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

A Conductimetric Study of the Interaction of Anionic Mucopolysaccharides and Cationic Dyes[1]

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RECEIVED JUNE 26, 1953

The electrical conductance of aqueous solutions of anionic mucopolysaccharides in the presence of cationic dyes is measured to compare the behavior of metachromatic and non-metachromatic dyes. The difference, Δ, between the specific conductance of a mixture of mucopolysaccharide and dye and the sum of their separate specific conductances is determined. For hyaluronate or chondroitin sulfate as anions, and either metachromatic dyes or those believed to be non-metachromatic as cations, Δ amounts to between 15 and 25% of the total conductance. There is no significant difference in Δ for any of the cationic dyes tested. A comparison is made between changes in Δ and changes in band intensities of the metachromatic dyes in both visible and ultraviolet regions, and a correlation is apparent. Contrary to previous belief, not all ultraviolet bands are constant over a wide range of chromotrope concentration. The bearing of these facts on the theory of metachromasy is discussed.

Quantitative studies on the interaction of polyanionic substances and cationic metachromatic dyes have been almost entirely restricted to spectrophotometric analysis in the visible region.[2-7] Regarding the nature of the interaction which produces a metachromatic color in the dye, several thoughts have been expressed. Michaelis and Granick[2] related the ability of dyes to dimerize with their production of metachromatic colors in colloidal solutions of suitable stainable substrates. This idea was extended by Wiame.[3] It implied a combination of stainable substrate and dye. Yet the color change was ascribed not to this combination, but to the dimerization of the dye thought to accompany it. More recently, the parallel behavior of anionic polysaccharides and anionic detergents gave rise to the thought that dye cations may be fixed by anionic micelles of the chromotrope.[5] There was need of a quantitative method to show that combination between dye and chromotrope occurs. An attempt was made to prove the existence of such combination in solution by equilibrium dialysis.[8] The results showed that the metachromatic dyes, methylene blue and crystal violet, were bound to a greater extent than the slightly metachromatic dye, methylene green. Reproducibility was poor by this method, and it was not further pursued.

A new method has now been explored which makes use of the electrical conductance of aqueous solutions of mixtures of anionic polysaccharides and cationic dyes to estimate the extent of their interaction. This method has been applied to four dyes, two known to be metachromatic and two believed to be non-metachromatic. The results of these measurements have been directly compared with the extinction coefficients of the dyes at relevant wave lengths in the same solutions. In this way the extent of metachromasy and the extent of interaction can be compared at a fixed dye concentration as the polysaccharide concentration is varied. The present study is further extended to include extinction coefficients of ultraviolet bands. These bands have been thought to be unaffected by chromotropes even in those dyes whose visible bands show metachromatic changes.[2]

(1) This investigation was supported (in part) by research grant A28(C) from the National Institute of Arthritis and Metabolic Diseases, of the National Institutes of Health, Public Health Service, and (in part) by the Masonic Foundation for Medical Research and Human Welfare.

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Experimental

The dyes were recrystallized two or three times by dissolving in hot water and chilling the filtered solutions. Potassium chondroitin sulfate was made from the crystalline calcium salt prepared from cartilage.[5] Potassium hyaluronate was made from umbilical cords by a method to be described elsewhere. Distilled water was twice redistilled in an all glass still with a spray trap, discarding head and tail fractions. The specific conductance was 4×10^{-6} at 0° and 5×10^{-6} at 24.6°.

The conductance cell was made with two bright platinum cones about 1 cm. in diameter and 1 cm. apart. The cell constant, determined with twice recrystallized potassium chloride, was 1.219. Resistance was measured with an a.c. bridge at a frequency of 2500. At the beginning of the work, all resistances were measured at two temperatures, 24.6 and 0°. Since no difference was found at these two temperatures in the quantities of primary interest, all the later work was done only at 24.6°.

One purpose of this work was to measure deviations from additivity of specific conductances of dye and polysaccharide when mixed over a wide concentration range of the polysaccharide. To do this most conveniently, a solution was made containing the polysaccharide at 0.006 period per liter and the dye, in most cases, at 0.000375 *M*. Successive dilutions of this solution were made by adding to 10 ml. an equal volume of dye solution also at 0.000375 *M*. To minimize errors a single pipet was used. Polysaccharide concentrations are calculated as periods per liter with period weights of 417 for potassium hyaluronate and 607 for potassium chrondroitin sulfate. Molar extinction values, ε, were calculated from optical densities measured in a 0.1-cm. silica cell with a Beckman spectrophotometer.

Results

Specific conductances of the mucopolysaccharides and Duponol are given in Table I, those for the dyes and potassium chloride in Table II. From these two tables can be calculated, by addition, the specific conductance that would be expected of any mixture if all ions present behaved independently. Our concern is with the difference, Δ, between such a calculated specific conductance for a mixture and that actually observed. The values of Δ are listed in Table III. The actual specific conductance of any mixture that was measured can be obtained by subtracting Δ from the calculated specific conductance. When the components of a mixture are

present in equivalent amounts, values of Δ that are of the order of 5% of the total conductance of the solution are not considered significant for our present purposes. In the case of mixtures involving polysaccharides or Duponol as one component, the mixtures have, almost invariably, lower specific conductances than the sums of the components. In the very few cases where the reverse is true, a minus sign is put before the Δ values of Table III.

TABLE I

SPECIFIC CONDUCTANCE OF CHROMOTROPES AT VARIOUS CONCENTRATIONS

Chromotrope concn. periods per l. × 10^3	Specific conductance, mhos × 10^4 Potassium hyaluronate 0°	24.6°	Potassium chondroitin sulfate 0°	24.6°	Duponol 0°	24.6°
12.0	6.495	11.020	8.847	16.090	...	4.332
6.0	3.311	5.773	4.502	8.221	...	2.716
3.0	1.669	2.929	2.282	4.186	0.737	1.633
1.5	0.864	1.482	1.185	2.157	.434	0.901
0.75	.445	0.771	0.612	1.139	.236	.474
.375	.228	.401	.320	0.610	.124	.242
.187	.118	.207	.167	.326	.068	.133
.0937	.061	.112	.084	.175	.031	.062
.0468	.033	.057	.043	.093	.018	.035

TABLE II

SPECIFIC CONDUCTANCE OF DYES AND SALTS

	Dye concn., mole/l. × 10^3	KCl concn., mole/l. × 10^3	Specific conductance, mhos × 10^4 0°	24.6°
Methylene blue	0.375	...	0.207	0.383
Methylene blue	.750	...	...	.734
Methylene blue + KCl	.375	0.375	.486	.882
Methylene blue + KCl	.375	1.50	...	2.467
Methylene green	.375	...	.196	0.373
Crystal violet	.375	...	.214	0.393
Methyl green	.375	...	.751	1.398
Biebrich scarlet	.375	...	.345	0.646
Potassium chloride	...	0.375	.302	0.544

These cases are not significant and reflect the range of experimental error when the differences are small.

In Table III are figures for four cationic dyes. Three of these dyes show values of Δ which rise to a plateau that is approximately reached when the polysaccharide concentration becomes equivalent to the dye concentration. After this, further increase in polysaccharide concentration causes no significant changes in Δ. Furthermore, Δ is, within the limits of error, the same for all three dyes, 0.2×10^{-4} mho. At the polysaccharide concentration equivalent to the dye concentration, Δ is 15 to 25% of the total conductance. The fourth dye, methylene green, is a divalent cation. In this case, the values of Δ increase progressively over the whole range of polysaccharide concentration and do not level off to a plateau. Despite this increase, when Δ is calculated as a percentage of the total conductance of the solution, it rises to a maximum and then falls as the polysaccharide concentration is increased. The maximum value of Δ as per cent. occurs at a polysaccharide concentration of 0.00075 period per liter, just equivalent to the dye at 0.000375 *M*. Under these conditions, Δ amounts to 15% of the total calculated conductance.

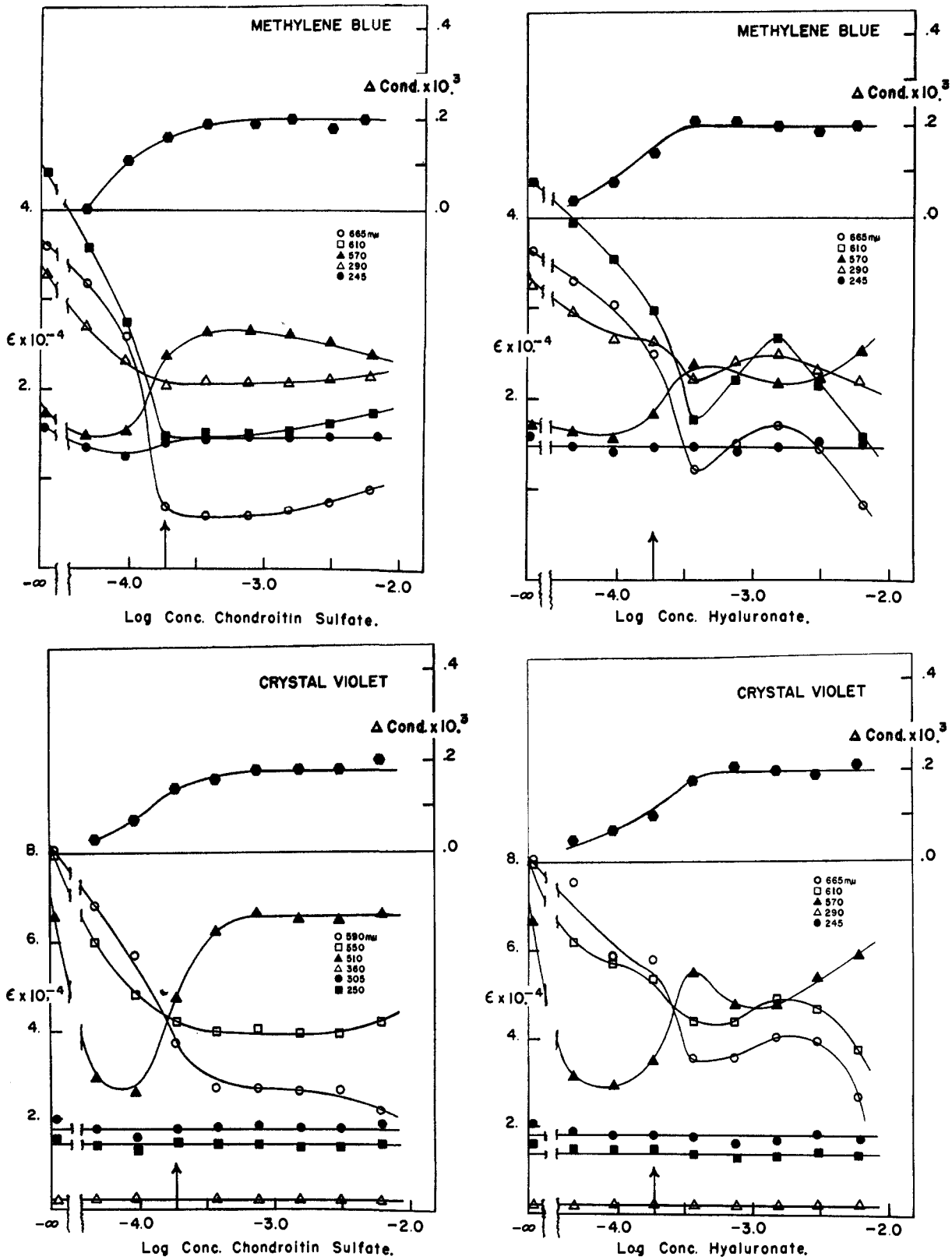
Potassium chloride added to the polysaccharide solutions also results in conductance values lower than those calculated from the sum of the components. The values of Δ are smaller than those for the cationic dyes and seem to be constant over almost the whole range of polysaccharide concentration. At equivalent concentrations of potassium chloride and polysaccharide Δ is 6% of the total conductance. The anionic dye, Biebrich scarlet, gives essentially constant values of Δ even smaller than those given by potassium chloride.

Some of the conductance measurements were run at two temperatures since metachromasy is known to increase at lower temperatures. It was thought that increased binding would be apparent by calculating Δ as percentage of total conductance at

TABLE III

VALUES OF Δ MHOS × 10^4 FOUND IN MIXTURES OF DYES, SALTS AND CHROMOTROPES

	Potassium hyaluronate					Potassium chondroitin sulfate								Duponol	
	Methylene blue	Methylene green	Crystal violet	Methyl green	KCl	Methylene blue	Methylene green	Crystal violet	Methyl green	KCl	Biebrich scarlet	Methylene blue + KCl	Methylene blue + KCl	Methylene blue	Methylene blue
Dye concn., m./l. × 10^3	0.375	0.375	03.75	0.375	...	0.375	0.375	0.375	0.375	0.375	0.375	0.375	0.375	0.750	0.375
Salt concn., m./l. × 10^3	...	...	...	...	0.375	...	...	...	...	...	...	.375	1.50	...	...
Polysac. concn., per l. × 10^3															
									24.6°						
6.0	.20	.21	.20	.67	.07	.20	.20	.20	.57	.07	.05	.44	.50	.36	.06
3.0	.19	.21	.18	.56	.05	.18	.16	.18	.47	.06	.05	.26	.40	.33	.13
1.5	.21	.20	.19	.44	.06	.20	.14	.18	.46	.06	.01	.27	.33	.30	.12
0.75	.21	.18	.20	.33	.05	.20	.12	.18	.40	.04	.04	.28	.28	.32	.16
.375	.21	.16	.17	.21	.06	.20	.14	.16	.28	.06	.03	.25	.23	.30	.15
.1875	.14	.12	.09	.12	.06	.17	.14	.14	.16	.06	.02	.17	.19	.18	.09
.0937	.09	.08	.06	.05	.07	.12	.08	.07	.07	.04	.02	.08	.11	.11	.04
.0468	.04	.06	.04	.03	.04	.01	−.01	.03	.02	.03	.02	.06	.06	.08	.01
									0°						
6.0	.09		.11	.34		.19			.25	.04	.02	.22			...
3.0	.09		.10	.28		.16			.22	.04	.00	.17			...
1.5	.12		.11	.23		.16			.23	.06	.03	.17			−.04
0.75	.13		.12	.16		.14			.16	−.02	.00	.13			.03
.375	.12		.09	.10		.14			.11	.03	.01	.12			.07
.1875	.08		.05	.06		.13			.06	.03	.01	.08			.08
.0937	.05		.03	.03		.09			.03	.02	.01				.05
.0468	.03		.02	.01		.00			.01	.02	.01				.02
															.01



Figs. 1–4.—In upper part of each figure are plotted values of Δ as ordinates; in lower part are plotted molar extinctions of visible and ultraviolet bands. Abscissa values for both parts are chromotrope concentrations as log periods per liter. The arrow on the abscissa axis marks the concentration at which the chromotrope concentration becomes equal to the dye concentration. This arrow should be at the abscissa value −3.4.

the equivalence point. Table III gives some values of Δ at 0°. These values are not significantly different from those at 24.6° when calculated as percentage of the total conductance.

Direct comparison of spectral changes accompanying changes in Δ in the case of methylene blue are plotted in Figs. 1 and 2. In each case the extinction values at the wave lengths of the five maxima of the dye are plotted against the log of the chromotrope concentration. The wave lengths chosen are the α and β bands at 665 and 610 mμ, the metachromatic or μ band at 570 mμ, and the peaks of the two ultraviolet bands at 290 and 245 mμ. The spectrographic data of these two figures differ from similar data previously presented[5] in that measurements were carried out at a much higher dye concentration, and data are included on the two ultraviolet bands of methylene blue. Contrary to expectations, the ultraviolet bands in some cases show variations in intensity with variations in chromotrope concentration. The tendency is clear in both Figs. 1 and 2 for the α and β bands to run parallel while both run counter to the μ band. The near ultraviolet band at 290 mμ tends to parallel the α and β bands though the extent of its variation is much smaller. The far ultraviolet band shows the smallest latitude in its variation and in the presence of hyaluronate it is practically constant.

Figures 1 and 2 show the relation between Δ and the extinction values of the absorption bands. In both figures, Δ and the extinction of the μ band rise to a plateau-like maximum at log concentration of chromotrope about -3.5; at about this same concentration, the α, β and near ultraviolet bands have fallen to minima. At higher chromotrope concentrations all these values are nearly level in the case of chondroitin sulfate, while with hyaluronate, extinction values show a set of maxima at a concentration of -2.8.

Similarly, Figs. 3 and 4 show the trend of Δ and extinction values of crystal violet with hyaluronate and chondroitin sulfate. In this case the μ band also runs counter to the α and β bands which are roughly parallel. The three ultraviolet bands of crystal violet are constant over the range of chromotrope concentration studied. The principal minima of α and β bands, maximum of the μ band and leveling off of Δ values all occur at about log concentration -3.4.

Previous work has shown that at a low dye concentration (10^{-5} M) the metachromatic spectrum of methylene blue in the presence of chondroitin sulfate changed to the normal spectrum on the addition of potassium chloride (10^{-3} M). In the present work the bands of methylene blue (3.75×10^{-4}) were found to vary with chondroitin sulfate in the presence of potassium chloride (1.5×10^{-3}) exactly as they do in the absence of potassium chloride as shown in Fig. 1. In Table III are values of Δ in which potassium chloride at two different concentrations is added to methylene blue and chondroitin sulfate. The effect is to give values of Δ which are even larger than those in the absence of potassium chloride and which increase progressively.

Discussion

In the past, the study of metachromatic dyes has been carried out mainly by histologists whose interest was to distinguish different components and conditions of tissues. Cationic dyes were called metachromatic when they stained mast cell granules or cartilage in a color different from the color of dilute aqueous solutions of the dyes. On this basis, Michaelis[9] called crystal violet metachromatic and methylene blue non-metachromatic. Later Lison[10] made a comprehensive study of metachromasy and decided that neither methylene blue nor crystal violet were metachromatic. Michaelis and Granick[2] used agar as a chromotrope and found that both methylene blue and crystal violet gave spectral changes similar to those of toluidine blue, whose metachromatic properties have never been questioned. Our own work[5,8] has also shown that the spectral properties of these three dyes are similar and that all must be called metachromatic. There are other cationic dyes with structures similar to those of the metachromatic dyes which have always been thought not to possess this property. Of these, methylene green[10] and methyl green[11] are examples.

The principal objective of the present work was to study a new method for examining the interaction of anionic chromotrope and cationic dye. The results, given as values of Δ, show no evidence of a difference in binding that could serve to classify cationic dyes as metachromatic and non-metachromatic.

Δ is not simply a measure of the extent of binding. Part of the Δ values is due to ionic interactions other than combination. An attempt to judge this empirically is made by measuring values of Δ when the polysaccharides are mixed with potassium chloride or Biebrich scarlet. That the cationic dyes give such large values of Δ may be considered to indicate binding of dye cation and polysaccharide anion.

Independent evidence of binding is afforded by previous work on equilibrium dialysis.[8] In that study methylene green was found to be bound to a smaller extent than methylene blue or crystal violet. In the present work carried out at a much higher dye concentration, these three dyes appear to be bound to about the same extent as judged by values of Δ.

Of theoretical importance is the variation in some ultraviolet band intensities with chromotrope concentration. These ultraviolet variations, when they occur in the case of methylene blue, run parallel with the α and β bands and counter to the μ band. The finding of variations in ultraviolet band intensities opens a new aspect of metachromasy that may help in the building up of the underlying theory.

The data of the figures are consistent with the view that cationic dyes combine with anionic polysaccharides or detergents. This view is supported by equilibrium dialysis and by the fact that at higher dye and polysaccharide concentrations, insoluble colored precipitates are produced. The concentration range of chromotrope over which the main drop in α and β bands and the rise in the μ band occurs, in the case of the metachromatic

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dyes, is about the same as that in which the main rise in Δ values occurs. This concentration range extends up to the value at which dye and chromotrope are approximately equivalent. At chromotrope concentrations above this point there is little change in Δ values except with methyl green and methylene blue in the presence of potassium chloride. Band extinction values above the equivalence point differ rather markedly in behavior depending on whether the chromotrope is chondroitin sulfate or hyaluronate. The course of the curves relating band intensities to chromotrope concentrations of the present work differs somewhat from similar curves presented previously.[8] This difference is an important part of the whole phenomenon of metachromasy and shows the effect of the dyestuff concentration on the course of these curves. On the basis of the present work, as well as of our previous work, it seems possible to adopt tentatively the view that the metachromatic color is due to binding of dye by chromotrope and is not due to dimerization or polymerization of the dye.

The fact that methylene green and methyl green, dyes which have been thought to obey Beer's law and to be unaffected by chromotropes,[11] give values of Δ as large as, or even larger than, those given by the metachromatic dyes methylene blue and crystal violet, has raised a suspicion that perhaps the band intensities of these dyes are not as constant as was thought. Work on these two and other non-metachromatic dyes is in progress to examine this point.

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The Size and Shape of Bovine Fibrinogen. Studies of Sedimentation, Diffusion and Viscosity[1]

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RECEIVED MAY 18, 1953

Sedimentation and diffusion measurements have been made on solutions of bovine fibrinogen, using several experimental methods. The resulting sedimentation constant, extrapolated to infinite dilution and corrected to standard conditions, was 7.80×10^{-13} sec.$^{-1}$ in the Spinco ultracentrifuge and 7.95×10^{-13} sec.$^{-1}$ in the oil-turbine ultracentrifuge. The diffusion constant at a concentration of 4.0 g./l. was 2.02×10^{-7} cm.^{2} sec.$^{-1}$, using a schlieren optical system, and 2.01×10^{-7} cm.^{2} sec.$^{-1}$, employing a Gouy interferometer optical system. Appropriate combination of these results gives a molecular weight of 330,000 ± 10,000 and a frictional ratio of 2.34. The intrinsic viscosity was found to be 0.25. Deductions as to the molecular shape and dimensions have been made by use of several hydrodynamic theories.

Introduction

The sedimentation and diffusion constants of fibrinogen were first studied by Holmberg.[3] The resulting molecular weight was 700,000. No other diffusion results have been reported, but several additional sedimentation studies have been made.[4-8] In some of these investigations the results were combined with viscosity and flow birefringence data to give a molecular weight of 500,000 and, later, 400,000. Other experimental methods have been applied to the study of this protein, including osmotic pressure[5,9,10] and light scattering.[11] These have generally given values of the molecular weight in the range of 400,000–500,000, or a little higher. Very recently, a light scattering investigation in this Laboratory gave a result[12] of 340,000. It was felt that a new investigation of the molecular weight as determined by the method of sedimentation and diffusion would be of considerable interest.

Materials and Methods

The fibrinogen was prepared from Armour bovine fraction I, preparation 128–163, following two different procedures, previously described.[12] The older method, a freeze–thaw procedure, gave a product called fraction I-C, containing 88–90% clottable protein. This material was used in the earlier sedimentation experiments. The other method, an ammonium sulfate procedure, gave a product called fraction I-L, generally containing 94–98% clottable protein. This material was used in the other sedimentation experiments and in all the diffusion experiments. Because of reports[13,14] that 3 to 4% of the initial fibrinogen is split off during conversion to fibrin, the practice will be followed of adding about 3 units to all designations of per cent. clottability when the actual percentage of fibrinogen in the protein is to be stated.

Stock solutions of fibrinogen were adjusted to the desired ionic strength and *p*H by dialysis at 0° for 24 hours against several changes of buffer, and clarified by filtration through washed filter pads. The solvent was a buffer of 0.45 ionic strength, of which 0.40 was contributed by sodium chloride and 0.05 by sodium phosphates. The *p*H was always 6.2. Fibrinogen concentration was determined by gravimetric assay,[15] and total protein concentration by Kjeldahl or by dry weight.

The sedimentation studies were made in both the Sved-

(1) This is Paper No. 26 of a series on "The Formation of Fibrin and the Coagulation of Blood" from the University of Wisconsin, supported in part by research grants from the National Institutes of Health, Public Health Service.

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