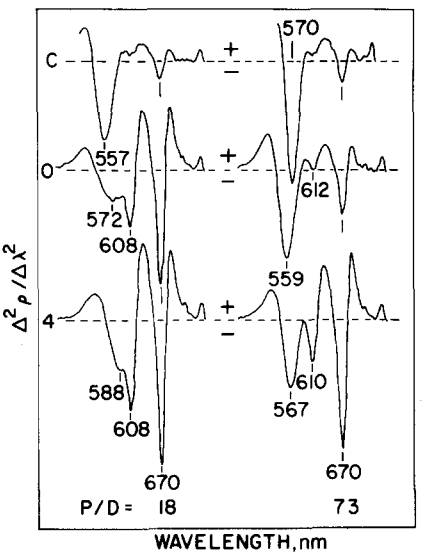


Fig. 6. Second derivative absorption ($\Delta^2\rho/\Delta\lambda^2$) spectra of MB-alginate solutions of $P/D = 18$ and 73 (see Table 3 or 4), made from untreated (C) alginate and alginate after brief (0) and 4 h treatment with 0·3 M HCl. Spectra were recorded on a Perkin-Elmer Hitachi 557 spectrophotometer, with operating parameters $\lambda \times 0{\cdot}5$ and $\Delta\lambda = 3$ nm, and a 2 mm slit. Range is 450 (left) to 750 nm; the peak at the right end of each trace is an artifact. The troughs at 670 and near 610 nm belong to the monomeric MB component.

Infrared spectra were recorded on films cast from acid-treated and untreated Na Alginate. The spectra were practically indistinguishable except in the range 750–1000 cm^{-1}, where bands characteristic of mannuronic and guluronic acid residues occur (Mackie, 1971), shown in Fig. 7. The peak of the mannuronate band shifts from 819 cm^{-1} in the untreated sample to 811 cm^{-1} after 8 h acid treatment, as compared to 810 cm^{-1} reported by Jayme & Tio (1968). Less distinct changes are suggested in the bands near 890 and 950 cm^{-1}. The comparative strengths of the mannuronate band and the guluronate band near 780 cm^{-1} suggest a man/gul ratio about 2 (Mackie, 1971), consistent with the copolymer composition analysis.

Bead formation

One of the outstanding properties of alginate solutions is their ability to form cohesive gels on contact with solutions of divalent metal ions,

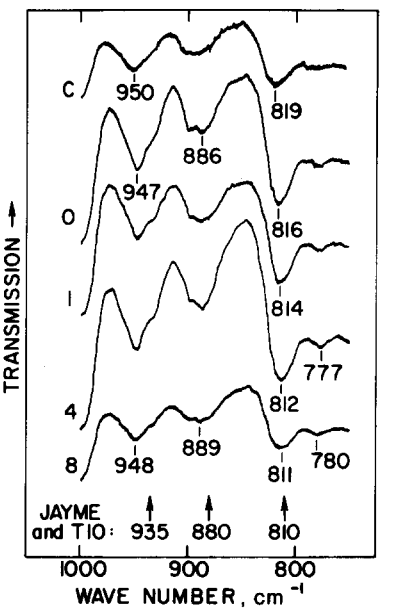


Fig. 7. Infrared spectra of films, cast from solutions of Na alginate samples, untreated (C), and pretreated for 0, 1, 4 and 8 h with 0·3 M HCl, in the range 1000–750 cm^{-1}. Positions of bands reported by Jayme & Tio (1968) are indicated at bottom.

such as Ca^{2+} or Sr^{2+}. These gels have been used to immobilize active chloroplasts (Gisby & Hall, 1980) and algae (Ochiai *et al.*, 1980), and in the form of beads have been stained with dyes to conduct photosensitized oxidation in heterogeneous phase systems (Seely & Hart, 1977). Coherent beads had formed from all commercial samples of alginate we tested earlier. It was therefore surprising to note, while purifying the alginate, that instead of beads a flocculent precipitate was formed with $CaCl_2$ solution.

We were naturally interested to determine at what stage of acid alteration the gel-forming property appears. Solutions of untreated and acid-treated Na alginate were added dropwise to 0·1 M $SrCl_2$ solution. Gel beads formed in every case, including the untreated control. However, this experiment was performed about a month after spectral data were obtained, and re-examination of dye-alginate spectra on control samples showed some alteration in the direction of increased metachromatic dye absorbance around 595 nm. It was concluded that solutions of alginate, and possibly also undissolved material, undergo a slow alteration in properties, even without acid treatment, that induces the property of forming coherent gels. Exhaustion of material has prevented confirmation of these observations.

DISCUSSION

Our investigation has shown that treatment with dilute acid at room temperature alters the stereospecific properties of alcohol-precipitated and presumably native alginate, as manifested by its ability to bind dyes in metachromatic forms. The alteration takes place in two stages, a fast one which appears to affect only part of the alginate, and a slow one which leads to profound changes in metachromasy and to near disappearance of the CD. The matter might seem of slight importance but for the following: (a) essentially all commercial alginate is prepared with the use of acid at some stage, (b) alginate is valued mainly for the viscosity and gelling properties it confers on solutions, (c) there is reason to believe that its gelling properties are altered by acid treatment along with its stereospecificity, (d) severity of acid treatment does not seem to have been recognized as an important process variable, and (e) spectra of MB bound to commercial alginate samples of different origin closely resemble those of the dye with samples of alginate treated

with acid for various lengths of time (Seely & Hart, 1979). Alginate for research purposes is also generally prepared *via* an acid-treatment step, as for example, after an overnight extraction of seaweed powder with 0·2 N H_2SO_4 (to leach out acid-soluble polysaccharides) in the standard procedure of Haug (1965). So far as we are able to determine, all samples of alginate for which data are reported in recent literature were prepared with the use of acid at some stage.

Alcohol-precipitated and presumably native alginate differs from commercial alginates in that it binds a larger fraction of dye metachromatically in optically active forms absorbing near 570 nm, it does not precipitate with dye in excess, and it appears not to form cohesive gel beads with $CaCl_2$ solution. Simply converting to alginic acid and back increases the amount of dye in the 595 nm form at $P/D = 22$ (Fig. 3a), and strengthens the Type B of CD spectrum seen at $P/D = 87$ (Fig. 4b). The strengthening of the CD spectrum appears connected with the increased absorbance around 560 nm (Fig. 4a) rather than with the changes around 595 nm. The same changes occur in neutral solutions of untreated alginate, but in a matter of weeks of storage at 5°C. The ability to form coherent gel with $CaCl_2$ appears to be coeval with these changes, and may be a property of the polymer segments to which 595 nm metachromatic MB is bound, since dye in this form is very easily displaced by divalent metal ions (Seely & Hart, 1979). These speculations require confirmation with fresh native alginate samples.

The nature of the changes induced by acid treatment is rather problematical. The mildness of the conditions under which they occur suggests conformational changes in polymer chain segments, rather than alterations in the primary structure. It would be very desirable in future work to examine the effect of acid treatment on the viscosity of alginate solutions, as an indicator of possible changes in chain configuration. The effect of acid treatment on the ability of alginate to sequester metal ions and to crosslink, and the effect of bound metal ions on the spectral properties of bound dyes, are also worthy of much more attention than it was possible to provide. Further studies with alginate from various sources should be directed toward these questions.

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