intermediates and free enzyme are at equilibrium concentrations given by the expressions

$$(X_\alpha) = K_\alpha{}^0 (E)(A) \quad \alpha = 1 \ldots f-1 \quad (18a)$$

$$(X_\beta) = K_\beta{}^0 \frac{(E)(A)}{(Q)} \quad \beta = f \ldots g-1 \quad (18b)$$

$$(X_\gamma) = K_\gamma{}^{n+1} (E)(R) \quad \gamma = g \ldots n \quad (18c)$$

$$(E) = \frac{(E)_0}{1 + (A)\sum_{\alpha=1}^{f-1} K_\alpha{}^0 + \frac{(A)}{(Q)}\sum_{B=f}^{g-1} K_\beta{}^0 + (R)\sum_{\gamma=g}^{n} K_\gamma{}^{n+1}} \quad (18d)$$

A* ⇄ R* Exchange.—Analogously to the previously discussed mechanisms, the A* ⇄ R* exchange rate is obtainable from the over-all steady-state rate law, *i.e.*

$$R_{AR} = \frac{(V_{AB}/K_{AB})(A)(B)}{\frac{(A)}{K_A} + \frac{(B)}{K_B} + \frac{(Q)}{K_Q} + \frac{(R)}{K_R} + \frac{(A)(B)}{K_{AB}} + \frac{(Q)(R)}{K_{QR}} + \frac{(A)(Q)}{K_{AQ}} + \frac{(B)(R)}{K_{BR}} + \frac{(B)(Q)}{K_{BQ}} + \frac{(A)(B)(Q)}{K_{ABQ}} + \frac{(B)(Q)(R)}{K_{BQR}}} \quad (19)$$

A* ⇄ Q* Exchange.—When the isotope is exchanged between A* and Q*, the only labeled intermediates are designated by the subscript α, *i.e.*, X^*_α. Applying the steady state condition to the rate of change of concentration of these intermediates and the equilibrium relations 18b–18d to the other intermediates and free enzyme yields a differential equation for the approach to isotopic equilibrium with an exchange rate R_{AQ} defined

$$R_{AQ} = \frac{(V_{AB}/K_{AB})(A)(B)}{\frac{(B)}{K_B} + \frac{(R)}{K_R} + \frac{(B)(R)}{K_{BR}} + \frac{(A)(B)}{K_{AQ}}} \quad (20)$$

where

$$K_{AQ} = \frac{1}{\sum_{\alpha=1}^{f-1}\sum_{s=0}^{f-1} \frac{K^s{}_\alpha}{k_{(s+1)}}} \quad (21)$$

Again it can be seen that

$$R_{AQ}, R_{BR} > R_{AR} \quad (22)$$

As a consequence of the application of the steady state condition in the above treatment, the information derivable from the exchange rates is in the main of the same type as that accessible from ordinary steady state kinetic studies. The appearance of certain new kinetic parameters in the expressions for some of the exchange rates does provide some ancillary information, however. Studies of exchange kinetics *alone* are less fruitful than ordinary steady-state kinetic studies in view of the fact that the latitude provided by the variation of the concentrations of the unlabeled species is restricted by the condition of over-all thermodynamic equilibrium.

The chief recommendation of such studies seems to lie in the opportunity to establish the sequence of combination of substrates with the enzyme through the anticipated inequalities in the exchange rates. This is particularly apparent for the dehydrogenase systems without ternary complexes where it has been pointed out previously that steady-state kinetic studies are insufficient to establish this order.[10]

The inequalities in exchange rates plainly derive only from the sequence of reaction of the various substrates and are in no way contingent on the number and steady-state concentrations of intermediates. Consequently, the contention that these inequalities provide a basis for *any* inferences about "rate-limiting steps"[5,8a,b] is seen to be without foundation.

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[CONTRIBUTION FROM THE DEPARTMENT OF MEDICINE, THE STUDY GROUP ON AGING, AND THE STUDY GROUP FOR RHEUMATIC DISEASES, NEW YORK UNIVERSITY SCHOOL OF MEDICINE, NEW YORK, N. Y.]

Measurement of the Stability of Metachromatic Compounds[1]

BY MEDINI KANTA PAL[2] AND MAXWELL SCHUBERT

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Two methods, ultracentrifuging and adsorption to $CaHPO_4$, which had previously been shown to remove from solution the metachromatic compound of chondroitin sulfate and methylene blue, have been extended to study metachromatic compounds of a wider variety of dyes and polyanions. Though in most cases the two methods are equivalent, the centrifugal method was found more generally effective than the adsorption method. An extension of this study to metachromatic solutions in mixtures of ethanol and water led to the development of a new method that makes possible a quantitative estimate of the relative stabilities of different metachromatic compounds in terms of the concentration of ethanol or urea that brings about their destruction. Results with ethanol and urea are nearly equivalent in twelve combinations of dye and polyanion and show it is possible to set up a single scale of stabilities of metachromatic compounds containing different dyes and polyanions. On such a scale it becomes clear that not only do polyanions differ markedly among themselves in the stabilities of the compounds they form with a single dye, but dyes also differ correspondingly. With any particular polyanion, methylene blue and crystal violet form much less stable metachromatic compounds than do toluidine blue and acridine orange.

In earlier work two independent methods were developed which showed that the metachromatic color produced in a solution containing chondroitin sulfate and methylene blue was mainly due to the formation of a single compound consisting of equiva-

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lent amounts of dye and polyanion. This was shown by actual removal of this compound from solution, determination of a difference spectrum that was purely metachromatic, and analysis of the compound removed for content of dye and polyanion (chromotrope). In one method (I) the metachromatic compound was removed from the solution by high speed centrifugation[3]; in the other method (II) it was removed by adsorbants which were shown to adsorb metachromatic compound but neither dye nor polyanion alone.[4] Thus for the first time it became possible to refer metachromatic color in solution to the existence of a single compound of dye and polyanion, in which polyanionic sites were saturated with dye cations. That such a single compound of cationic dye and polyanion could exist in solution in the presence of an excess of polyanion over ten times that of the dye showed that dye cations are not distributed at random over anionic sites of all the polyanion in solution but tend to select adjacent sites on a single polyanion chain. Such a selection of adjacent sites could reasonably be related to the known tendency of the metachromatic dyes to polymerize. The formation of metachromatic compound would involve not only a free energy term for the cation–anion ion pair interaction but also a free energy term for the interaction of neighboring cations along a single polyanion chain.

Since the work described had been done with only two dyes and a single polyanion, the first need seemed to be to determine whether the two methods could be applied to a wider set of combinations of dyes and polyanions, and whether in a wider application the two methods would always agree. This has been done and the results are presented here. In the course of this work a further application of the two methods was explored; an attempt was made to measure the progressive decrease in metachromatic compound formation in the presence of increasing concentrations of ethanol. Marked differences were found in the concentrations of ethanol at which different metachromatic compounds were destroyed. Out of this there was developed a new, independent, and purely spectrophotometric method to measure the stabilities of metachromatic compounds composed of different dyes and polyanions in terms of the ethanol concentration required to destroy a fixed fraction of each metachromatic compound. An additional finding is that urea can be used in place of ethanol with quite similar results. With this method it is possible to set up a single scale to measure the stabilities of metachromatic compounds containing different dyes and polyanions.

Experimental

The dyes used were the purest grades from the National Aniline Co. Each was recrystallized at least once and on chromatographing showed only a single component.

Methylene blue (10 g.) was dissolved in HCl solution (0.1 *M*, 160 ml.) at 95°, filtered hot, and the filtrate chilled to 5°. The crystals were separated by centrifuging, washed with chilled ethanol and ether, and dried *in vacuo;* yield 8.3 g.

Crystal violet (10 g.) was dissolved in water (200 ml.) at 95°, filtered hot, and the filtrate chilled to 5°. The crystals were separated, washed, and dried at above; yield 5.2 g.

Toluidine blue (6 g.) was dissolved in HCl solution (0.03 *M*, 100 ml.) at 50°, filtered hot, and to the filtrate concentrated HCl (10 ml.) slowly added while the filtrate was chilled to 5°. The crystals were separated, washed, and dried as above; yield 4.0 g.

Acridine orange (6 g., as a double salt with $ZnCl_2$) was dissolved in water (200 ml.). The solution was stirred four times with the potassium form of Dowex-50 (12 g. each time) to remove Zn ions. The solution was concentrated *in vacuo* to 20 ml., and ethanol (100 ml.) was added precipitating KCl which was removed. To the clear solution ether (160 ml.) was added and on chilling the dye crystallized as its chloride. The crystals were separated, washed, and dried as above; yield 2.2 g. The product was recrystallized by dissolving in ethanol (100 ml.), adding ether (50 ml.), and chilling; yield 1.0 g.

Alginic acid, λ-carrageenan and κ-carrageenan were kindly supplied by Marine Colloids, Inc., New York. Each was reprecipitated and some head and tail fractions removed. Alginic acid (5 g.) was dissolved in water (550 ml.) containing $KHCO_3$ (2.8 g.), and acetic acid (0.3 ml.) and potassium acetate (5 g.) were added. Ethanol was added to 25% (v./v.) and insoluble material discarded. From the clear solution, addition of ethanol to 37% precipitated the fraction used (3.6 g.). λ-Carrageenan (4 g.) in water (600 ml.) containing potassium acetate (12 g.) was fractionated by addition of ethanol, retaining the fraction precipitating between 30 and 45% (v./v.) ethanol, which amounted to 2.7 g. λ-Carrageenan was precipitated from aqueous solution by addition of potassium acetate and ethanol. No fractionation was achieved since the entire sample was recovered.

A commercial sample of heparin (Fisher Scientific Co.) was dissolved in 0.1 *M* NaCl (1 g. in 100 ml.) and reprecipitated by addition of ethanol (150 ml.); yield 0.9 g.

Chondroitin sulfate and one of its naturally occurring compounds with protein (called PP-L) were prepared by methods described elsewhere.[5]

Hyaluronate was prepared from acetone-dried umbilical cords (10 g.) by extraction twice, each time with an aqueous solution (150 ml.) of KCl (30%) and K_2CO_3 (1%). The filtered extract was dialyzed, and to it there was added potassium acetate (10 g.) and ethanol (1 liter). The precipitated product of mixed polysaccharides was centrifuged, washed with ethanol and ether, and dried *in vacuo*. The product (0.42 g.) was extracted twice, each time with a solution of potassium acetate (20 g.) in water (20 ml.). Under these conditions chondroitin sulfates dissolve readily but hyaluronate does not. The undissolved residue was dissolved in water (30 ml.), the solution filtered with suction, and potassium acetate (2 g.) and ethanol (80 ml.) were added precipitating crude hyaluronate (1.5 g). For purification to remove all chondroitin sulfate this crude hyaluronate was dissolved in water (120 ml.) and KCl (0.9 g.) and methylene blue (96 mg.) added. The solution was centrifuged at 40,000 r.p.m. for 0.5 hour and the sediment was discarded. The supernatant solution was stirred twice, each time with Dowex-50 (K form, 8 g.), to remove methylene blue. To the solution was added potassium acetate (5 g.) and ethanol (240 ml.) and the precipitated product washed with ethanol and ether and dried. The yield was 83 mg. and the product was free of sulfur.

Ribonucleic and deoxyribonucleic acids were commercial products used without further purification. Since the nucleic acids gave obvious precipitates with many of the dyes at the high concentration used ($1.25 \times 10^{-4} M$) they were omitted from most of the present studies.

The ultracentrifugal(I) and adsorption(II) methods, including measurement of spectra and difference spectra, and analysis of pellets and supernatant solutions for both dye and chromotrope have been described in recent work.[3,4] Some simplifications in the analysis of pellets (method I) were developed. Pellets drained of most adhering solution were dissolved by stirring at 50° with 5 ml. of a solvent, 0.08 *M* NaCl in 33% (v./v.) ethanol in water. For determination of chromotrope, 2 ml. of this solution decolorised with Dowex-50 was evaporated to dryness in a hot water-bath, the residue was dissolved in 2 ml. of water containing NaCl and 1

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(4) M. K. Pal and M. Schubert, *J. Histochem. Cytochem.*, **9**, 673 (1961).

(5) B. R. Gerber, E. C. Franklin and M. Schubert, *J. Biol. Chem.*, **235**, 2870 (1960).

ml. of 0.3% cetylpyridinium chloride was added. To obtain maximum turbidity the amount of NaCl was adjusted for each chromotrope so its final concentration after addition of the cetylpyridinium chloride was as follows: 0.15 *M* for chondroitin sulfate and alginate, 0.08 *M* for heparin, 0.04 *M* for hyaluronate. Standard curves were determined for each chromotrope under the same conditions.

Results

A direct comparison of the two methods (I and II) which remove metachromatic compounds from solution was first made on a set of 32 combinations of four dyes and eight chromotropes. Earlier use of these two methods with the particular combination methylene blue and chondroitin sulfate had shown that after removal of the metachromatic compound the supernatant solution was orthochromatic. In the wider application of these two methods this is not always the case. With ribonucleate, deoxyribonucleate, chondroitin sulfate, alginate and hyaluronate the supernatant solutions are all orthochromatic. With λ-carrageenan, κ-carrageenan and heparin the supernatant solutions are generally but not always metachromatic. Table I records the percentage of dye removed as metachromatic compound in each case. Consider first the degree of agreement between the two methods for any particular combination of dye and chromotrope as measured by the difference in percentage of dye removed; this difference is less than 10 in 18 of the combinations; it lies between 10 and 20 in 9 of the combinations, and is greater than 20 in 5 of the combinations (those underlined in Table I). In these last 5 combinations the

TABLE I

PERCENTAGE OF DYE REMOVED AS METACHROMATIC COMPOUND BY CENTRIFUGATION (I) OR BY ADSORPTION TO $CaHPO_4$ (II)

Initial dye concentration, 1.25×10^{-4} *M*; initial chromotrope concentration, 1.50×10^{-4} equiv./l.; NaCl, 0.004 *M*

Method	Acridine orange		Toluidine blue		Methylene blue		Crystal violet	
	I	II	I	II	I	II	I	II
λ-Carrageenan	95	92	95	88	89	74	90	95
κ-Carrageenan	89	86	94	70	81	54	80	96
Heparin	81	95	89	91	80	40	73	82
Ribonucleic acid	95	97	96	94	86	85	81	77
Deoxyribonucleic acid	94	96	96	94	88	70	75	73
Chondroitin sulfate	82	96	94	86	86	74	74	87
Alginate	76	76	94	77	72	41	60	44
Hyaluronate	73	77	83	59	15	15	24	24

centrifugal method always removed a higher percentage of the dye than the adsorptive method. Considered in terms of the dye used, agreement between the two methods is best with acridine orange, worst with methylene blue. It appears that in a great many of the cases tried the two methods are equivalent, but that in the small number of cases where there is no agreement it is the adsorptive method which fails to remove some of the metachromatic compound. It is surprising that by the centrifugal method there is so little variation in the amount of metachromatic compound sedimented. In only 3 of the 32 combinations examined was the amount of dye removed less than 70% of the initial dye. The amount of dye removed is not necessarily the amount initially present in the metachromatic form, since removal of the metachromatic compound initially present may result in a rapid shift in equilibria forming more metachromatic compound which is then likewise sedimented.

Table II records data on the compositions of metachromatic compounds removed by the centrifugal method. The parentheses following the figures in column 4 indicate whether the composition of the sediment was determined directly on the sediment or indirectly by analysis of the supernatant solution. In most of the combinations examined the equivalence ratio, E.R., (the ratio in equivalents of chromotrope to dye) is close to 1.0 as it was in the case of chondroitin sulfate and methylene blue examined under a more varied range of conditions.[3,4] In the case of heparin with either toluidine blue or crystal violet the ratio is persistently found higher, averaging 1.4.

Ethanol has been thought to destroy metachromasia produced by some chromotropes and not to destroy that produced by others, but a systematic study of this phenomenon in solution has never been attempted. The techniques used to compile Table I seemed to offer a way to study the stabilities of a series of metachromatic compounds with respect to ethanol. Results of such experiments are summarized in Table III, giving the percentage of dye removed as metachromatic compound in combinations of seven chromotropes and two dyes by each of the two methods at two concentrations of ethanol. Without exception, in all combinations of dye and chromotrope the amount of metachromatic compound removed drops as the concentration of ethanol is raised. The extent of the drop varies widely for different combinations of dye and chromotrope. Also without exception the extent of the drop is greater by the adsorption (II) than by the centrifugal method (I). Thus it appears even more markedly in Table III than in Table I that $CaHPO_4$ often fails to remove metachromatic compound that can be removed by centrifugation, and the difference is often very great. The centrifugal method to remove metachromatic compound seems more effective than the adsorption method. Considering only the centrifugal method, there are quite marked differences in the ethanol concentration at which the most marked drop in the amount of metachromatic compound removable occurs. In the case of toluidine blue, there is no great drop in the amount of metachromatic compound sedimented with λ-carrageenan, but there is with alginate or hyaluronate. In the case of methylene blue, ethanol reduces more markedly the amounts of metachromatic compounds sedimented with all chromotropes. The results suggest that ethanol destroys metachromatic compounds of all chromotropes and that differences among chromotropes in their behavior with ethanol is one of degree rather than of kind as the terms "true" and "false" metachromasia might indicate.

The concentration of dye is another variable whose effects are worth investigating, particularly in an attempt to relate the present studies to conditions that may exist in tissue staining. All comparisons so far described were made at $1.25 \times$

TABLE II

COMPOSITION OF METACHROMATIC COMPOUNDS REMOVED BY THE CENTRIFUGATION METHOD (I) IN TERMS OF EQUIVALENCE RATIO E. R. (EQUIV. CHROMOTROPE/EQUIV. DYE)

Chromotropes	Dyes	E.R. at start	E.R. in sediments	Dye sedimented, %
Heparin	Toluidine blue	1.2	1.13 (Sup)	92
Heparin	Toluidine blue	1.2	1.35 (Sed)	..
Heparin	Toluidine blue	1.2	1.60 (Sed)	86
Heparin	Toluidine blue	2.4	1.40 (Sup)	86
Heparin	Toluidine blue	2.4	1.55 (Sed)	..
Heparin	Toluidine blue	2.4	1.50 (Sed)	77
Heparin	Toluidine blue	3.6	1.31 (Sed)	77
Heparin	Toluidine blue	4.8	1.21 (Sed)	..
Heparin	Toluidine blue	6.0	1.38 (Sed)	78
Heparin	Toluidine blue	9.6	1.65 (Sed)	..
Heparin	Toluidine blue	9.6	1.25 (Sed)	86
Heparin	Crystal violet	1.2	1.16 (Sup)	76
Heparin	Crystal violet	2.4	1.44 (Sup)	72
Chondroitin sulfate	Toluidine blue	1.2	1.24 (Sed)	95
Chondroitin sulfate	Toluidine blue	4.8	1.09 (Sed)	93
Chondroitin sulfate	Toluidine blue	9.6	0.98 (Sed)	94
Chondroitin sulfate	Toluidine blue	20.	1.13 (Sed)	94
Chondroitin sulfate	Toluidine blue	30.	1.18 (Sed)	93
Chondroitin sulfate	Toluidine blue	40.	1.17 (Sed)	92
Chondroitin sulfate	Toluidine blue	50.	1.20 (Sed)	90
Alginate	Toluidine blue	1.2	1.20 (Sup)	95
Alginate	Toluidine blue	1.2	1.20 (Sup)	95
Alginate	Toluidine blue	2.4	0.83 (Sup)	58
Alginate	Toluidine blue	2.4	0.90 (Sup)	61
Hyaluronate	Toluidine blue	1.2	1.00 (Sup)	80
Hyaluronate	Toluidine blue	2.4	1.04 (Sup)	81
Hyaluronate	Acridine orange	1.2	1.05 (Sup)	65
Hyaluronate	Acridine orange	2.4	0.85 (Sup)	54
Hyaluronate	Crystal violet	1.2	0.96 (Sup)	22

TABLE III

EFFECT OF ETHANOL ON THE PERCENTAGE OF DYE REMOVED AS METACHROMATIC COMPOUND BY CENTRIFUGATION (I) OR BY ADSORPTION TO $CaHPO_4$ (II)

Initial dye concentration, 1.25×10^{-4} *M*; initial chromotrope concentration, 1.50×10^{-4} equiv./l.; NaCl, 0.004 *M*

	Toluidine blue						Methylene blue					
Method	I			II			I			II		
Alcohol, %	0	15	40	0	15	40	0	15	40	0	15	40
λ-Carrageenan	95	90	71	88	80	61	89	85	52	74	36	34
κ-Carrageenan	94	84	53	70	45	33	81	77	36	54	18	18
Heparin	89	74	46	91	26	23	80	64	22	40	22	12
Protein compd. of chondroitin sulfate (PP-L)	80	70	18	89	23	9	80	52	14	80	10	0
Chondroitin sulfate	94	82	6	86	18	4	86	63	19	74	22	10
Alginate	94	80	6	77	56	5	72	36	19	41	14	10
Hyaluronate	83	35	8	59	33	0	15	12	12	15	5	10

10^{-4} *M* dye. Effects at higher dye concentrations are recorded in Table IV for toluidine blue, the dye most commonly used in metachromatic staining of tissues, and only for the chromotropes chondroitin sulfate and hyaluronate, said to produce "true" and "false" metachromasia, respectively. In increasing the dye concentration the chromotrope concentration is of course also increased to maintain the ratio constant. It is apparent that at higher dye and chromotrope concentrations the effect of ethanol in decreasing the percentage of dye present as metachromatic compound is weakened to such an extent that at the highest dye and chromotrope concentration even 50 to 60% ethanol fails to reduce very much the percentage of dye sedimented as metachromatic

TABLE IV

PERCENTAGE OF DYE REMOVED AS METACHROMATIC COMPOUND AT VARIOUS CONCENTRATIONS OF ETHANOL AND DYE

Concn. of chromotrope, 1.2 × concn. of dye (all in equivalents); NaCl = 0.004 *M*

	Toluidine blue, $M \times 10^4$	Ethanol concentration, % (v./v.) 0	15.	30.	40.	50.	60.
Chondroitin sulfate	1.25	94	82	41	6	..	..
	10.0	99	..	92	..	73	70
	100.	100	..	98	..	92	..
Hyaluronate	1.25	83	35	10	8	..	.
	10.0	94	88	..	25	..	13
	100.	99	..	..	86	..	78

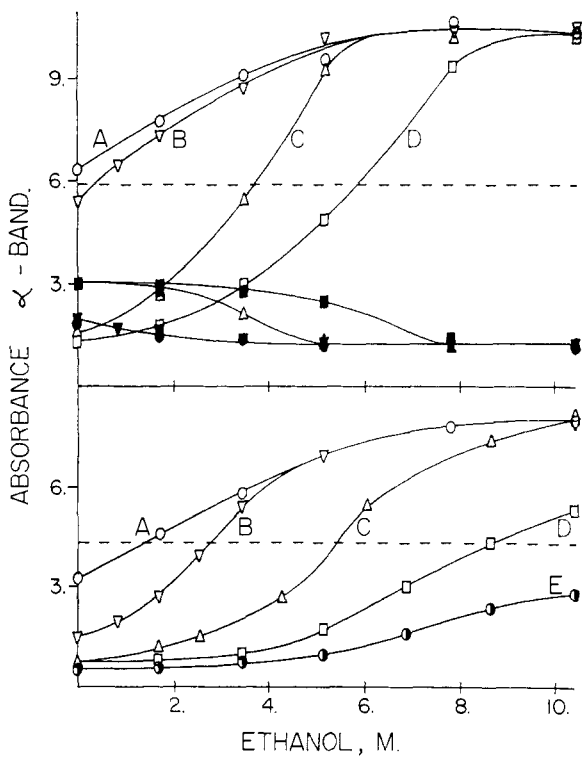


Fig. 1.—Absorbance at the α-bands of dyes *vs.* molarity of ethanol in the presence of various chromotropes; dye, 1.25×10^{-4} *M*; chromotrope, 1.50×10^{-4} equiv./l.; NaCl, 0.0025 *M*. Upper half, methylene blue, α-band at 665 mμ; A, dye alone; B, with hyaluronate; C, with chondroitin sulfate; D, with heparin; (corresponding solid symbols are absorbances at the μ-band, 570 mμ). Lower half, toluidine blue, α-band at 635 mμ; symbols as above: E, with λ-carrageenan.

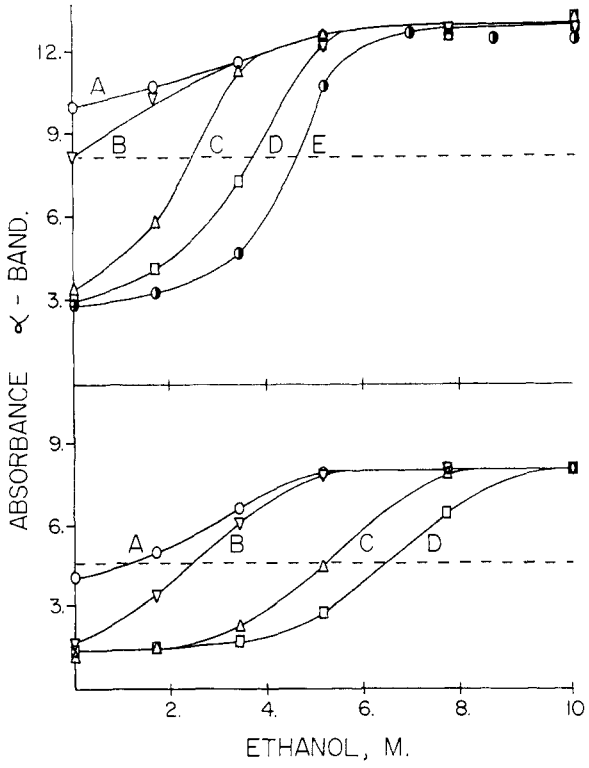


Fig. 2.—Absorbance at the α-bands of dyes *vs.* molarity of ethanol in presence of various chromotropes; dye, 1.25×10^{-4} *M*; chromotrope, 1.50×10^{-4} equiv./l.; NaCl, 0.0025 *M*: upper half, crystal violet, α-band at 590 mμ, symbols same as in Fig. 1; lower half, acridine orange, α-band at 495 mμ, symbols same as in Fig. 1.

compound. It is also apparent that at the intermediate dye concentration (10^{-3} *M*) and an ethanol concentration of 60% most of the metachromatic compound of chondroitin sulfate remains and is sedimentable while only a small fraction of the metachromatic compound of hyaluronate remains.

The data of Tables III and IV show the progressive destruction of metachromatic compounds, as ethanol concentration is increased, by methods which selectively remove the metachromatic compounds from solution. Another way to follow the extent of destruction of metachromatic compounds by ethanol might be to follow the increasing absorbance of solutions of dye and chromotrope at the α-band or the decreasing absorbance at the μ-band as a function of ethanol concentration. This method has never before been used quantitatively. Experience gained in collecting data presented in Figs. 1 and 2 shows it offers a rather precise as well as rapid method to compare the stability of different metachromatic compounds. In the upper part of Fig. 1 are plotted absorbances at the α-band with methylene blue alone (curve A) and in the presence of three different chromotropes (curves B, C, D) at a series of ethanol concentrations. In all four cases the absorbance at the α-band rises to the same plateau values with increasing ethanol concentration. In each case, over the same range of ethanol concentration, the absorbance at the μ-band drops. Since the drop in the μ-band is so much smaller than the rise in the α-band, and since in all cases studied these two changes occur over the same concentration range of ethanol, only the α-band has been plotted in subsequent figures. The abscissa value at which each of the curves of α-band absorbance reaches the level of their common plateau indicates the ethanol concentration at which all metachromatic compound is destroyed, and all dye cations are freed of association with each other. A sharper measure of the stabilities of these metachromatic compounds is the ethanol concentration at which they appear to be half destroyed, the ethanol concentration at which the α-band absorbance attains half its plateau value. The horizontal dotted lines in the Figs. 1 and 2 represent the absorbance at half the plateau values for each of the dyes studied. The half plateau value is taken half way between the upper plateau level approached or reached for each family of curves at high ethanol concentration and the lower level of absorbance approached at low ethanol concentration in the presence of the strongest chromotropes (heparin or λ-carrageenan) which represents mainly the end absorbance of the metachromatic or μ-band. The families of curves in Figs. 1 and 2 show the differences among the chromotropes with respect to the stabilities of their metachromatic compounds with each of four dyes. For each dye the order of

the stabilities of the metachromatic compounds with a series of chromotropes is similar. Hyaluronate is a feeble chromotrope, indicated in all cases by the position of its curve B, very close to curve A, that for the dye alone. In all cases, at zero ethanol concentration, the curves B start at absorbance levels already near the half plateau value. The successive curves B (hyaluronate), C (chondroitin sulfate), D (heparin) and E (λ-carrageenan) cross the half plateau value at successively higher ethanol concentrations, in the same order for all four dyes. Corresponding to the qualitative impression from histochemical studies that chromotropes differ in "strength," the half plateau values provide a quantitative measure of these different chromotropic strengths. Just as the chromotropes can be arranged in order by their half plateau values, so can the dyes, and the dyes can be said to differ in their metachromatic strengths. For any chromotrope the order of metachromatic strength of the dyes is toluidine blue > acridine orange > methylene blue > crystal violet, with one exception that in the case of chondroitin sulfate, acridine orange is slightly stronger than toluidine blue. These half plateau values are more readily compared by the numbers read from the figures and summarized in Table V. In combinations of the strongest chromotropes and the strongest metachromatic dyes, complete destruction of metachromasia is not achieved even at the highest ethanol concentration used (60% by volume or about 10 *M*). Above such ethanol concentrations precipitation of chromotropes sometimes begins. To the extent that this occurs with bound dye cations the precipitate may retain metachromatic color, but if metachromatic compound has been completely destroyed the precipitate will be colorless. An example of this was observed at 70% ethanol; the precipitate produced with the combination toluidine blue and the carrageenans was metachromatically colored, while that produced with the combination toluidine blue and alginate was colorless.

TABLE V

MID-PLATEAU VALUES OF FIGS. 1, 2, 3, 4 AS ETHANOL OR UREA CONCENTRATION

	Concentration, *M*			
	TB	AO	MB	CV
		Ethanol		
Hyaluronate	2.6	2.4	0.3	0.0
Chondroitin sulfate	5.3	5.6	3.6	2.4
Heparin	8.5	6.3	5.6	3.6
		Urea		
Hyaluronate	1.6	1.5	...	...
Chondroitin sulfate	4.8	6.4	2.7	2.3
Heparin	7.8	9.0	4.2	3.4

The thought that dye polymerization might be in part due to formation of hydrophobic bonds suggested that urea might also be capable of destroying metachromatic compounds. Urea has been used to break hydrophobic bonds in a study of its effect on the critical micelle concentration of dodecyltrimethylammonium ions.[6] The data

(6) W. Bruning and A. Holtzer, *J. Am. Chem. Soc.*, **83**, 4865 (1961).

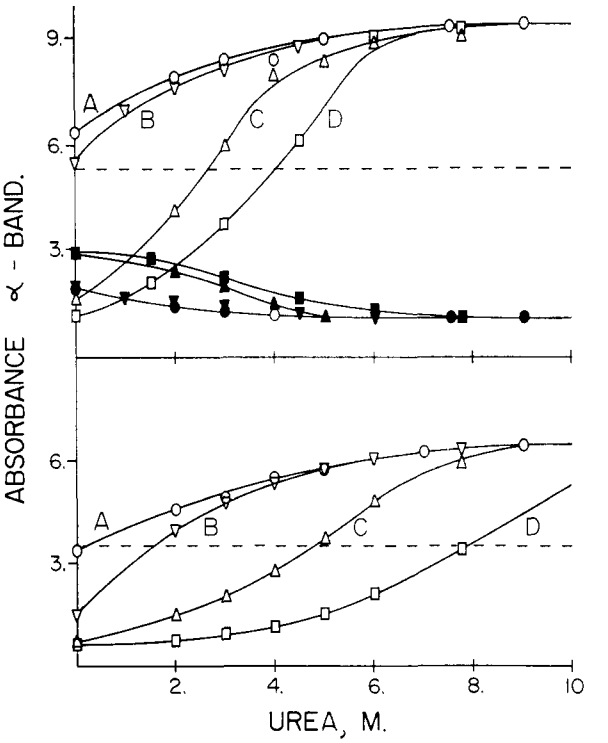


Fig. 3.—Absorbance at the α-bands of dyes *vs.* molarity of urea in the presence of various chromotropes; dye, 1.25 × 10^{-4} *M*; chromotrope, 1.50 × 10^{-4} equiv./l.; NaCl, 0.0025 *M*: upper half, methylene blue, α-band at 665 mμ, symbols as in Fig. 1 (corresponding solid symbols are absorbances at the μ-band, 570 mμ); lower half, toluidine blue, α-band at 635 mμ, symbols as in Fig. 1.

of Figs. 3 and 4 show that with increasing concentration of urea there is a destruction of metachromatic color strikingly similar to that caused by ethanol. The chromotropes all lie in the same order with respect to the urea concentration at which metachromatic color is half destroyed as they did with ethanol. Table V summarizes the half plateau values found with urea and allows direct comparison with those found with ethanol.

Other approaches to the study of the stability of metachromatic compounds are illustrated in Fig. 5. The destruction of metachromatic color by the addition of neutral salt is shown by curve B. With increasing salt concentration in a solution containing toluidine blue and chondroitin sulfate the α-band rises to a plateau as it does with ethanol, but the rise is only a fifth as great. In the absence of chondroitin sulfate, addition of NaCl to toluidine blue solution causes a drop in the α-band absorbance to the same plateau (curve A, Fig. 5). Because the total change in absorbance is so much smaller by this method, it was not further investigated. In a few cases addition of NaCl causes precipitation rather than destruction of metachromatic compound. With toluidine blue and heparin, for example, at NaCl over 0.06 *M*, a purple precipitate forms.

Excess chromotrope can also destroy metachromatic color. Curve E of Fig. 5 shows the initial drop followed by a rise in the α-band, while curve F shows the initial rise followed by a drop in

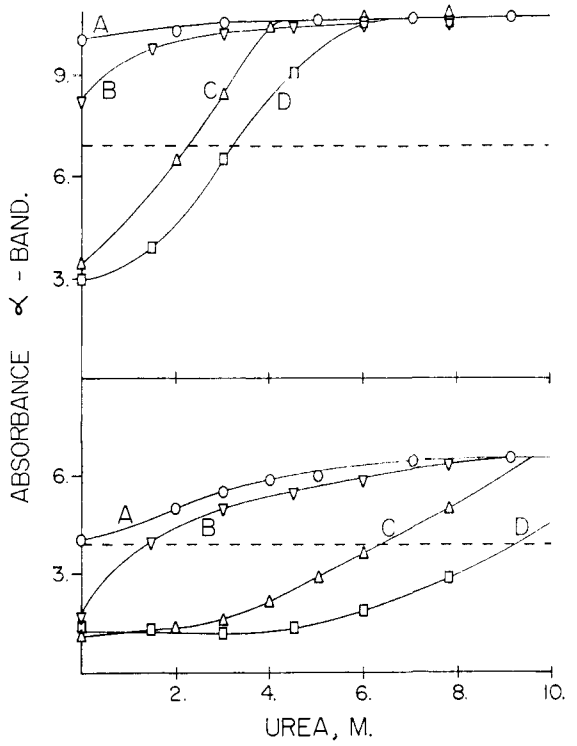


Fig. 4.—Absorbance at the α-bands of dyes *vs.* molarity of urea in the presence of various chromotropes; dye, 1.25×10^{-4} *M;* chromotrope, 1.50×10^{-4} equiv./l.; NaCl, 0.0025 *M:* upper half, crystal violet, α-band at 590 mμ, symbols as in Fig. 1; lower half, acridine orange, α-band at 495 mμ, symbols as in Fig. 1.

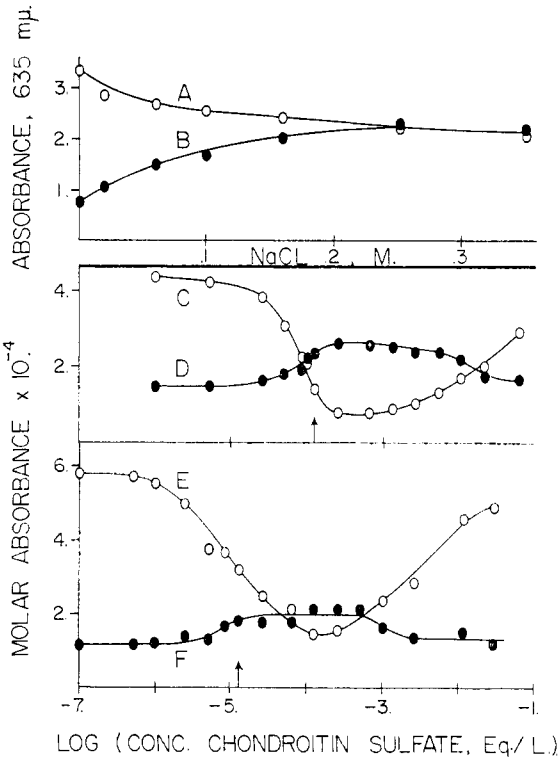


Fig. 5.—Top section, absorbance at 635 mμ (α-band) *vs.* molarity of NaCl: A, toluidine blue alone in water, 1.25×10^{-4} *M;* B, the same with chondroitin sulfate, 1.50×10^{-4} equiv./l.: Mid and lower section: molar absorbance at 665 mμ (α-band) and 570 mμ (μ-band) of methylene blue *vs.* log (chondroitin sulfate concentration): C, α-band; D, μ-band with methylene blue 1.25×10^{-4} *M;* E, α-band; F, μ-band with methylene blue $1.25 \times 10^{5-}$ *M.* Arrows on the abscissa of the mid and lower sections locate the concentrations of chondroitin sulfate which are exactly equivalent to the respective amounts of dyes.

the μ-band with increasing concentration of chondroitin sulfate. The metachromatic color is most pronounced (α-band minimum) at a concentration of chondroitin sulfate about ten times greater than that at equivalence (marked by the arrow on the abscissa), and at higher concentrations of chondroitin sulfate the α-band rises and the μ band drops. At higher dye concentration (curves C and D), the same changes occur but only at higher concentrations of chondroitin sulfate. Since even at the highest chromotrope concentration the α-band has not clearly reached its plateau value, this method was not further pursued.

Discussion

For the general theory of metachromasia the techniques that involve removal of the metachromatic compound from solution are of unique value because they allow actual determination of the proportion of dye and chromotrope in the metachromatic compound separated. The analysis can be made either directly with the pellet sedimented, or by difference with the supernatant solution. The results of such analyses (Table II) show that for several combinations of dye and chromotrope the equivalence ratio in the metachromatic compound is close to 1.0. However in the case of heparin the ratio was found persistently high, averaging 1.4, indicating that some anionic sites of heparin are not associated with dye cations. This raises the question whether the appropriate equivalent weight of heparin was used. The charge density is higher and the equivalent weight is lower for heparin than for any other chromotrope used in the present work. The most significant form of the charge density for present purposes is the number of potential anionic charges per unit of chain length, or its reciprocal the average distance between charges. The repeating unit for heparin consists of four pyranose units (two glucosamine and two glucuronate). This period carries seven potential anionic charges (two carboxylate, three ester sulfate and two amidosulfate groups), and the average distance along the chain between charges is 2.9 Å. The distance between dye molecules in dimeric methylene blue has been estimated to be 3[7] or 4.[8] It is possible that use of all the ionic sites of heparin by toluidine blue would crowd the dye molecules too closely. If it is assumed that only five can be accommodated per period their distance apart would be 4 Å. This would correspond to an equivalent weight of 275, and give an equivalence ratio of 1.0. The deviation of E.R. from unity for heparin in Table II may thus reflect a difference between potential anionic sites per

(7) E. Rabinowitch and L. F. Epstein, *J. Am. Chem. Soc.*, **63**, 69 (1941).

(8) S. E. Sheppard and A. L. Geddes, *ibid.*, **66**, 2003 (1944).

period and the maximum number suitable for binding toluidine blue.

In no case studied does formation of metachromatic compound appear to be complete; there is always free dye and chromotrope in equilibrium with the metachromatic compound. Metachromatic equilibria are rapidly established and only metachromatic compound is removed in the methods used. It is then to be expected that removal of the metachromatic compound initially present would result in rapid readjustment of equilibria forming more metachromatic compound which would also be removed. An attempt was made to estimate the amount of metachromatic compound sedimented in addition to the amount initially present in a metachromatic solution. The method used is illustrated in Fig. 6 and compares the shapes of difference spectra produced by weak and strong chromotropes on the same dye. Curve A is the observed spectrum of a solution containing toluidine blue and chondroitin sulfate. It has a single sharp peak at 545 mμ, the position of the μ band of the dye. The solution represented by curve A was centrifuged at 100,000 *g*, the spectrum of the orthochromatic supernatant solution was measured and subtracted from curve A giving curve B, the difference spectrum. If only metachromatic compound initially present was sedimented then curve B represents its absorption spectrum. If during centrifuging some free dye combines with chromotrope and is also sedimented there will be a disappearance of absorption at 635 mμ (α-band) which will appear on the difference curve B as a shoulder at 635 mμ. There is only a suggestion of such a shoulder and from the independently determined absorbance value of free toluidine blue at 635 mμ this could represent no more than 2% of the total dye initially present in the solution represented by curve A. Therefore it can be said that at least 98% of the metachromatic compound sedimented in the case of toluidine blue and chondroitin sulfate was initially present in the metachromatic solution. The situation is different in the case of toluidine blue and hyaluronate as shown by the corresponding pair of curves C and D. There is a far more distinct shoulder on the difference curve D. From this shoulder it was estimated that about 10% of the total dye initially present in the metachromatic solution (curve C) may have been converted during the process of centrifuging from free dye to metachromatic compound and sedimented.

The results summarized in Table I show that the centrifugal method, which seems more generally reliable than the adsorption method, does not distinguish very finely among the set of combinations of dyes and chromotropes examined. In 23 cases of the 32 examined centrifugally the fraction of dye sedimented as metachromatic compound lies in the range 80 to 95%. The phenomenon of readjustment of equilibria discussed in connection with Fig. 6 contributes to diminish the difference in the total amount of metachromatic compound sedimented in most cases. The results in Table III suggest that a finer distinction among a number of combinations of dye and chromotrope can be made

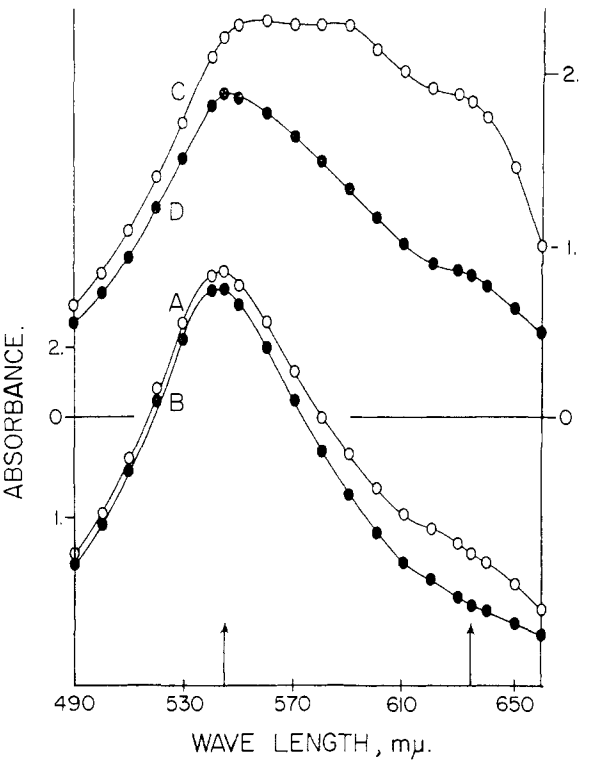


Fig. 6.—Absorption spectra of solutions of toluidine blue with chromotrope and corresponding difference spectra; dye, 1.25×10^{-4} *M*; chromotrope, 1.5×10^{-4} equiv./l.; NaCl, 0.0025 *M*: A, dye and chondroitin sulfate, initial solution; B, difference spectrum (spectrum A—absorption spectrum of supernatant solution left after centrifuging the solution of curve A at 40,000 r.p.m.); C, dye and hyaluronate, initial solution; D, the corresponding difference spectrum as defined above. Arrows on the abscissa locate the μ-band, 545 mμ, and the α-band, 635 mμ, of the spectra. Left ordinate for A and B; right ordinate for C and D.

by working in solutions containing some ethanol. For example, the fractions of toluidine blue sedimented as metachromatic compounds in water with seven chromotropes all lie in the range 80 to 95%, but in 15 or 40% ethanol the range becomes much broader. At higher dye concentrations this range is again diminished even in the presence of higher ethanol concentrations (Table IV). This suggests that at appropriate dye concentrations different combinations of dye and chromotrope might show widely differing sensitivities to ethanol concentration.

Such considerations led to the development of the method of Figs. 1 and 2 as a technique capable of distinguishing more subtle differences in the stabilities of metachromatic compounds composed of different dyes and chromotropes. This method avoids the separation of the metachromatic compound with consequent shifting of equilibria. At a constant dye and chromotrope concentration it simply measures absorbance of the solution at the α-band of the dye at a series of ethanol concentrations. The shapes of plots of absorbance *vs.* ethanol concentration suggest that they represent dissociation of the metachromatic compounds with liberation of free monomeric dye. For each

dye and a series of chromotropes there was obtained a series of curves all lying between two limiting plateaus: a low plateau corresponding to maximum formation of metachromatic compound and end absorbance of its μ-band, and a high plateau corresponding to complete destruction of the metachromatic compound and the absorbance of the free dye in ethanol. The abscissa of the point of intersection of each curve with the absorbance level half way between these two plateaus may be called the mid-plateau value and becomes a convenient measure of the stability of each metachromatic compound, the molar ethanol concentration at which about half the metachromatic compound is destroyed. The mid-plateau value corresponds only approximately to half destruction of the metachromatic compound because of the several equilibria involving at least the species: polymeric, dimeric and monomeric dye cations, D_n^{+n}, D_2^{+2}, and D^+; the metachromatic compound, in which E.R. is 1.0; and other forms of association of dye cations with chromotrope in which E.R.>1 and which occur in solution mainly in the presence of excess chromotrope. In Table V are collected mid-plateau values of a number of metachromatic compounds. It seems that both dyes and chromotropes can be graded with respect to relative stabilities of the metachromatic compounds they form; the lowest mid-plateau value is that shown by crystal violet and hyaluronate, a weakly metachromatic dye and a weak chromotrope; the highest mid-plateau value, that of toluidine blue and λ-carrageenan, a strongly metachromatic dye and a strong chromotrope, is not even reached at 60% ethanol (Fig. 1, curve E).

The thought that dye dimerization as well as metachromatic compound formation may in part be due to hydrophobic bond formation between individual dye ions led to the trial of urea as an agent to destroy metachromatic compounds in solution. The results using urea are altogether parallel to those using ethanol. With urea the upper plateau values are in all cases (Figs. 3 and 4) lower than those that result from ethanol (Figs. 1 and 2). The mid-plateau values obtained by this urea treatment are collected in Table V. Comparison of mid-plateau values obtained with ethanol and those obtained with urea shows they follow the same trends and in many cases are nearly identical. In all cases but two the mid-plateau values resulting from urea treatment are slightly lower than those resulting from ethanol treatment. These results indicate that the mechanism of metachromatic compound destruction by ethanol and urea are similar. If the destruction of metachromatic color were mainly due to breaking of hydrophobic bonds it would be expected that urea would have a much weaker effect than ethanol. Actually on a molar basis it is generally slightly more potent. This could indicate either that the particular hydrophobic bonds involved in metachromatic compound formation happen to be more readily split by urea than other hydrophobic bonds,[6] or that such bonds are not involved at all in metachromatic compound formation. Such a depolymerizing effect might also be expected of non-metachromatic dyes that obey Beer's law but are known to polymerize. Simple experiments show that congo red, dianil blue and benzopurpurin 4B dissolved in water do not dialyze through cellophane but when dissolved in 10 M urea or 10 M ethanol solutions they do dialyze slowly.

The need for quantitative methods to serve as a basis for discussion of metachromasia is emphasized by the wide diversity of opinion that continues to exist even with respect to such questions as which cationic dyes are metachromatic and which polyanions are chromotropes. Jacques,[9] in a discussion of mast cells, says methylene blue gives no metachromasia whatever and toluidine blue only a trace, and that chondroitin sulfate is not a chromotrope in the same sense as heparin. There is a striking contrast between these views and the uniformities in the graded behavior of spectra, summarized in Figs. 1 and 2, of what are here considered metachromatic compounds. There are also divergent opinions expressed as to whether hyaluronate is a chromotrope. Data of Table I and Figs. 1 and 2 agree with the views of Sylven and Malmgren[10] that hyaluronate is a feeble chromotrope. At low dye concentration it can form a sedimentable metachromatic compound, but this compound is destroyed at low ethanol concentration. Yet hyaluronate at high dye concentration can form a stable metachromatic compound as is apparent from Table IV where the metachromatic compound is resistant to destruction by high concentrations of ethanol. Another problem that arises in histochemical applications is the significance of the distinction between true and false metachromasia.[11] A physiochemical basis for this distinction is apparent in Figs. 1 and 2. In some cases the metachromatic compound is completely destroyed at low concentrations of ethanol and at higher concentrations colorless chromotrope is precipitated, in other cases where the metachromatic compound is stable to high enough concentrations of ethanol it is the colored metachromatic compound itself which precipitates. The former cases correspond to false, the latter cases to true metachromasia. Yet in which class a chromotrope will fall may depend on the concentration of dye and chromotrope.

Another approach to the study of metachromasia is the use of excess polyanion to destroy the metachromatic compound. Figure 5, curves C and E, show the rise in the α-band at concentrations of chromotrope above that corresponding to the minimum of the α-band. Bradley and Wolf[12] used this method and have developed a theory that allows calculation of a stacking coefficient, a measure of the tendency of dye molecules to occupy adjacent sites on the polymer chain.

The data of Figs. 1 to 4 and the summary of Table V open a new area for the study of metachromatic compounds. What the significant variable is that changes as ethanol or urea is added

(9) L. B. Jacques, *Can. J. Biochem. Physiol.*, **39**, 643 (1961).

(10) B. Sylven and H. Malmgren, *Lab. Invest.*, **1**, 413 (1952).

(11) A. G. E. Pearse, "Histochemistry," Little, Brown and Co., Boston, Mass., 1960, pp. 248–254.

(12) D. F. Bradley and M. D. Wolf, *Proc. Natl. Acad. Sci.*, **45**, 944 (1959).

and brings about destruction of the metachromatic compound is not clear. That it may not involve destruction of hydrophobic bonds was pointed out above. That it is not dielectric constant seems likely from the quantitatively similar results with ethanol and urea. While no interpretation of the curves of Figs. 1 to 4 can be offered beyond their representation of the destruction of metachromatic compounds, they offer a simple and quantitative method to measure stabilities of metachromatic compounds, and in a wider field a new technique for the study of polyanions or cationic dyes in solution.

[CONTRIBUTION FROM RIAS, 7212 BELLONA AVENUE, BALTIMORE 12, MARYLAND]

The Effect of Substitution on the Ionization Potentials of Free Radicals and Molecules. III. Estimation of the Ionization Potentials of Cycloalkyl Radicals and Cyclic Amines by δ_K Values

BY JOYCE J. KAUFMAN

RECEIVED APRIL 20, 1962

It has been shown previously by the author (Parts I and II) that the effect of substituent groups on ionization potentials of alkylamines (Y_3N) and alkyl free radicals (Y_3C) were almost identical. From measured photoionization potentials of amines a new set of constants, δ_K values, which quantitatively reflect changes in ionization potential with substituent groups, were derived and using these, ionization potentials of alkyl free radicals could be estimated to within the experimental error of their measurements. In the present article δ_K values obtained from linear and branched alkyl substituents are shown to be extendable to cyclic substituents in which the carbon from whence the unpaired electron was being withdrawn was itself part of the ring. Using the original δ_K values it is now possible to estimate the ionization potentials of cyclic free radicals and cyclic amines.

δ_K values, a new set of constants which quantitatively measure the effect of substitution on ionization potentials, have been previously derived by the author[1,2] from measurements on substituted amines and shown to hold true also for calculation of ionization potentials of substituted linear and branched alkyl radicals. The recently published values for the ionization potentials of cycloalkyl radicals[3] enable one to verify that δ_K values have equal validity for the estimation of the ionization potentials of cycloalkyl radicals—a fact which would not have been immediately apparent.

The symbols δ_K(or $\delta_K^{(1)}$), $\delta_K^{(2)}$, $\delta_K^{(3)}$ are the changes in ionization potential caused by substituting one, two or three identical groups, respectively, for H atoms (on the *same* central atom of the radical or molecule from which the electrons are being withdrawn in the ionization process). Since it has been shown that there is a saturation effect on ionization potentials, another type of δ_K value is the difference in ionization potential found by adding a second identical substituent group when a first substituent group is already present. $\delta_K^{(1-0)}$ (or δ_K or $\delta_K^{(1)}$) and $\delta_K^{(2-1)}$ are the differences in ionization potential between mono- and unsubstituted, and between di- and monosubstituted molecules or radicals (with identical substituent groups).

Using the method outlined in ref. 1 and the table of δ_K values contained therein, the cycloalkyl radical ionization potentials are calculated as:

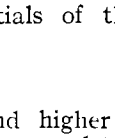
Cyclopropyl: $I(\mathrm{Me}) - \delta_K^{(2)}(\mathrm{Me}) = 9.96$ ev. $- 1.91$ ev.
$= 8.05$ ev. (calcd.)
8.05 ± 0.1 ev. (exptl.)

Cyclobutyl:
(1) $I(\mathrm{Me}) - \delta_K^{(1)}(\mathrm{Me}) - \delta_K^{(2-1)}(\mathrm{Et}) = 9.96$ ev. $- 1.18$ ev. $- 0.85$ ev.
$= 7.93$ ev. (calcd.)
or
(2) $I(\mathrm{Me}) - \delta_K^{(1)}(\mathrm{Et}) - \delta_K^{(2-1)}(\mathrm{Me}) = 9.96$ ev. $- 1.29$ ev. $- 0.73$ ev.
$= 7.94$ ev. (calcd.)
7.88 ± 0.05 ev. (exptl.)

Cyclopentyl:
(1) $I(\mathrm{Me}) - \delta_K^{(2)}(\mathrm{Et}) = 9.96$ ev. $- 2.14$ ev.
$= 7.82$ ev. (calcd.)
or
(2) $I(\mathrm{Me}) - \delta_K^{(1)}(\mathrm{Me}) - \delta_K^{(2-1)}(\mathrm{Pr}) = 9.96$ ev. $- 1.18$ ev. $- 0.94$ ev.
$= 7.84$ ev. (calcd.)
7.79 ± 0.03 ev. (exptl.)

Cyclohexyl:
(1) $I(\mathrm{Me}) - \delta_K^{(1)}(\mathrm{Et}) - \delta_K^{(2-1)}(\mathrm{Pr}) = 9.96$ ev. $- 1.29$ ev. $- 0.94$ ev.
$= 7.73$ ev. (calcd.)
or
(2) $I(\mathrm{Me}) - \delta_K^{(1)}(\mathrm{Me}) - \delta_K^{(2-1)}(\mathrm{Bu}) = 9.96$ ev. $- 1.18$ ev. $- 1.02$ ev.
$= 7.76$ ev. (calcd.)
7.66 ± 0.05 ev. (exptl.)

The calculated and experimental values are in excellent agreement, better than the agreement with values calculated by much more tedious methods.[3] The best expression to use for the ionization potentials of the cycloalkyl radicals for



cyclobutyl and higher members Y_1—Y_2, where Y_1 and Y_2 correspond to the substituent groups R_1 and R_2 with one less hydrogen (Y_1 is preferably equal to Y_2 or else it is the closest smaller one to Y_2) is $I(\text{cycloalkyl}) = I(\mathrm{Me}) - \delta_K^{(1)}(R_1) - \delta_K^{(2-1}$

(1) Joyce J. Kaufman and W. S. Koski, *J. Am. Chem. Soc.*, **82**, 3262 (1960).

(2) Joyce J. Kaufman and W. S. Koski, paper presented before the Section on Physical Chemistry, Structure and Reactivity of Small Molecular Species, 18th International Congress of Pure and Applied Chemistry, Montreal, August 1961.

(3) R. F. Pottie, A. G. Harrison and F. P. Lossing, *J. Am. Chem. Soc.*, **83**, 3204 (1961).