[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY AND THE STUDY GROUP ON RHEUMATIC DISEASES, NEW YORK UNIVERSITY COLLEGE OF MEDICINE]

Metachromatic Effects of Anionic Polysaccharides and Detergents[1]

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A quantitative comparison has been made of the chromotropic action of six anionic polysaccharides and two anionic detergents on two cationic metachromatic dyes of different structures. All the chromotropes studied produce effects on the α-, β- and μ-bands of both dyes which show similar trends, though each shows quantitative differences. The possible significance of the similarity in chromotropic behavior is discussed with reference to micelle formation. An attempt has been made by equilibrium dialysis to get independent evidence for the binding of some of these dyes by two anionic polysaccharides. The results indicate that methylene blue and crystal violet, which are metachromatic, are bound by the polysaccharides. Methylene green, which is scarcely metachromatic, is bound to a much smaller extent if at all. The presence of salt, which destroys metachromasy, also destroys the binding of the dyes by polysaccharides as measured by equilibrium dialysis whereas high chromotrope concentration destroys metachromasy but not dye binding.

Michaelis,[2] discussing the effects produced on the colors of aqueous solutions of metachromatic dyes by different substances, considered three classes. First, substances such as nucleic acid and alcohol slightly increased the intensity of the long wave length band (the α-band) of the dye and produced no metachromatic color; second, chromotropes, such as agar, greatly decreased the intensity of the α-band and produced the metachromatic color; finally, some substances, such as detergents, behaved like agar at low concentrations and like nucleic acid at high concentrations. These changes in band intensity were interpreted in terms of the state of polymerization of the dye. It recently has been shown that nucleic acids do produce a metachromatic color.[3]

In a previous report from this Laboratory,[4] the effect of varied concentrations of chondroitin sulfate on the absorption spectra of metachromatic thiazine dyes was studied and the results presented by a simple and quantitative method. The extinction values at one or more critical wave lengths were measured and plotted against the chromotrope concentration. In this way it was shown that with increasing concentrations of potassium chondroitin sulfate, both the α- and β-bands of methylene blue are first depressed and then rise again to their values in the absence of chrondroitin sulfate, while the μ-band first rises and then falls.

This raises the question whether different chromotropes, when studied with a single dye such as methylene blue, will produce similar or related curves and, in particular, whether the different classes of chromotropes will differ in their behavior. In the past, comparisons have been made only qualitatively and then only with crude chromotropes. In the present study, a comparison is made of six anionic polysaccharides of animal, plant and bacterial origin and two anionic detergents. In all cases, except heparin, attempts have been made to purify the chromotropes as far as current methods allow.

Having such data raises a second question. To what extent will these results apply to a metachromatic dye of a different class? A study was then made of the effects of the same chromotropes on crystal violet, a dye with an absorption spectrum similar to that of methylene blue, but with a different structure.

Finally, equilibrium dialysis experiments were begun in order to study the interaction between dyes and chromotropes by an independent method.

Experimental

The dyes used in this study were commercial products each being recrystallized two to three times from water. Chondroitin sulfate was used as the potassium salt derived from the crystalline calcium salt.[4] The equivalent weight used was 293 for the potassium salt. Hyaluronates, prepared from human umbilical cord and Group A, type 36 streptococcus, were used as the potassium salts. The equivalent weight was assumed to be 417. Heparin was used as the isotonic solution of the sodium salt supplied in the commercial product Liquamin containing approximately 10 mg. per ml. Assuming an equivalent weight of 191, this solution was regarded as containing 0.05 meq. per ml. Sodium alginate was prepared as follows from the commercial product Kelacid.[5] To 10 g. of Kelacid suspended in water (1 l.), enough saturated NaOH solution (4 ml.) was added dropwise to dissolve it completely. The solution was filtered and the sodium salt precipitated by addition of an equal volume of alcohol. The flocculent product was centrifuged and washed with absolute alcohol and absolute ether. The dry material, 10.8 g., was easily soluble in water. It had an ash as sulfate of 23.3% and its equivalent weight was assumed to be 198. Agar was purified by dissolving crude commercial material (5 g.) in hot water (3.5 l., about 70°) and filtering with suction through Filtercel. To the clear solution was added potassium oxalate (0.5 g.) and potassium acetate (10 g.) and after several hours it was filtered from a small deposit. The solution was then acidified with acetic acid (5 ml.) and stirred with three successive portions of kaolin (10 g. each), being filtered each time. The clear solution, shaken with amyl alcohol and chloroform, produced almost no gel. After dialysis, the solution was evaporated *in vacuo* to 180 ml. and excess potassium acetate (10 g.) added. The product was precipitated with six volumes of absolute alcohol and yielded 1.3 g. of dry material. The equivalent weight was based on analysis by Jones and Peat[6] and was assumed to be 1738. Analysis of our material gave the following results: Crude agar: N, 3.3; S, 1.0; ash as sulfate, 3.5. Purified agar: N, 0.2; S, 1.1; ash as sulfate, 18.7.

Potassium myristate was prepared from myristic acid and recrystallized twice from alcohol. It had an ash of 29.3% and its equivalent weight was assumed to be 266. The Duponol used was prepared from the commercial preparation, Duponol C, which had an ash of 22.5%. This was dissolved in boiling ethanol (6 g. in 300 ml.) and the solution, after filtering from some undissolved residue, was chilled at 0° for several days. The crystalline product was centrifuged cold and washed with cold alcohol and ether. The

(1) This work was supported in part by the United States Public Health Service and in part by the Masonic Foundation for Medical Research and Human Welfare.

(2) L. Michaelis, *J. Phys. Colloid Chem.*, **54**, 1 (1950).

(3) N. Weissman, W. H. Carnes, P. S. Rubin and J. Fisher, THIS JOURNAL, **74**, 1423 (1952).

(4) A. Levine and M. Schubert, *ibid.*, **74**, 91 (1952).

(5) Kelacid was kindly supplied by the Kelco Co. of New York.

(6) W. G. M. Jones and S. Peat, *J. Chem. Soc.*, 225 (1942).

yield was 2.7 g. with an ash of 20.5%. Its equivalent weight was assumed to be 288. Cetyltrimethylammonium chloride was used as the commercial product without further purification.

Since previous work has shown that acids and salts are effective in destroying metachromasy, all studies were made in unbuffered solutions. The dye and chromotrope made up in stock solutions were mixed to give a final dye concentration of 1.25×10^{-5} M and the desired concentration of chromotrope. The absorption spectra of the dye solutions were then determined at a series of chromotrope concentrations. Each solution was read in a Beckman model DU spectrophotometer using 1-cm. corex cuvettes. The optical densities were determined not more than two hours after the solutions were prepared.

The unit of chromotrope concentration chosen differs from that used in the previous study. Instead of the period weight, the equivalent weight is used which is the weight of the repeating unit divided by the number of anionic groups per unit. At any chromotrope concentration, the number of anionic groups per unit volume will therefore be the same for different chromotropes.

A series of equilibrium dialyses was carried out in cellophane tubing with a diameter of about 20 mm. suspended in stoppered 250-ml. erlenmeyer flasks. Inside the tubing were placed 14 ml. of methylene blue, crystal violet or methylene green at a concentration of 1.25×10^{-5} M together with potassium chondroitin sulfate or sodium alginate and with or without potassium chloride at the desired concentration. Outside the bag were 90 ml. of dye solution also at 1.25×10^{-5} M and potassium chloride when desired. Equilibration was brought about by gentle mechanical shaking for 24 hours. In the course of these dialysis experiments, some dye is fixed by the cellophane tubing and flasks. The amount varies with conditions and has been measured by separate elution with dilute HCl. The dye fixed by the cellophane varies between 10 and 50% of the total dye in the system while that fixed by the flasks varies between 5 and 10%. The dye fixed by cellophane and glass is considered not to affect the equilibrium between the dye in solution inside and outside the bag.

In these experiments, the dye concentrations cannot be determined from the optical density of the solutions at a given wave length until the metachromatic color resulting from the presence of a chromotrope is destroyed. This was done by the addition of one drop of concentrated HCl or, in the case of crystal violet, approximately 8 mg. KCl, to 6 ml. of dye solution after removal from the bag or flask. As standards, a series of dye–chromotrope mixtures were made up covering the range of concentrations used during the equilibrium dialysis. To each of these, HCl was added as above before determining the optical density. The resulting curves, relating optical density and dye concentration, were practically independent of the amount of chromotrope present. The deviations from Beer's law were small enough to be neglected over the concentration range involved.

Results

The α-, β- and μ-bands of methylene blue have been derived from Fig. 1 of our previous work[4] and correspond, respectively, to the wave lengths 665, 610 and 570 mμ. These wave lengths are the positions at which maxima most frequently appear as the chromotrope concentration is varied. The α-, β- and μ-bands of crystal violet have been derived from a similar set of measurements on the absorption spectra in the presence of increasing concentrations of chondroitin sulfate and have been found at wave lengths 590, 550 and 510 mμ, respectively.

Examples of molar extinctions of methylene blue and crystal violet at their respective α-, β- and μ-bands are plotted against the log of the chromotrope concentration in Figs. 1 to 4. All six anionic polysaccharides as well as the two anionic detergents studied behave qualitatively alike. In general, the effect of increasing the chromotrope concentration causes a drop followed by a rise in the extinction of the α- and β-bands, and in most cases a rise followed by a drop in the extinction of the μ-bands. The only exception is bacterial hyaluronate which produces no effect on the extinction of the β-band of methylene blue at any concentration as indicated in Table I. At the highest chromotrope concentrations reached, the heights of some of the bands have returned to the neighborhood of their values in the absence of chromotrope, while in the case of Duponol C, the α-band of methylene blue has risen to levels well above that value.

TABLE I

PER CENT. CHANGE IN EXTINCTION OF METHYLENE BLUE AND OF CRYSTAL VIOLET AT THEIR α, β- AND μ-BANDS PRODUCED BY OPTIMAL CONCENTRATIONS OF DIFFERENT CHROMOTROPES

	α-Band		β-Band		μ-Band	
Chromotrope	Methylene blue	Crystal violet	Methylene blue	Crystal violet	Methylene blue	Crystal violet
Heparin	−83	−65	−43	−40	+130	+96
Agar	−60	−42	−38	−21	+75	+87
Alginate	−62	−30	−30	−13	+52	+28[a]
Chondroitin sulfate	−53	−51	−33	−28	+80	+125
Umbil. cord hyaluronate	−56	−33	−27	−21	+90[a]	+56
Bacterial hyaluronate	−37		0		+60	
Myristate	−69	−59	−57	−37	+10[a]	+9[a]
Duponol	−63	−52	−30	−26	+70	+23
Cetyltrimethylammonium bromide	+15		+16		+10	

[a] When no absolute maximum is present, per cent. rise in the μ-band is calculated at the concentration at which the minima of the other bands appear.

The similarity in the behavior of all the chromotropes is subject to two kinds of variation. The position along the abscissa at which the greatest depression in extinction occurs varies somewhat but lies in all cases between the values −2.5 and −5. The concentration of the dyes expressed in similar units is −4.90. Thus, maximum metachromasy is produced at a chromotrope–dye concentration ratio lying in the range from 1 to 300. There is, of course, some uncertainty with respect to the equivalent weights chosen for the chromotropes, but in all cases except agar, this would produce no noticeable effect on the abscissa values which are logarithmic. The second kind of variation is the maximum extent of depression of the α- and β-bands or elevation of the μ-band. These data are summarized in Table I and show the spread in these values for the same bands with different

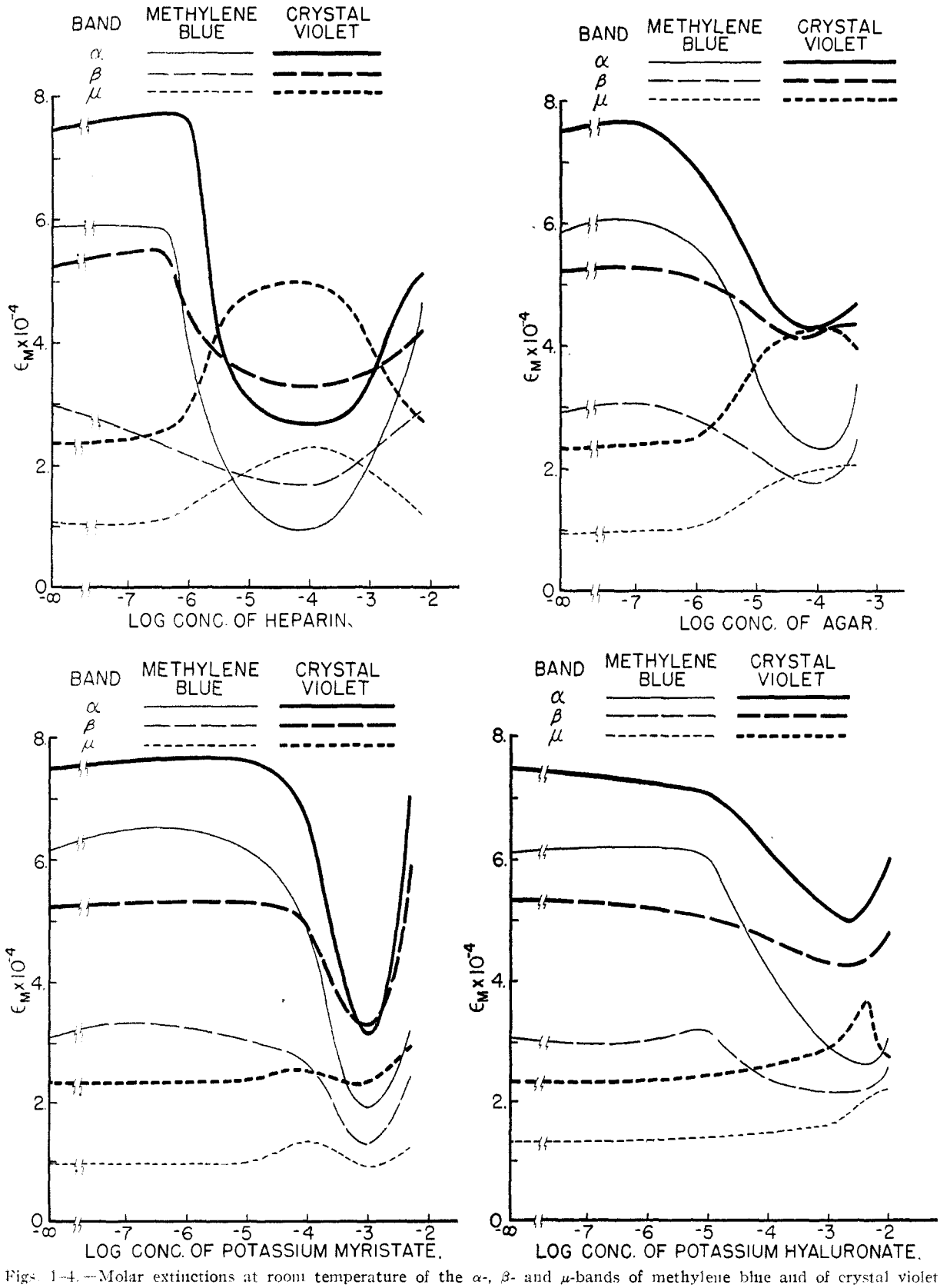
TABLE II

RESULTS OF EQUILIBRIUM DIALYSIS EXPERIMENTS

M.B. = methylene blue; C.V. = crystal violet; M.G. = methylene green.

Chromotrope concn. inside, meq./l.	Dye used	KCl concn., mole/l.	Final dye concn. inside, meq./l.	Final dye concn. outside, meq./l.	Dye inside / Dye outside
Chromotrope, potassium chondroitin sulfate					
0.00	M.B.	0	0.0078	0.0055	1.4
0.27	M.B.	0	.0297	.0032	9.3
2.00	M.B.	0	.0471	.0019	24.8
0.00	M.B.	.0005	.0091	.0090	1.0
0.27	M.B.	.0005	.0317	.0062	5.1
0.00	M.B.	.01	.0114	.0121	0.9
0.27	M.B.	.01	.0133	.0121	1.1
0.00	C.V.	0	.0092	.0086	1.1
0.27	C.V.	0	.0500	.0047	10.6
2.00	C.V.	0	.0576	.0022	26.2
0.00	M.G.	0	.0118	.0092	1.3
0.27	M.G.	0	.0166	.0081	1.8
2.00	M.G.	0	.0370	.0053	7.3
Chromotrope, sodium alginate					
0.00	C.V.	0	0.0092	0.0086	1.1
0.13	C.V.	0	.0137	.0071	1.9
2.00	C.V.	0	.0502	.0038	13.2[a]

[a] Dye-chromotrope preciptates at this concentration.



Figs. 1–4.—Molar extinctions at room temperature of the α-, β- and μ-bands of methylene blue and of crystal violet (1.25×10^{-5} *M*) plotted against increasing concentrations of chromotrope as log equivalents per liter.

chromotropes. About the only correlation that it seems possible to draw is that the extent of the effect on the β-band is almost always about half that on the α-band. Except for bacterial hyaluronate and myristate this ratio lies in the range 0.43 to 0.64.

In contrast to the effect of the anionic detergents is that

of the cationic detergent, cetyltrimethylammonium bromide. At no concentration does this substance produce a depression of the α- or β-bands of methylene blue; on the contrary it produces a rise amounting to only 15% at the α- and β-bands and a still smaller rise at the μ-band.

The results of the equilibrium dialysis experiments are summarized in Table II. With increasing concentrations of chondroitin sulfate inside the bag to a value of 2.0 meq./l., there is an increase in the ratio of dye inside to dye outside to a value of 25 for either methylene blue or crystal violet. Sodium alginate produces a similar though perhaps less marked effect with crystal violet; the extent of this effect is rendered doubtful because of the beginning of precipitation of crystal violet with the top concentration of alginate. Methylene green is also a cationic dye but shows no deviations from Beer's law and scarcely any metachromatic spectral changes in the presence of chondroitin sulfate. The data in Table II show that methylene green becomes concentrated inside the bag in the presence of chondroitin sulfate to a ratio of about 30% of that of methylene blue or crystal violet.

In our previous work it was shown that salts at concentrations of 0.01 M completely destroy metachromasy. In Table II are figures showing that 0.0005 M KCl cuts to half the concentration ratio of methylene blue inside and outside the bag when chondroitin sulfate is present, while 0.01 M KCl suppresses the concentrating effect entirely.

Discussion

Our experiments have shown that the anionic polysaccharides and detergents all have essentially parallel effects on the α-, β- and μ-bands of methylene blue and crystal violet. For any one chromotrope, the curves for both dyes often show a striking coincidence of maxima and minima of each band along the abscissa. This simple generalization is true only in dilute aqueous solutions in the absence of added salts. It was not expected from previously existing data based on the action of metachromatic dyes on heterogeneous tissue sections. Even when studies in more or less homogeneous solutions or agar gels were made, there was little systematic variation of conditions such as concentration in comparing different chromotropes. Buffers were commonly used in concentrations as high as 0.1 M, but we now know that salts in general at concentrations as low as 0.0005 M produce great changes in metachromasy.

The effects of all chromotropes used in the present study are similar to those of the third class described by Michaelis.[2] At low concentrations they depress the α-band of metachromatic dyes, while at high concentrations they tend to elevate it to or even above its value in the absence of chromotrope. None of the substances we have studied behaves in the manner of the first two classes. Even in his own work (Fig. 3 of reference 2), Michaelis shows a series of absorption spectra of toluidine blue in the presence of increasing concentrations of nucleic acid in which both the α-band (640 mμ) and the β-band (590 mμ) show first a depression followed at concentrations above 0.003% by a rise in the α-band to a level above that in the absence of nucleic acid. There is in fact little significant difference between this set of curves and the corresponding set for sodium oleate (Fig. 8 of reference 2).

There is one chromotrope for which our results appear to differ somewhat from those of Michaelis. In Fig. 4,[2] Michaelis shows a progressive drop in the α-bands of toluidine blue as the agar concentration is increased to 0.5%. In our results at higher agar concentrations, there is the beginning of a rise in the α- and β-bands and the beginning of a drop in the μ-band, showing the return of the dyes to their colors in the absence of chromotrope. That there is only the beginning of a change in these curves may be due to the fact that the agar curves of Fig. 3 are carried to a log concentration of only -3.3 while all other chromotropes are carried to concentrations at least ten times as high. Another difference in our work is that we have used what we believe to be a purified agar which gives clear homogeneous solutions at the concentrations used. Log concentrations above -3.3 were not used because such solutions of agar were turbid and spectrophotometric readings would not be accurate. Crude agar at the upper concentrations used by Michaelis gives stiff gels. We have avoided this condition in comparing chromotropes quantitatively since, in a gel, a reasonable question may be raised as to how much agar is really in solution.

Corrin, Klevens and Harkins[7] made a study of the effect of soaps at various concentrations on the absorption spectrum of pinacyanol chloride, another cationic dye that in aqueous solution shows deviations from Beer's law and is metachromatic.[2] From their detailed data for potassium laurate, we have plotted the extinction for the α-band (620 mμ) against the log of the soap concentration. The resulting curve is similar in shape to those for the α-bands of methylene blue or crystal violet in the presence of potassium myristate or Duponol, but the minimum lies at a higher chromotrope concentration (1 log unit). This might be due to the higher dye concentration used by these workers or to a difference in critical concentration of micelle formation between laurate and myristate.

Though there is a rough parallelism in the extent of effect of any one chromotrope on the α-, β- and μ-bands of both dyes, it has not been possible to relate this to any structural features of the chromotropes. Agar which has a single ester sulfate anion for every nineteen or more galactose residues gives almost the same curves as alginate which has a carboxylate anion on each pyranose residue. Hyaluronate, which has a carboxylate anion for every twelve carbon atoms produces the smallest changes in band heights, while myristate which has a carboxylate anion for every fourteen carbon atoms produces the greatest change in band heights. Comparison of the two detergents shows that while in both cases very sharply defined minima of the α- and β-bands occur, those of myristate are found at ten times the concentration as those of Duponol yet these two detergents have almost identical critical concentrations.

The results of the equilibrium dialysis experiments with methylene blue and crystal violet show strong evidence for the binding of dye by chromotrope. It is important to recognize that while high chromotrope concentration in most cases causes suppression of the μ-band and disappearance of metachromasy, it does not result in suppression of dye binding by chromotrope as measured by equilibrium dialysis. On the other hand, the presence

(7) M. L. Corrin, H. B. Klevens and W. D. Harkins, *J. Chem. Phys.*, **14**, 480 (1946).

of 0.01 *M* salt which has been previously shown to suppress metachromasy also suppresses dye binding measured by equilibrium dialysis. This would indicate two different mechanisms of suppression of metachromasy. From the results of our previous work, we believed that high chromotrope concentrations destroyed metachromasy by acting as a salt. Equilibrium dialysis experiments indicate that this idea may not be correct. Of particular interest in looking for a connection between dye binding and metachromasy is the case of methylene green, a dye similar to methylene blue but showing almost no deviations from Beer's law and almost no metachromatic effect. In equilibrium dialysis the extent of its binding by chondroitin sulfate is only about 30% of that of methylene blue or crystal violet as indicated by the ratios in the last column of Table II. The extent to which methylene green becomes concentrated in the bag may be considered as a measure of the Donnan effect. The increased concentration effect with methylene blue or crystal violet over that of methylene green may then be a measure of the binding of these dyes by chromotrope. The work of Kurnick and Mirsky[8] using dialysis shows binding of methyl green by desoxyribonucleate. Methyl green, a close relative of crystal violet, also obeys Beer's law and seems to show no metachromasy.

Another factor which must be considered in the interaction of dye and chromotrope is the formation of precipitates such as that of toluidine blue by heparin.[9] This precipitation occurs at dye and chromotrope concentrations higher than those used in the present study. It is important to note that the dye–chromotrope complex prepared in our laboratory, when washed and redissolved in water, does not produce a metachromatic spectrum but one similar to that of a dilute toluidine blue solution. This precipitate of dye and heparin thus has spectral properties which may account for the rise in the α- and β-bands of metachromatic dyes at high chromotropic concentrations. It might be assumed that two separate conditions of the dye can exist which produce the same spectrum.

It seems possible to correlate the metachromatic properties of polysaccharides and detergents in the more dilute solutions, as follows. At chromotrope concentrations 10^{-6} and below there is no marked evidence for interaction between dye and chromotrope. At these low concentrations the individual polysaccharide molecules are assumed to be linearly disposed and the detergent molecules to be separated and ionized. In the concentration range from 10^{-6} to about 10^{-4} where α- and β-bands are depressed and μ-bands rise, it is assumed that micelle formation of the chromotropes occurs to produce in the solution micro regions of high anion density. The occurrence of such micelles in soap solutions has been extensively studied and the critical concentration of micelle formation has been found[7,10] for various soaps to lie in the range from 10^{-4} to 0.5 *M*. The work of Fuoss and Strauss[11] similarly indicates that high molecular weight linear polyelectrolytes change their molecular shapes with increasing concentration from one of extended chains to one of highly folded or coiled chains. Thus, in more concentrated solutions of both anionic detergents and polysaccharides, micelle-like configurations seem to be formed. Such micelles may account for the results of both metachromatic and dialysis experiments though the mechanism is still obscure.

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The Conversion of Fibrinogen to Fibrin. X. Light Scattering Studies of Bovine Fibrinogen[1]

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Light scattering measurements have been made on solutions of bovine fibrinogen, using two types of purified fractions and three different solvent compositions. The angular scattering distribution was measured in a conical cell, and the dissymmetry and absolute intensity were measured with high precision in a semi-octagonal cell. From the results, the molecular weight is calculated to be 340,000 ± 20,000; the length is 520 Å. on the basis of a thin rod and 650 Å. on the basis of a thin ellipsoid. These values are not perceptibly affected by the presence of 0.50 *M* hexamethylene glycol.

Introduction

The arrested polymerization of fibrinogen by thrombin in the presence of certain inhibitors has been studied by sedimentation,[2,3] viscosity,[2,3] and flow birefringence.[4–6] Much valuable additional

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