The "Isoextraction" Method and the Study of the Self-Association of Methylene Blue in Aqueous Solutions[1]

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Abstract: Because of the extreme dilutions, 10^{-6}–10^{-4} M, to which measurements have to be made to study the self-association of many ionic dyes, most equilibrium methods are insufficiently sensitive. Nonequilibrium methods, such as the popular spectral method, are difficult to apply to multiple equilibria, because of the need to obtain an additional parameter, for example. the molar extinction coefficient, along with the equilibrium constant of association for each associated species. In this paper a new experimental approach based on partition between solvents, called the "isoextraction" method, is described. It is an equilibrium method, and its sensitivity is limited only by the sensitivity of analytical concentration determinations. The general principle of this method is to determine, at partition equilibrium of the dye salt D^+A^- (where D^+ represents the dye cation and A^- the anion) between an aqueous phase and an organic phase, various combinations of the *total* equivalent dye concentration in the aqueous phase (B) and the anion concentration ($[A^-]_W$) such that, for each combination, the organic phase has the same concentration and, therefore, the same activity of the dye salt D^+A^-. The condition of equilibrium, along with some reasonable assumptions, then leads to the constancy of the concentration product $b[A^-]_W$, called $K_{A(0)}$, in all the aqueous solutions under isoextraction conditions, where b is the equilibrium dye *monomer* concentration. The experimentally accessible product $B[A^-]_W$, called K_A, gives $K_{A(0)}$ on plotting against B and extrapolating to infinite dilution of the dye, where $b = B$. Therefore, since $K_A/K_{A(0)} = B/b$, b can be determined as a function of B. The $b(B)$ data can then be analyzed to study the nature of the self-association. The method, modified slightly for experimental convenience, has been applied to the problem of the self-association of methylene blue, using chloroform as the organic solvent. It is shown that the ratio B/b increases rapidly with concentration. From completely general considerations for self-association systems, the average number of monomers in the average aggregate, $\bar{\nu}$, has been derived as a function of B. At infinite dilution, it has the expected theoretical value of 2 for systems showing dimerization. $\bar{\nu}$, however, increases rapidly to nearly 5 at higher concentrations. The self-association thus cannot be described by dimerization alone as is frequently assumed. Further analysis reveals that the self-association equilibria involve stepwise association to dimers, trimers, tetramers, and higher multimers.

The nature of the self-association of dyes in aqueous solutions, aside from its intrinsic interest, is important in the understanding and interpretation of a great variety of problems, such as dyeing of fibers, tissue staining in biology, spectral change and energy transfer studies, adsorption, and photography. Dye association is also one of the simplest examples of "stacking" interactions. Unfortunately, although a large number of studies have been made, neither the nature of the equilibria nor the causes behind the association are known to any degree of satisfaction. The major obstacle has been the lack of suitable methods of investigation, a general problem for systems showing a strong tendency toward self-association.[4] Many dyes show appreciable association at concentrations as low as 10^{-6}–10^{-4} M, and the qualitative evidence from many studies suggests that a series of multimers is formed. The low concentrations effectively rule out a large number of experimental approaches. Of the ones in use, the popular spectral method,[5–7] the conductivity method,[8] and the recent polarographic method[9] are nonequilibrium methods for which, in the general case, not only the equilibrium constants of association for a set of multimers but also a set of constants characteristic of the various multimers (such as molar extinction coefficients, ϵ) have to be estimated. The need to evaluate a second set of parameters other than the equilibrium constants severely limits the utility of these methods. Quantitative results have been derived usually on the assumption of only dimerization taking place. This assumption may be grossly erroneous. On the other hand, very few of the existing equilibrium methods can cope successfully with the problem of the suspected multiple equilibria in dye association.

In this paper, we will indicate the principles of a new partition method, named the "isoextraction" method,[10] for studying complicated association equilibria in general and present our results on the self-association of methylene blue studied by the new method.

The Isoextraction Method

Methods based on the distribution between two phases (for example, an aqueous (W) and an organic (O) phase) for studying equilibria in one phase, W, are, in principle, quite versatile:[4] the partition equilibrium is generally attained rapidly, a variety of solvents can be used as phase O, and, since only concentration mea-

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(1) Based in part on the doctoral dissertation of A. K. Ghosh, Calcutta University, 1963; presented in part at the 152nd National Meeting of the American Chemical Society, New York, N. Y., Sept 1966.

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surements are involved, very dilute solutions can be studied by using radioactive tracers, in general, spectrophotometry for the highly absorbing dyes, and other suitable analytical methods. Association equilibria involving the monomer can be investigated from the concentration dependence of the partition coefficients.

Unfortunately, the method is difficult to use in practice for many systems, particularly ionic systems, because the behavior of the extracting species in the O phase is not known. Consider an ionic dye D^+A^-, where D^+ is the dye ion and A^- the counterion. To study the self-association of D^+ in water, it is necessary to know not only the partition coefficient of D^+A^-, but all association and hydration equilibria of the D^+ and A^- species in the O phase.

The isoextraction method obviates the need for such additional information about the O phase. The principle of this method is to maintain the total concentration of the dye in the O phase constant by adding to the aqueous phase suitably increasing amounts of a salt such as Na^+A^-, which does not itself extract significantly into the O phase, as the concentration of the dye in the aqueous phase is lowered. We, therefore, obtain experimentally various values $[A^-]_W$ as a function of the total *equivalent* concentration of the dye in the aqueous phase, B, such that for each value of B and the corresponding $[A^-]_W$, the concentration in the O phase is the same. If we make the very reasonable assumption that the changes in composition of the dilute aqueous phase, and the resultant minute changes in the water activity, do not alter the activity coefficients of the various species in the O phase, then the activity product $(a_{D^-})_O(a_{A^-})_O$ in the O phase must be the same under these isoextraction conditions. Therefore, the activity product $(a_{D^-})_W(a_{A^-})_W$ in the various aqueous solutions at equilibrium must also be the same. If we can assume now that the activity coefficients are constant, and that the A^- ions in the aqueous phase do not participate in any association, the product $b[A^-]_W$ should be constant in all the aqueous phases under isoextraction conditions, b being the concentration of the dye monomer. The experimentally accessible $B[A^-]_W$, called K_A, will be a function of B if $B \neq b$, *i.e.*, if there is any self-association. However, the extrapolated value of K_A at infinite dilution of B, $K_{A(0)}$, must be equal to $b[A^-]_W$ since at infinite dilution $B = b$. $K_A/K_{A(0)}$, therefore, gives the ratio of B/b as a function of B. Thus, irrespective of the nature of the solute in the O phase, it is possible by this method to estimate the free monomer concentration as a function of the total concentration in the aqueous phase, perhaps the most useful information for studying association equilibria.

The basic principle of the isoextraction method is, thus, to furnish a contant activity of a particular species and is thus similar to the use of a solid salt as an equilibrium phase. The isoextraction method is, however, considerably more flexible, since many combinations of the counterion and the O phase can be used, and the chosen reference concentrations can be altered considerably. The method should, therefore, be of considerable general applicability.

The isoextraction method can be used to determine activity coefficients of extracting species.[10,11] After completing our studies,[1] we learned that a method based on the above principle has been previously used to determine activity coefficients in some inorganic systems.[12] To our knowledge, it has not been used previously for studying association equilibria. For our experiments with methylene blue, we found it convenient to use a slight modification of the isoextraction method, as described later.

Experimental Section

Materials. Two samples of methylene blue chloride (MBCl) were used. Sample A was E. Merck's reagent variety dye, used after three recrystallizations from twice-distilled ethanol. Sample B was E. Merck's "guaranteed" reagent, used without further purification.

Methylene blue perchlorate ($MBClO_4$) was precipitated from hot solutions of MBCl (purified) by adding $KClO_4$. After thorough washings with distilled water, the precipitate was recrystallized twice from ethanol.

The $KClO_4$ used was E. Merck's "guaranteed reagent" variety.

Pure quality chloroform from E. Merck was thoroughly and freshly washed with distilled water five or six times and used without drying.

Apparatus. A Hilger Uvispek spectrophotometer was employed. The cell compartment was thermostated, when necessary, within ±0.2°.

Partition Experiments. Pyrex centrifuge tubes with glass stoppers were used. After the solution had equilibrated in water thermostats controlled to within ±0.05°, and settled, the upper aqueous layer was removed with a syringe, the tube was cooled below room temperature to balance the heating during the next centrifugation step used for clarification, and the absorbance of the chloroform layer was measured at room temperature at the band maximum. Reproducible results were obtained by this procedure.

Methylene Blue Solutions. Solutions were prepared from dye samples which had been thoroughly ground, dried for 1 hr at 100°, and cooled in a desiccator.

The procedure does not lead to complete or reproducible drying, which seems to be difficult to attain at even higher temperatures.[6] Relative concentrations could be determined conveniently within 1% by absorbance measurements in 3.2 N HCl at 745 mμ. At such acidities, MB is primarily in the doubly charged protonated state,[13] and it was found that Beer's law is accurately obeyed.

To determine absolute concentrations, two different methods were employed. It was found that $[ClO_4^-]_W$ concentrations in the dilute range ($\sim 10^{-5}$ M) can be determined by the partition of $MBClO_4$ between chloroform and water in the presence of an excess of MBCl (after blank corrections due to MBCl). Dilute solutions of $MBClO_4$ were thus calibrated against standard $KClO_4$ solutions and intercompared with MBCl solutions by absorbance measurements in HCl. In a second method, a pure sample of sodium lauryl sulfate (NaLS) was used as the reference substance. LS^- ions were exhaustively extracted into chloroform by using MBCl in excess,[14] and the absorbance in chloroform (in which Beer's law is obeyed) was compared to that of a similar extract of MBCl using excess NaLS. The comparison, after suitable blank corrections, leads to a measure of the absolute concentration of MBCl. The two methods agreed to within about 1%, but differed by 3-12% from the weight concentrations. Exhaustive extractions of aqueous MB by $KClO_4$ into chloroform gave a constant value of 1.00 ± 0.01 (× 10^5) for ϵ in chloroform, and provided a further check on the concentrations.

Stock solutions were preserved in containers covered with black paper. Solutions in 0.001 N HCl showed no change in concentration over a period of 2 months.

Good criteria for establishing the purity of methylene blue are not available. The literature values of ϵ for MBCl differ widely[5-7,15,16] and may reflect difficulties in drying. Our value in ethanol at the band maximum (9.8 × 10^4) is appreciably higher than previously

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reported values.[15,16] After our work was completed, Bergmann and O'Konski[6] suggested the possibility of the presence of trimethyl thionine, TMT, obtained from demethylation of MB, in commercial samples. On applying their criterion based on spectral shape, it seems that a few per cent of TMT was present in our samples. Since both in its extractivity into chloroform from our acid solutions and the tendency toward self-association, TMT should be only slightly less efficient than MB,[17a] and since our calculations on self-association are based on relative values of K_A, the errors due to the presence of small amounts of TMT in our estimates of the association constants should be small.

Modified Isoextraction Method. Instead of using a combination of MB^+A^- and a nonextractable salt containing the common A^- in the isoextraction method, we found it convenient, for reasons to be discussed shortly, to add $KClO_4$ to MBCl solutions in the aqueous phase. $MBClO_4$ has a high partition coefficient in favor of chloroform. The extractability of MBCl is much smaller and was corrected from blank experiments using MBCl alone. Moreover, 0.001 *M* HCl was added to all the aqueous solutions. $KClO_4$ itself was found to have negligible extractivity into chloroform.

The rationale behind this modified procedure was provided by some experiments in which the partition of $MBClO_4$ was studied as a function of added $KClO_4$ concentration to high and nearly constant concentrations of MBCl in the aqueous phase. Figure 1 shows that the curves are linear within experimental error and pass through the blanks. Thus, the extractions of MBCl and $MBClO_4$ are additive. The concentration of the dye in chloroform, after subtracting the blank due to MBCl, was proportional to $[ClO_4^-]_W$ for a constant value of *B*, within the experimental error of 1-2%. It can be shown from this result that both MBCl and $MBClO_4$ are nearly completely in the ion-pair form in chloroform, which is not unexpected. Chloroform has a low dielectric constant, 4.6, and the Fuoss theory of ion association[17b] predicts that for the reasonable distances of closest approach of 5 and 4 Å, the dissociation constants are 8.5×10^{-11} and 3.9×10^{-13} mol/l. The blank correction decreases rapidly in value at lower dye concentrations. The isoextraction concentration was chosen to correspond to an absorbance of 1.00 of $MBClO_4$ in chloroform (1.00×10^{-5} *M*). In comparison, in presence of 0.001 *M* HCl, the blank absorbance values were less than 0.01 for 10^{-5}*M* MBCl in water, 0.035 at 10^{-4} *M*, and 0.13 at 10^{-3} *M*. The concentration dependence of the blank values themselves was consistent with the partition behavior expected if MBCl in chloroform is completely in the ion-pair form.

The advantages of the modified procedure were several. (a) The adsorption of the cationic dye on glass surfaces, which are usually negatively charged,[18] is an important and obstinate source of error in dilute solutions. The adsorption was considerably reduced in 0.001 *M* HCl, and reproducible results were obtained at aqueous concentrations as low as 1×10^{-5} *M*. The acid also prevented the base-catalyzed demethylation of MB.[6] (b) Extremely small amounts of extractable anionic impurities can cause significant errors. The blank values occasionally showed some scatter and systematic differences between stock solutions. The blank correction, determined for each experiment each day, corrected for this impurity effect to a large extent. (c) The concentration of $KClO_4$ necessary to obtain the isoextraction condition was low and decreased rapidly with increasing dye concentration. Taking the 0.001 *M* HCl present into account, the ionic strength in the aqueous solutions remained low (0.0012–~0.004) and fairly constant over the whole concentration range. (d) The proportionality of the concentration of $MBClO_4$ in chloroform to $[ClO_4^-]_W$ at constant *B* (Figure 1) allowed us to interpolate and extrapolate from absorbance values close to but different from the chosen isoextraction value of 1.00. The overall average scatter of k_A-*B* plots at three temperatures was about 1-2%, which is expected from the known sources of error. Thus, no substantial error due to the interpolations or extrapolations is indicated.

Calculation of K_A. The concentration of MB in the chloroform layer was calculated from the total absorbance, and that for ClO_4^- was obtained after subtracting the blank due to MBCl. A correction of 0.5% was applied to the volume of chloroform because of its slight solubility in water. Aqueous concentrations of ClO_4^- and MB (*B*) were determined from material balance. From the observed proportionality of $MBClO_4$ concentration in chloroform to $[ClO_4^-]_W$ at a constant *B* (Figure 1), the value of $[ClO_4^-]_W$ for any *B* which corresponds to the isoextraction concentration, 1.00×10^{-5} *M* $MBClO_4$ in chloroform, was calculated from the experimentally determined $[ClO_4^-]_W$ and the corresponding $MBClO_4$ concentration in chloroform. The product $B[ClO_4^-]_W$ corresponding to the isoextraction condition is the previously defined K_A.

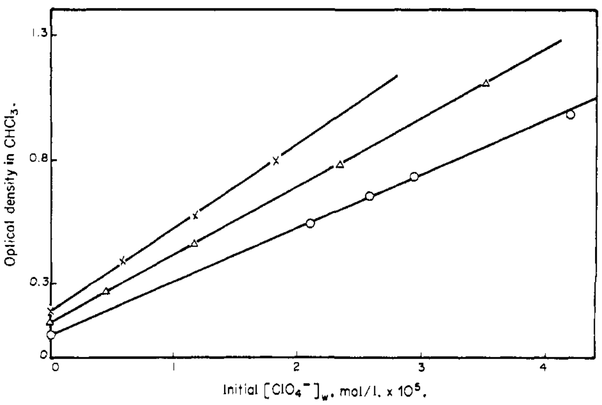

Figure 1. Partition of mixtures of MBCl and $KClO_4$ between equal volumes of water and chloroform at 34.9°. Initial MBCl concentration in water, equiv/l.: ○, 4.21×10^{-4}; △, 9.0×10^{-4}; and ×, 1.76×10^{-3}.

The uncertainty in K_A was higher both for the highly dilute MBCl (~1×10^{-5} *M*), where up to 50% of the dye extracted, and at the highest concentrations of MBCl, where high percentages of ClO_4^- extracted. The data in the middle region (2×10^{-5}–5×10^{-4} *M* MBCl) were the most reliable.

As K_A varies linearly with *B* at low concentrations, the $K_{A(0)}$ values could be obtained reliably by linear regression analysis.[19]

Results

Table I gives the K_A-*B* data at 25.1° for our experimental series 1, covering a factor of about 300 in concentration. The data, plotted in Figure 2, were obtained with sample A. When the experiments were repeated 1 year later (series 2), primarily with sample B but also sample A for several experiments, the K_A values obtained were higher by 8–10%. This difference is probably due to the presence of a larger fraction of demethylated MB in the older sample used in the series 2 experiments, which is expected to extract less into chloroform and show a higher value of K_A. However, the ratio $K_A/K_{A(0)}$ changed much less. The association

Table I. Isoextraction Data at 25.1° (Series 1)[a]

B, equiv/l.	$K_A \times 10^9$, (equiv/l.)^2	*B*, equiv/l.	$K_A \times 10^9$, (equiv/l.)^2
7.64×10^{-6}	3.59	2.59×10^{-4}	7.28
1.383×10^{-5}	3.64	3.24×10^{-4}	8.24
2.243×10^{-5}	3.69	3.88×10^{-4}	9.54
2.453×10^{-5}	3.72	4.84×10^{-4}	9.98
3.48×10^{-5}	3.92	6.56×10^{-4}	12.44
6.73×10^{-5}	4.40	1.023×10^{-3}	16.68
1.165×10^{-4}	5.39	1.342×10^{-3}	20.30
1.21×10^{-4}	5.27	2.05×10^{-3}	29.7
1.556×10^{-4}	5.91	2.05×10^{-3}	37.4
1.976×10^{-4}	6.39		

[a] $K_{A(0)} = 3.41 \times 10^{-9}$ (equiv/l.)^2.

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[17] (a) According to Rabinovitch and Epstein,[5] thionine, the completely demethylated form of tetramethylthioine (methylene blue), has a dimerization constant lower than that of methylene blue by a factor of only 3.6. (b) R. M. Fuoss, *J. Amer. Chem. Soc.*, 80, 5059 (1958).

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[19] A. K. Ghosh and P. Mukerjee, *J. Amer. Chem. Soc.*, **92**, 6408 (1970).

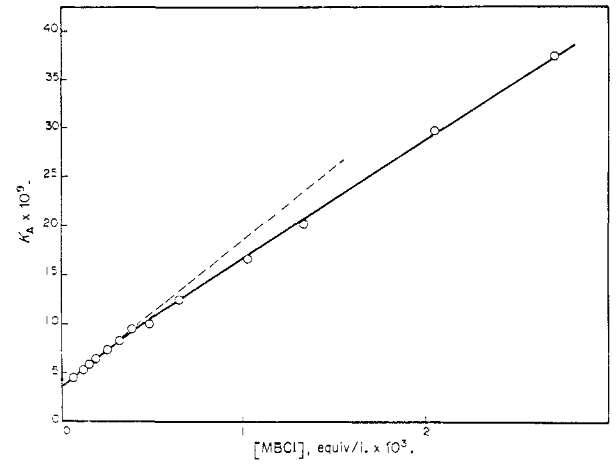

Figure 2. Variation of K_A [(equiv/l.)^2] with B (equiv/l.) at 25.1°. Not all data in dilute solutions are plotted.

constants derived from the two sets of data agreed to within 4% at 35° and 2% at 25°. As the series 1 data show less scatter and were more extensive, our analysis is based entirely on this series. The data of Table I can be used directly to evaluate b at any value of B, from the ratio $K_A/K_{A(0)}$. The B/b ratio increases with concentration to a value of more than 10, showing that association is extensive at higher concentrations.

Effect of Dissolved Chloroform. The absorption spectrum of a moderately concentrated solution of MBCl, 1.69×10^{-4} M, in water was compared to that in water saturated with chloroform. At such concentrations, the spectrum of MB is highly sensitive to the degree of association.[5-7,10] The two spectra, however, agreed within experimental error, suggesting that the dissolved chloroform does not affect the association equilibria.

Qualitative Nature of the Self-Association of MB

The B-b data make it possible to analyze the nature of the self-association of MB to a considerable depth.[20] This analysis is simplified to a great extent if two assumptions are made: (a) the self-association of MB does not involve any counterions, as indicated by work reported later,[11,21] and (b) the activity coefficients of all species are constant and close to unity. This assumption should be satisfactory for our isoextraction data, because of the low values and small variations in the ionic strength. In the association of charged species, moreover, the activity coefficients of the reactants and products cancel to a large degree.[11,19] The equilibrium constants below are defined in terms of concentrations.

The equilibrium constant β_q for the formation of any multimer b_q, containing q monomers b_1 can be expressed as

$$\beta_q = \frac{[b_q]}{b^q} \tag{1}$$

The stepwise association constant for the reaction

$$b_{q-1} + b_1 = b_q \tag{2}$$

is defined as K_q, so that $\beta_2 = K_2$, $\beta_3 = K_2K_3$, and so on.

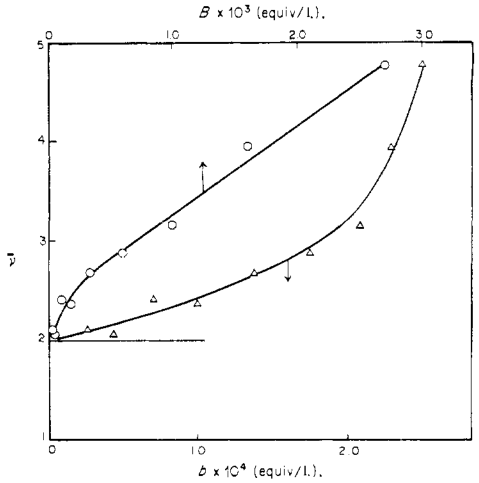

Figure 3. Variation of the average degree of association, $\bar{\nu}$, with the monomer concentration, b, and the total concentration, B, at 25.1°.

The total equivalent concentration, B, is given by the sum

$$B = b + 2\beta_2 b^2 + 3\beta_3 b^3 + \ldots \tag{3}$$

The total *molar* concentration, S, is given by the sum

$$S = b + \beta_2 b^2 + \beta_3 b^3 + \ldots \tag{4}$$

Differentiation of eq 4 shows that irrespective of which or how many multimers form

$$\mathrm{d}S/\mathrm{d}b = 1 + 2\beta_2 b + 3\beta_3 b^2 + \ldots = B/b \tag{5}$$

S, as a function of b, can be determined from our $B(b)$ data, using Kreuzer's[22] integrated form of eq 5, by graphical integration.

$$S' - S = 2.303 \int_b^{b'} B \mathrm{d} \log b \tag{6}$$

The qualitative nature of self-association is best investigated through a function $\bar{\nu}$, defined as

$$\bar{\nu} = \frac{B - b}{S - b} \tag{7}$$

where $\bar{\nu}$ is the average number of monomers in all multimers produced at any particular value of B. If a single multimer forms, $\bar{\nu}$ should have a constant value. For example, in a study of the dimerization of fatty acid anions,[4] it was found that $\bar{\nu}$ has a constant value of 2 for the palmitate system as the B/b ratio varied from a value close to unity to a value well above 10.

From eq 3 and 4 and the definition of $\bar{\nu}$, it can be shown that when dimers are present

$$\bar{\nu} = 2 + \frac{\beta_3}{\beta_2} b + \frac{2\beta_2\beta_4 - \beta_3^2}{\beta_2} b^2 + \ldots \tag{8}$$

Thus, if dimerization as well as higher equilibria are involved, $\bar{\nu}$ must have a theoretical limiting value of 2 at infinite dilution.

Figure 3 shows the calculated value of $\bar{\nu}$ for MB as a function of both B and b. $\bar{\nu}$ approaches the value of 2 in very dilute solutions, establishing the presence of dimers and arguing for the reliability of the isoextrac-

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tion technique, in that the theoretical limiting value of $\bar{\nu}$ is obtained. $\bar{\nu}$, however, increases continuously, the highest value observed being 4.8. Clearly, therefore, multimers at least as high as pentamers must be present.

For further analysis, we must distinguish between two models of self-association: (a) that involving dimers and some large multimers, as is probably the case for some micelle-forming systems,[23] or (b) the formation of an extended series of multimers, dimers, trimers, tetramers, etc. As the finite slope of the $\bar{\nu}$–b curve in Figure 3 at low b values and the positive curvature observed indicate, according to eq 8, that trimerization and tetramerization are significant, it seems that the model of an extended and continuous series of multimers is to be preferred. Considering that the flat and inflexible MB molecule is most likely to associate in some form of a stack, this sequential association seems physically plausible. This model is supported further by its applicability to a different dye, Orange II,[19] and to the self-association of MB studied by a completely different equilibrium method.[21]

Comparison with Previous Models. It has been suspected for a while that the frequently used description of the self-association of dyes in terms of dimerization alone is inadequate.[5,7,24–27] Indeed, the concentration-dependent absorption spectra of MB, in plots of ϵ against wavelengths, do not show an isosbestic point in any study covering a wide concentration range. A recent study by Braswell[7] is a good example. This fact invalidates any association model involving a single multimer. An association model using an extended series of multimers has been found useful for "stacking"-type interactions of nucleic acid bases and nucleosides,[28] and has been mentioned as a possibility for interpreting some spectral data for thionine.[27]

Braswell's study of the spectra of MB[7] led him to suggest that trimers must exist. This accords with our conclusion. Braswell's further conclusion that the self-association involves only dimers and trimers is in conflict with our results. Braswell used the nonequilibrium spectral method requiring estimates of ϵ values for the dimer and the trimer as well as β_2 and β_3. The description of the spectral data was far from satisfactory. The supporting evidence from vapor pressure osmometry, suggesting a limiting association number of 3, is not consistent with the analysis of the spectral data. At a concentration of 0.017 M MB, the osmometric measurements give an average degree of association of only 1.1, which is far too low when compared to estimates based on the β_2 and β_3 values of 2×10^3 l./mol and 6×10^6 l.^{2}/mol^2 found necessary to fit the spectral data. The reasons for this extraordinary result are not clear. Because of these uncertainties, we feel that our qualitative model of self-association merits further analysis.

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