tures such as $CH_2CO{=}CH_2$. It is very tempting to assume that ethylene, propylene, etc., are formed as the result of a reaction of methylene with these diradicals. Against this, however, is the definite evidence[1] that light intensity has no effect on the course of the reaction in ketene–ethylene mixtures. We have now repeated previous work on this subject arriving at the same conclusion. Therefore, we are led to the proposition that metathetic reactions of two such diradicals account for the olefinic hydrocarbons, whereas their association leads to the polymer. The rearrangement of the first of the two radicals readily accounts for the trace of acrolein observed in previous work,[1] whereas the oxygen of the second diradical should be quite reactive and therefore may be removed (perhaps by methylene) to yield allene.

CAMBRIDGE 38, MASS. RECEIVED JUNE 14, 1951

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

# Metachromasy of Thiazine Dyes Produced by Chondroitin Sulfate[1]

BY ANNE LEVINE AND MAXWELL SCHUBERT

A spectrophotometric study has been made of the appearance and disappearance of metachromasy in solutions of two thiazine dyes as a function of increasing chromotrope concentration. Purified but amorphous potassium chondroitin sulfate and crystalline calcium chondroitin sulfate were used. The resulting progressive changes in extinction at two critical wave lengths have been compared with the corresponding changes that occur in aqueous solutions of thiazines as their concentration is increased. The results rule out the possibility that metachromasy is due to dye dimerization, but could be interpreted to offer slender support to the theory of dye polymerization. An alternative view is suggested. The effect of electrolytes in destroying metachromasy is also studied. A difference in the chromatropic behavior between amorphous and crystalline chondroitin sulfate is found to occur at high concentrations.

Metachromasy[1a] is the change in color of dilute aqueous solutions of some dyes caused by the addition of certain high molecular weight polyelectrolytes. Lison[2] pointed out that the common substances of plant and animal tissues which cause metachromasy are half sulfate esters of high molecular weight polysaccharides, but it was subsequently observed that hyaluronate, hexametaphosphate[3] and silicates[4] also produce this effect.

Holmes first noticed that the basic dyes which do not conform to Beer's law are capable of producing metachromasy in appropriate tissues, and that this metachromatic color is similar to the color of the more concentrated dye solutions.[5] In the cases of toluidine blue and brilliant cresyl blue, Lison[2] observed a gradual shift in the absorption maxima with increasing dye concentrations approaching the maxima of the dyes in their metachromatic states. Michaelis and Granick,[6] in an extensive study of the absorption of a number of cationic dyes in the presence of agar, concluded that a parallelism exists between the production of metachromasy in dyes and their deviation from Beer's law in aqueous solution. A great advance was made by the work of Lison[2] and of Michaelis and Granick[6] who measured extinction curves in agar gels in contrast to all previous work which had been confined to qualitative observations on stained tissues.

If the metachromatic color change is caused by a mechanism similar to that which causes the color change with increasing dye concentration, then it might be expected that gradually increasing the chromotrope concentration, at a fixed dye concentration, would produce a similar progressive effect on the extinction curve of the dye. The use of chondroitin sulfate to induce metachromasy lends itself to such a study better than most known chromotropes since it has been made in quite pure form and has been crystallized as a calcium salt.[7] It yields perfectly clear, stable solutions that do not gel even at concentrations up to 10% and for quantitative study, therefore, has several advantages over agar. With this substance, a detailed study has been made of the metachromasy produced in aqueous solutions of a few thiazine dyes under a variety of conditions. It is now possible to compare quantitatively the effects due to metachromasy with those due to deviations from Beer's law. Most of the work has been done with methylene blue since deviations from Beer's law have been more intensively studied with this dye than with any other thiazine. Methylene blue does not show metachromasy in histological applications but its metachromatic spectral changes are similar to those of toluidine blue or of thionin which show obvious color changes.

## Experimental

Methylene blue was prepared from commercial material by recrystallization three times from hot water. For comparison a preparation was synthesized according to a method already described.[8] Extinction curves of both were the same and were affected similarly by chondroitin sulfate. The position of the peak of the band persistently occurred

(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.

(1a) The word "metachromasy" first appears in English in the work of Holmes (ref. 5). The word "metachromasia" first appears in *C. A.*, (1934) in an abstract of a paper in French by Lison. Only in 1940 does an original paper appear in English using the word "metachromasia," Hempelman, *Anal. Record*, **78**, 197 (1940). Therefore, in spite of common current usage of "metachromasia" in histochemical works the word "metachromasy" is retained here.

(2) L. Lison, *Arch. Biol.*, **46**, 599 (1935).

(3) J. M. Wiame, THIS JOURNAL, **69**, 3146 (1947).

(4) R. C. Merrill and R. W. Spencer, *ibid.*, **70**, 3683 (1948).

(5) W. C. Holmes, *Stain Technol.*, **1**, 116 (1926).

(6) L. Michaelis and S. Granick, THIS JOURNAL, **67**, 1212 (1945).

(7) J. Einbinder and M. Schubert, *J. Biol. Chem.*, **191**, 591 (1951).

(8) L. Michaelis, M. Schubert and S. Granick, THIS JOURNAL, **62** 204 (1940).

at 665 mμ in contrast to the values of 650 mμ reported by Vickerstaff and Lemin[9] and 656 mμ reported by Rabinowitch and Epstein.[10] This maximum was checked on three different Beckman spectrophotometers and the position of the peak did not vary with methylene blue concentration or addition of chondroitin sulfate. High salt concentration caused a shift to 670 mμ.

Toluidine blue was prepared from commercial samples by dissolving in hot water (1 g. of toluidine blue in 18 ml. of water), filtering and slowly stirring in 1.5 volumes of alcohol. The product separates in dense rosettes of blue needles after chilling on ice.

Methylene green was recrystallized three times by dissolving in hot water (1 g. in 18 ml. of water), filtering and chilling on ice.

Dilute solutions of methylene blue in water show small downward drifts in optical density with time. Rabinowitch and Epstein[10] found it possible to minimize this effect by the addition of small amounts of hydrochloric acid. This procedure could not be used because of the great influence that small amounts of electrolytes have on metachromasy, as will be apparent in the results. It was necessary, therefore, to measure the extinction of the control solution, methylene blue in water without chondroitin sulfate, in the absence of added hydrochloric acid. The magnitude of the drift was measured at 665 mμ over a period of an hour. It lies within the spread of independent determinations of the extinction at zero concentration of chondroitin sulfate as plotted in Fig. 2.

The amorphous potassium salt of chondroitin sulfate was extracted from beef nasal cartilage and purified as described elsewhere.[11] From this material the crystalline calcium salt was made after further purification.[7] In plotting data, the unit of weight used to describe chondroitin sulfate concentration is the period weight rather than the less readily measurable molecular weight. For the potassium salt a period corresponds to $C_{14}H_{19}O_{14}NSK_2 \cdot 4H_2O$ or a weight of 607.

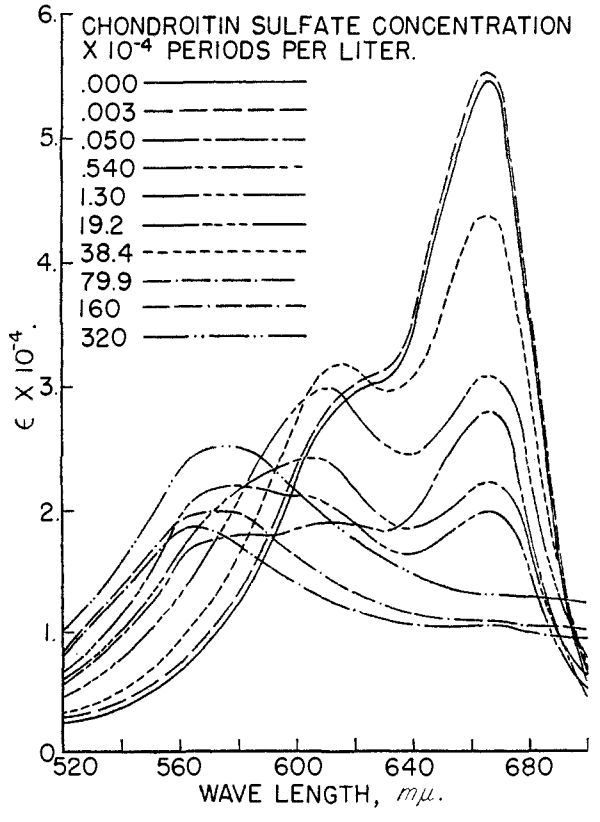


Fig. 1.—Absorption spectra of methylene blue ($1.25 \times 10^{-5}$ *M*) at several concentrations of potassium chondroitin sulfate.

## Results

In Fig. 1 is shown a set of extinction curves for solutions containing $1.25 \times 10^{-5}$ *M* methylene blue and amorphous potassium chondroitin sulfate at a series of concentrations between 0 and 0.032 period per liter. An increase in the concentration of mucopolysaccharide tends to depress the α band without shifting the wave length of its peak at 665 mμ until, at sufficiently high chondroitin sulfate concentrations, it ceases to be a maximum. Closer examination shows that there are ranges of concentration where this tendency is reversed. This has never been described before. It is brought out more clearly in Fig. 2 in which extinction values at 665 mμ are plotted against increasing chondroitin sulfate concentrations. Figure 2 also shows the spread of values obtained with independent preparations of chondroitin sulfate and of methylene blue.

The second band of importance is called the metachromatic band[6] which at low concentrations of chondroitin sulfate either does not exist at all, or is part of the shoulder on the short wave length side of the α band. At higher concentrations of chondroitin sulfate it may emerge as a distinct peak whose wave length position varies somewhat or it may appear as a broad plateau with no clearly marked peak. However, over most of the range, the metachromatic band lies between 610 and 570 mμ. The extinction at 610 mμ is of particular interest because this is the location of the β band. In Fig. 2 are also plotted curves of the extinction values at 610 mμ and at 570 mμ as a function of the chondroitin sulfate concentration. The curve at 610 mμ shows ranges of rising and falling values as does the curve for the α band. In fact, rather unexpectedly, it appears that the two curves tend to run roughly parallel, at least to the extent of falling and then rising together. A further point of interest in these curves is that the concentration range of chondroitin sulfate where the minima occur, includes a concentration equal to that of the methylene blue. This is marked on the abscissa axis by an arrow. That this may not be merely fortuitous gains weight from the corresponding curves of Fig. 6 in which extinction values at 665 and 610 mμ are plotted against chondroitin sulfate concentration, this time at a methylene blue concentration almost a hundred times higher than that of Fig. 2. Again the minimum extinction values fall at a chondroitin sulfate concentration roughly equal to that of the methylene blue. At this high concentration of dye, dark blue amorphous precipitates are formed in the presence of sufficiently high concentrations of chondroitin sulfate. No trace of precipitation occurs in the concentration range where the minima of Fig. 2 and Fig. 6 occur.

In contrast to the curves of Fig. 2 are those of Fig. 3. Values of the extinctions at 665 mμ and at 610 mμ are plotted against increasing methylene blue concentration in the absence of chondroitin sulfate. Under these conditions, the relations are much simpler as has already been shown by Rabinowitch and Epstein[10] and by Sheppard and Geddes.[12] With increasing methylene blue concentration, the extinction at 665 mμ decreases while that at 610 mμ rises. Curves obtained by stabilization of the solutions by addition of hydrochloric acid to bring the *p*H to 4.5 are also included for comparison. These curves express the deviation from Beer's law of methylene blue. The principal experimental result to appear from a comparison of Fig. 2 and Fig. 3 is that increasing concentration of chromotrope produces a succession of extinction changes at the two critical wave lengths quite different from those produced by increasing dye concentration.

In order to facilitate further study of the metachromatic effect produced by chondroitin sulfate, it seemed advisable to adopt a definition of metachromasy that could be measured by a single number. For this purpose the definition already used by Wiame[8] is of value. The degree of metachromasy of methylene blue under any set of experimental conditions is defined as the ratio, *R*, of the extinction at 610 mμ to the extinction at 665 mμ. *R* has its lowest value in dilute aqueous dye solutions and increases in value under those conditions where metachromasy is recognized as becoming pronounced. For dye stuffs other than methylene

(9) T. Vickerstaff and D. R. Lemin, *Nature*, **157**, 373 (1946).

(10) E. Rabinowitch and L. F. Epstein, THIS JOURNAL, **63**, 69 (1941).

(11) J. Einbinder and M. Schubert, *J. Biol. Chem.*, **187**, 725 (1950).

(12) S. E. Sheppard and A. L. Geddes, THIS JOURNAL, **66**, 1995 (1944).

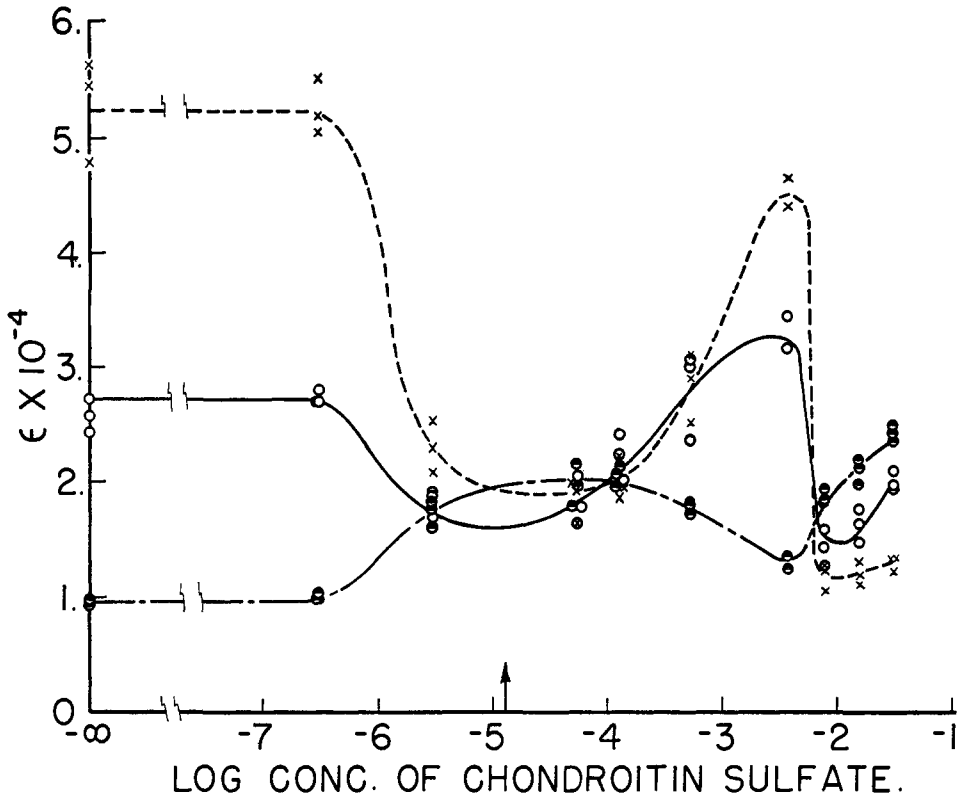


Fig. 2.—Extinction values of methylene blue ($1.25 \times 10^{-5}$ *M*) at three wave lengths plotted against increasing concentrations of potassium chondroitin sulfate expressed as periods per liter; ◓, 570 mμ; O, 610 mμ; ×, 665 mμ. Arrow indicates abscissa at which chondroitin sulfate concentration equals methylene blue concentration.

blue the same definition may be used though the particular pair of wave lengths will differ. In Fig. 4 are plotted values of the ratio, *R*, as a function of the chondroitin sulfate concentration. For comparison, an *R* curve plotted from the data of Fig. 3 would show that increasing methylene blue concentration produces a continuous increase in the degree of metachromasy. Curve 1 of Fig. 4 shows a more complicated structure since the degree of metachromasy does not

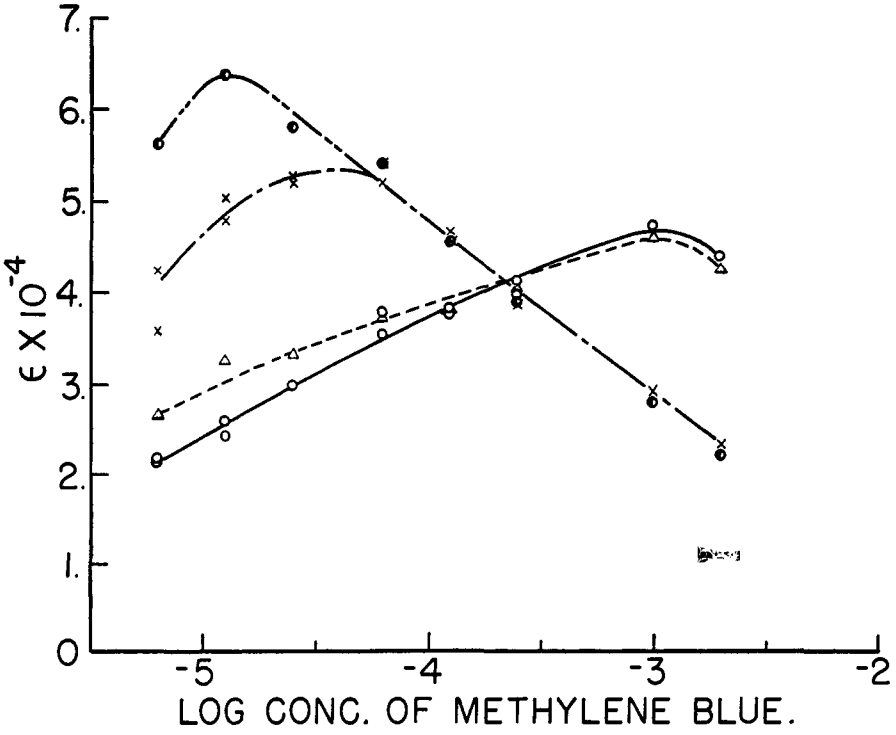


Fig. 3.—Extinction values of methylene blue at two wave lengths plotted against increasing molar concentrations of methylene blue: O, in water at 610 mμ; ×, in water at 665 mμ; △, in dil. HCl at 610 mμ; ◐, in dil. HCl at 665 mμ.

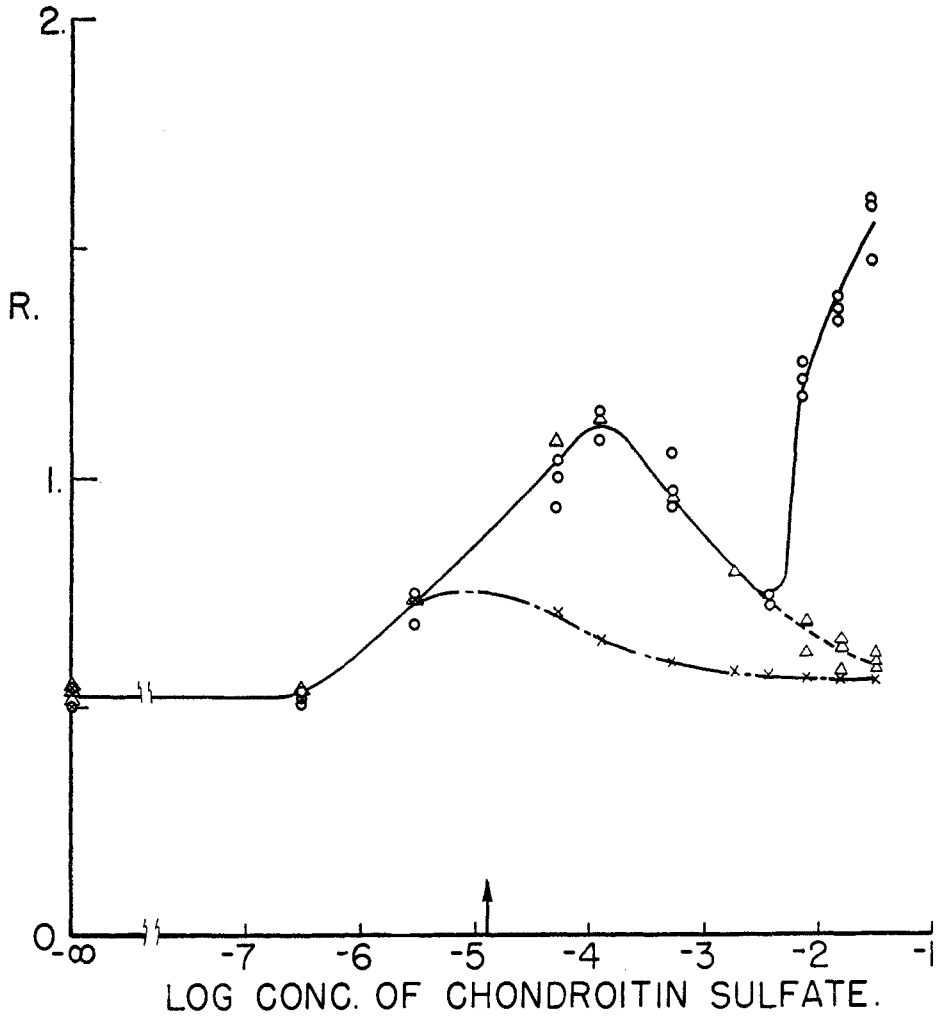


Fig. 4.—Extinction ratio, $R$, of methylene blue ($1.25 \times 10^{-5}$) $M$, $\epsilon$ at 610 m$\mu$/$\epsilon$ at 665 m$\mu$, plotted against increasing concentrations of chondroitin sulfate: curve 1, O, amorphous potassium chondroitin sulfate; curve 2, ×, crystalline calcium chondroitin sulfate; curve 3, Δ, potassium chondroitin sulfate prepared from crystalline calcium salt. Arrow indicates abscissa at which chondroitin sulfate concentration equals methylene blue concentration.

uniformly increase with increasing chondroitin sulfate concentration. There are three distinct segments, the first rising, the second falling and the third sharply rising. This suggests the possibility that three separate causes are operating, each one being dominant in the concentration range of each of the segments.

A clue to one of these causes was found when the observation was made that the presence of electrolytes in the concentration range $10^{-3}$ to $10^{-1}$ $M$ completely abolishes metachromasy. This was found to be true of the neutral salts, KCl, $CaCl_2$ and $(NH_4)_2SO_4$, as well as acetate and citrate buffers and HCl more acid than $p$H 4.5. In Fig. 5, this effect is shown as a function of calcium chloride concentration. The other salts show similar effects though they do so only at about twice the molar concentration. In the presence of a constant concentration of chondroitin sulfate, the values of R for both methylene blue and toluidine blue rapidly drop to their values in the complete absence of chondroitin sulfate. Also included in Fig. 5 are $R$ curves showing the complete lack of effect of salt at low concentrations in the absence of chondroitin sulfate at either low or high concentrations of methylene blue.

Salts at a sufficiently high concentration also produce a metachromatic effect. This happens both in the presence and the absence of chondroitin sulfate and at low and high methylene blue concentrations as shown in the curves of Fig. 5. In no case does the effect become pronounced until salt concentrations approach 1 $M$.

**Calcium salts have a more pronounced effect than potassium salts in suppressing metachromasy.** This must be kept in mind in considering the metachromatic effect of crystalline calcium chondroitin sulfate which is shown in Curve 2 of Fig. 4 and in Fig. 6. It is clear that this calcium salt never produces values of $R$ as high as those produced by the potassium salt. In order to compare the effect of the chondroitin sulfate anion of the crystalline calcium salt directly with that of the cruder potassium salt, the calcium salt was converted to a potassium salt by adding an equivalent amount of potassium oxalate and, after several hours, centrifuging off the calcium oxalate. The metachromatic effect of the resulting solution of potassium chondroitin sulfate is shown in curve 3 of Fig. 4. This curve now coincides with that of the cruder potassium chondroitin sulfate up to a concentration of $3.8 \times 10^{-3}$ period per liter. At higher concentrations, however, the two curves diverge sharply.

Toluidine blue is one of the dyes most commonly used for metachromatic staining of tissues. The variation in its absorption spectrum with increasing dye concentration has been studied by Lison.[2] In Fig. 7 is a set of curves showing the variation of the absorption spectrum in the presence of increasing concentrations of chondroitin sulfate. In toluidine blue the $\alpha$-peak appears to be lower in extinction and to merge with shorter wave bands. The change in shape of the absorption curve with increasing chondroitin sulfate concentration is only in part similar to the change in the methylene blue curve. An important difference is that at the highest concentration of chondroitin sulfate, the curve at all wave lengths lies above that for toluidine blue alone. The resulting $R$ curve in Fig. 8 differs from the corresponding

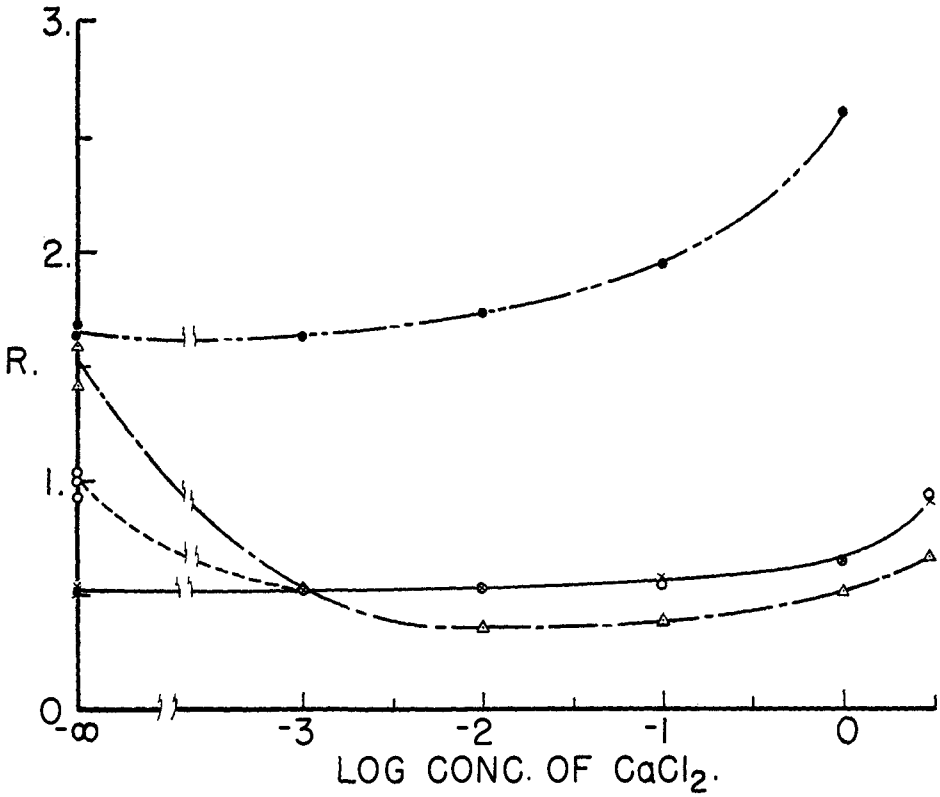


Fig. 5.—Extinction ratios, $R$, of methylene blue and of toluidine blue plotted against increasing molar concentrations of $CaCl_2$: ×, Mb $1.25 \times 10^{-5}$ $M$; O, Mb $1.25 \times 10^{-5}$ $M$ and potassium chondroitin sulfate $1.3 \times 10^{-4}$ periods per liter, ●, Mb 1. $\times 10^{-3}$ $M$; Δ, Tb $1.3 \times 10^{-5}$ $M$ and potassium chondroitin sulfate $1.3 \times 10^{-4}$ periods per liter. $R$ for methylene blue is $\epsilon$ at 610 mμ/$\epsilon$ at 665 mμ; for toluidine blue it is $\epsilon$ at 550 mμ/$\epsilon$ at 625 mμ.

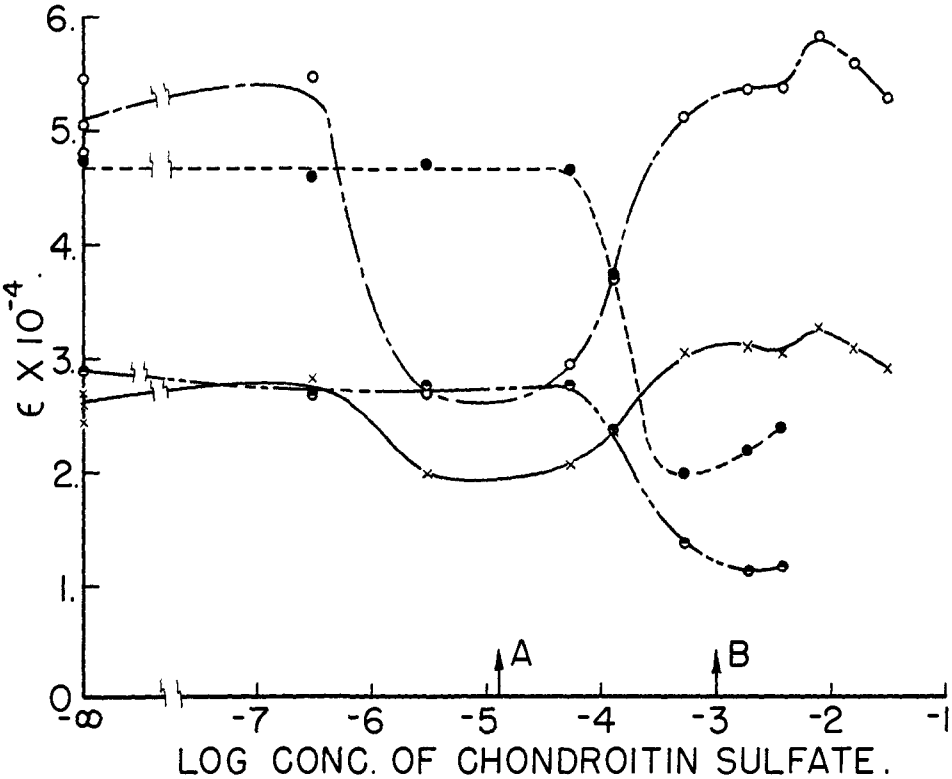


Fig. 6.—Extinction values of methylene blue at two wave lengths plotted against chondroitin sulfate concentration: crystalline calcium salt and Mb $1.25 \times 10^{-5}$ $M$, O, 665 mμ; ×, 610 mμ; amorphous potassium salt and Mb 1. $\times 10^{-3}$ $M$, ⊖, 665 mμ; ●, 610 mμ. Arrow A indicates abscissa at which chondroitin sulfate concentration equals dilute Mb. Arrow B indicates abscissa at which chondroitin sulfate concentration equals concentrated Mb.

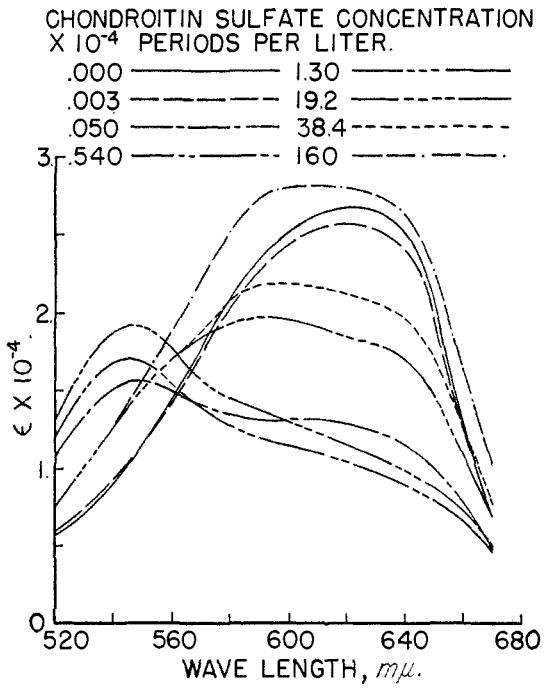


Fig. 7.—Absorption spectra of toluidine blue ($1.31 \times 10^{-5}$ $M$) at several concentrations of potassium chondroitin sulfate.

methylene blue curve mainly in the higher values reached by $R$ and in the absence of the sharply ascending spur at high chondroitin sulfate concentrations. The metachromasy of toluidine blue solutions is as sensitive to salt as that of methylene blue. In Fig. 5 is shown the sharp drop in the value of R with calcium chloride.

Methylene green deviates only slightly from Beer's law and the metachromasy induced by chondroitin sulfate is correspondingly slight. A hundred-fold increase in dye concentration, from $10^{-6}$ to $10^{-4}$ $M$, causes only a 12% decrease in the extinction at 620 mμ. Addition of $10^{-2}$ period per liter of chondroitin sulfate to $10^{-5}$ $M$ dye causes a 15% increase in extinction at 620 mμ.

## Discussion

Deviations from Beer's law have been ascribed to increasing dimerization of dye cations with increasing concentration, the heights of the α- and β-bands being assumed to represent the amounts of monomer and dimer, respectively. The dimerization theory rests not only on spectrophotometric data, but also conductance data of Robinson[13] and distribution data of Scheibe,[14] the latter relating to polymethine dyes. At high concentrations of dye in water, the spectrum approaches the metachromatic spectrum of a dilute dye solution in the presence of agar. The natural conclusion seemed to be that the dye in its metachromatic condition was dimerized. However, the results of the present study show conclusively that the metachromatic spectrum cannot be due to formation of dimeric dye cations since the extinctions at 665 and 610 mμ fall and rise together.

An attempt has been made to find support for the suggestion of Michaelis and Granick[6] that metachromasy is due to the formation of higher polymers of dye cations whose absorption bands lie to the short wave length side of the dimeric band.

(13) C. Robinson, *Trans. Faraday Soc.*, **31**, 245 (1935).
(14) G. Scheibe, *Kolloid-Z.*, **82**, 1 (1938).

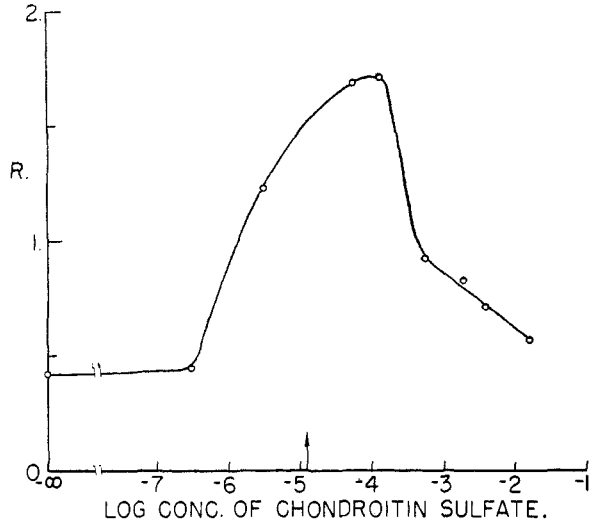


Fig. 8.—Extinction ratio, $R$, of toluidine blue ($1.31 \times 10^{-5}$ $M$), ε at 550 mμ/ε at 625 mμ, plotted against increasing concentrations of chondroitin sulfate. Arrow indicates abscissa at which chondroitin sulfate concentration equals toluidine blue concentration.

In Fig. 2 are plotted extinction values at 570 mμ as a function of chondroitin sulfate concentration. This curve differs from that at 610 mμ principally in that over most of the range it runs counter to the curve at 665 mμ. In this respect it is at least qualitatively in agreement with a theory of dye polymerization. In the absence of independent evidence it can offer only slender support to such a theory. Michaelis[15] has also expressed doubts as to the validity of the polymerization theory of metachromasy.

An interesting parallel to the dip and rise in the extinction of the α-band of methylene blue with increasing chondroitin sulfate concentration has been described by Rawson[16] for a different system. This occurs in the extinctions of the maxima of the acid azo dyes, Evans blue and Niagara sky blue 6B, in the presence of increasing concentrations of serum albumin. Anionic analogs to the cationic metachromatic dyes have received scarcely any attention.

It is further shown in the present study that with rising chromotrope concentration there is at first an appearance of metachromasy followed by its disappearance. This can be seen visually in the case of toluidine blue and can be followed spectrophotometrically by values of $R$ with both methylene blue and toluidine blue. Previous studies made with agar have not shown this phenomenon. This may be due to the limited solubility of agar. Hexametaphosphate, studied by Wiame,[3] is the only chromotrope for which this has ever been mentioned before.

The effect of neutral salts, in the concentration range $10^{-3}$ to $10^{-1}$ $M$, in depressing the metachromasy of methylene blue induced by chondroitin sulfate offers a plausible reason why concentrations of chondroitin sulfate at $10^{-3}$ also depress metachromasy. The more pronounced effect of calcium salts in suppressing metachromasy seems to account

(15) L. Michaelis, *J. Phys. Colloid Chem.*, **54**, 1 (1950).
(16) R. A. Rawson, *Am. J. Physiol.*, **138**, 708 (1943).

for the smaller metachromatic effect of the calcium chondroitin sulfate as compared with the potassium salt. Neutral salts at high concentrations produce an increase in values of $R$ either in the presence or absence of chondroitin sulfate. This is the effect Michaelis and Granick referred to as molecular aggregation when the salting out point is approached.

The locations of the minima in the extinctions at 665 and 610 m$\mu$ of Figs. 2 and 6 suggest that there is a loose binding of dye cations and chromotrope polyanions. Adsorption of dye ions to colloidal surfaces may cause a change in their color. Fajans and Hassel[17] showed this with anionic fluoresceins adsorbed to silver halides. Scheibe[14] showed that a shift in absorption maxima of some polymethine dyes occurs on adsorption to surfaces such as mica. Since chondroitin sulfate is polyanionic, it may be expected to have some of the properties of the water soluble synthetic linear polycations described by Fuoss and Strauss.[18] In particular its solutions are likely to have micro regions of high and low anionic density. Regions of high anionic density will tend to fix dye cations. If the dye is one whose energy levels are easily influenced by external electrical fields, then energy level differences and absorption band positions will be likely to change. Addition of neutral salts to a concentration of $10^{-3}$ $M$ would supply competing cations that could displace most of the dye cations ($10^{-5}$ $M$) and metachromasy would disappear. Increasing chondroitin sulfate concentration would tend to level out the anionic density throughout the solution and at the same time increase the average cation density and again metachromasy would disappear. Why dyes which show Beer's law deviations also show metachromasy cannot be accounted for except to suggest that in these dyes energy levels are more sensitive to external electrical fields. That the metachromatic effect is quite different from the Beer's law deviation effect has been sufficiently emphasized.

There is left for discussion the difference between the amorphous potassium chondroitin sulfate and the corresponding salt made from crystalline calcium chondroitin sulfate. The difference appears at concentrations of chondroitin sulfate above $10^{-4}$ period per liter. It might indicate the presence of a chromotropic component in the amorphous potassium salt that has been removed in the further purification leading to the crystallization of the calcium salt. Work is in progress dealing with this point.

The results of this study of metachromasy in dilute aqueous solutions of a purified chromotrope are not directly applicable to the interpretation of results of histological metachromatic staining. The latter is carried out on heterogeneous systems and is subject to far more complex influences.

(17) K. Fajans and O. Hassel, *Z. Elektrochem.*, **29**, 495 (1923).
(18) R. M. Fuoss and U. P. Strauss, *J. Polymer Sci.*, **3**, 246 (1948).

NEW YORK 116, N. Y. RECEIVED JUNE 26, 1951

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[CONTRIBUTION NO. 1557 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

# The Kinetics of the $\alpha$-Chymotrypsin-Catalyzed Hydrolysis of Chloroacetyl- and Trifluoroacetyl-L-tyrosinamide in Aqueous Solutions at 25°[1]

BY HENRY J. SHINE AND CARL NIEMANN[2]

The kinetics of the $\alpha$-chymotrypsin-catalyzed hydrolysis of chloroacetyl- and trifluoroacetyl-L-tyrosinamide have been determined in aqueous solutions at 25° and at the optimum *p*H for each substrate; *i.e.*, between 7.7 and 7.8 for chloroacetyl-L-tyrosinamide, and between 7.8 and 7.9 for trifluoroacetyl-L-tyrosinamide. The kinetic constants so obtained appear to support the proposition that the principal forces involved in the combination of the enzyme with specific substrates and competitive inhibitors are van der Waals forces rather than intermolecular hydrogen bonds.

Results of investigations on the nature of the $\alpha$-chymotrypsin-catalyzed hydrolysis of amino acid derivatives have led to the hypothesis[3,4] that an $\alpha$-amino acid derivative of the general formula $R_1CHR_2R_3$ which can combine with the enzyme at its catalytically active site does so *via* combination with three centers which are complementary to the three prominent structural features of the attached molecule, *viz.*, $R_3$, the functional derivative of the carboxyl group; $R_2$, the $\alpha$-amino acid side chain; and $R_1$, the remaining substituent of the $\alpha$-carbon atom. It is fortunate that the nature of the group $R_1$ can be varied over rather wide limits without causing a critical loss of those properties which are characteristic of experimentally useful specific substrates. Thus, with an appropriate choice in respect to the nature of $R_2$ and $R_3$, an examination of the effect of variation in the character of $R_1$, where $R_2$ and $R_3$ remain invariant within a given series, might be expected to yield information relative to the nature of the forces involved in the combination of the enzyme with the various specific substrates and, possibly, to the mechanism of the subsequent hydrolytic process.

The immediate objective of the present investigation was to determine the effects on the course of the reaction due to the presence of electron-attracting groups in an amide type $R_1$ substituent. The specific substrates selected for study were chloroacetyl-L-tyrosinamide and trifluoroacetyl-L-tyrosinamide. The *p*H-activity relationship was determined for each of these specific substrates, and the optimum *p*H for the hydrolysis of chloroacetyl-L-tyrosinamide was found to be between 7.7 and 7.8, and for trifluoroacetyl-L-

(1) Supported in part by a grant from Eli Lilly and Company.
(2) To whom inquiries regarding this article should be sent.
(3) H. Neurath and G. W. Schwert, *Chem. Revs.*, **46**, 69 (1950).
(4) H. T. Huang and C. Niemann, THIS JOURNAL, **73**, 3223 (1951).