of the D–H theories are of very limited use, since ion-size parameters and short-range interactions for dye ions cannot be estimated with any precision. However, the activity coefficients of the dimer and the monomers should continue to cancel to a great extent in K_2. We have, therefore, attempted to extend eq 3 to our higher ionic strength data. This can be done conveniently since essentially the same $f_\pm$ is involved in the dependence of $K_{A(0)}$ on μ (eq 2) and K_2 on μ (eq 3), both in NaF solutions. The two equations together predict a linear variation in log K_2 with log $K_{A(0)}$, when both are evaluated at the same μ, with a slope of unity. Figure 3 shows that this predicted relationship, expected to be valid in dilute solutions, is actually obeyed over the whole concentration range, with an average deviation of about 1% in K_2.

The above correlation may be fortuitous. However, the experimental fact that K_2 increases by only 33% as μ increases by a factor of 40 from 0.0025 to 0.102, suggests strongly that the self-association of MB, at least for the smaller multimers, is controlled mainly by changes in $f_\pm$ when μ is varied, and that counterion participation in the association equilibria is small, at least for the fluoride ion. Otherwise, an increase in counterion concentration by a factor of 40 would have resulted in considerably greater association at 9.5×10^{-4} M MB, where the fraction of total dye in the associated form is 75–80%. The previous assumption of no counterion participation[4,5] in the self-association of MB is thus justified.

The present conclusions are in agreement with some recent findings of Padday,[9] published after the present work was completed. Padday[9] has shown by electrometric measurements that counterion association does not play a very important part in dye association and metachromatic interactions, contrary to some recent suggestions.[6–8]

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Study of the Self-Association of Methylene Blue from Protonation Equilibria[1]

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Abstract: The self-association of methylene blue (MB^+) has been investigated by a new method based on the study of the protonation equilibrium of MB^+. The concentration of MBH^{2+} can be estimated by spectrophotometry at 745 mμ. The experiments have been performed in 0.200 M HCl. The apparent proton dissociation constant, $K_{pd(A)}$, of the species MBH^{2+}, defined as $B[H^+]/[MBH^{2+}]$, where B is the total equivalent concentration of all non-protonated dye species, varies by a factor of 9 as B varies from 2.26×10^{-5} to 1.27×10^{-3} M. The extrapolated value of $K_{pd(A)}$ at $B = 0$ gives the true proton dissociation constant, $K_{pd(0)}$, defined as $b[H^+]/[MBH^{2+}]$, where b is the concentration of dye monomers. The ratio $K_{pd(A)}/k_{pd(0)}$ thus equals B/b, and b can be obtained as a function of B. The estimated association of MB^+ with MBH^{2+} leads to a small correction. The $B(b)$ data are qualitatively very similar to those obtained by using the "isoextraction" method and are described well by the association model previously found satisfactory for methylene blue at low ionic strengths. The present method thus provides independent confirmation of previous conclusions about the self-association of MB^+. The dimerization constant, K_2, is somewhat higher than expected on the basis of a long extrapolation from our previous study of its ionic strength dependence. If the difference is attributed to ion pairing of the dimer with Cl^-, the derived equilibrium constant for ion pairing is low enough to provide strong support for the assumption of negligible counterion participation in our low-ionic-strength studies.

In previous papers[3–5] we have reported on our study of the self-association of methylene blue (MB^+) by the new "isoextraction" technique. Although the experimental method and the data and their interpretations are felt to be reliable,[3–5] an independent study of the self-association of MB^+ using an entirely different equilibrium method appeared to be highly desirable. The present paper outlines the principles of such a method, based on the protonation reaction of MB at high acid concentrations, and the results obtained therefrom. This new method is also expected to be useful for other systems.

In a broad sense, the principle of the present method is similar to that of the isoextraction method[3] and the solubility method[4] in that an equilibrium reaction involving the monomers is studied as a function of the total concentration so that the competitive equilibrium reactions in the self-association of the monomer can be disentangled.

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

(2) Life Science Dept., Harcourt Butler Technological Institute, Kanpur-2, India; requests for reprints may be sent to P. Mukerjee, School of Pharmacy, University of Wisconsin, Madison, Wisconsin 53706.

(3) P. Mukerjee and A. K. Ghosh, *J. Amer. Chem. Soc.*, **92**, 6403 (1970).

(4) A. K. Ghosh and P. Mukerjee, *ibid.*, **92**, 6408 (1970).

(5) A. K. Ghosh and P. Mukerjee, *ibid.*, **92**, 6413 (1970).

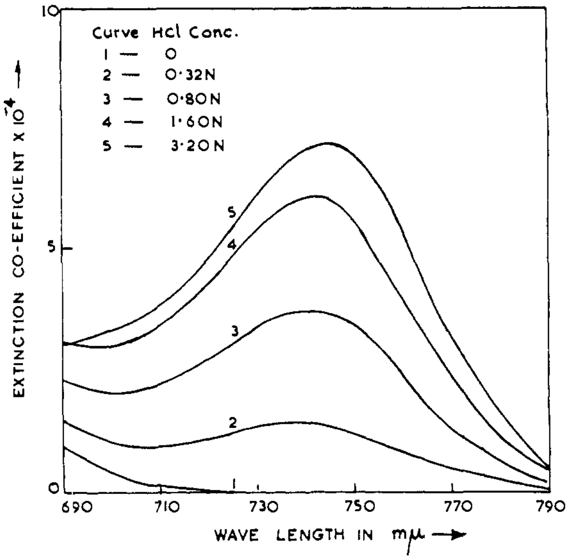

Figure 1. The effect of protonation on the spectrum of MBCl (2.16×10^{-5} *M*) in HCl solutions.

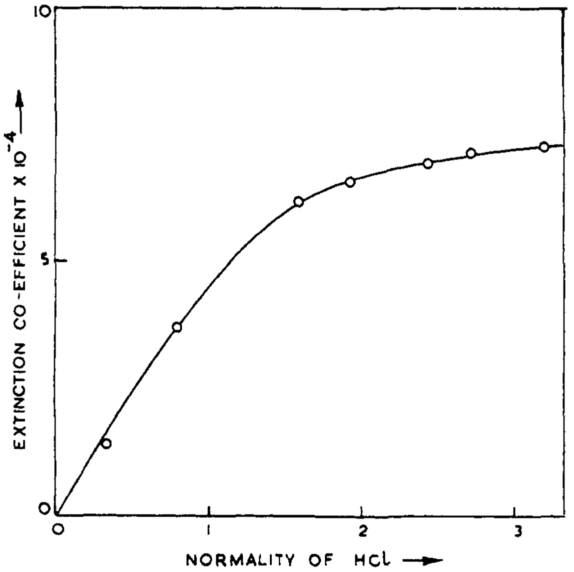

Figure 2. The effect of HCl concentration on the molecular extinction coefficient of MBCl (2.16×10^{-5} *M*) at the band maximum.

Methods

The apparatus and chemicals used and methods for determining the absolute and relative concentrations of MBCl have been described previously.[3]

Protonation of Methylene Blue. MB^+ reacts with hydrogen ions at high acid concentrations.[6] The absorption spectrum of the monoprotonated dye, MBH^{2+}, is different from that of monomeric MB^+, which has a band maximum at about 660 mμ, or its aggregates, which absorb at lower wavelengths.[7,8] Figure 1 shows the effects of HCl on the spectrum of a dilute solution of MBCl. The absorption band of $MBH,^{2+}$ with a peak at about 745 mμ, gradually develops on increasing the acid concentration. Figure 2 shows the apparent molecular extinction coefficient, ε, of the absorption peak of MBH^{2+} as a function of the concentration of HCl. The initial rise is sharp but the curve flattens out at about 3 *N* HCl. At still higher acidities, the apparent ε gradually decreases because of the formation of doubly or triply protonated species.[6]

Determination of the Proton Dissociation Constants. If the reaction between MB^+ and H^+ is represented as

$$MBH^{2+} \rightleftharpoons MB^+ + H^+ \qquad (1)$$

the proton dissociation constant, $K_{pd(0)}$, is defined as

$$K_{pd(0)} = \frac{b[H^+]}{[MBH^{2+}]} \qquad (2)$$

where b is the concentration of monomeric MB^+. The corresponding experimentally accessible quantity, called the apparent proton dissociation constant, $K_{pd(A)}$, is described by

$$K_{pd(A)} = \frac{B[H^+]}{[MBH^{2+}]} \qquad (3)$$

where B is the total equivalent concentration of all *nonprotonated* dye species in the system, and is, therefore, the same as previously defined.[3,4] If $K_{pd(A)}$ is determined over a range of B, its extrapolation to the infinite dilution of the dye furnishes $K_{pd(0)}$ because, at infinite dilution, $B = b$. Evidently, then, at any concentration

$$\frac{K_{pd(A)}}{K_{pd(0)}} = \frac{B}{b} \qquad (4)$$

The $b(B)$ data thus obtained provide detailed information on the nature of the self-association.[3,4]

Because of the high value of $K_{pd(0)}$ for MB, the protonation study is practical only at a high acid concentration, and, therefore, a high ionic strength, μ. After some preliminary experiments, an acid concentration of 0.200 *M* HCl was considered suitable. The change in μ on adding MBCl was negligibly small, so that all activity coefficients and the concentration of the chloride counterion could be considered constant.

The long-wavelength edge of the spectrum of nonprotonated MBCl extends into the spectral region of the protonated species studied, 740–750 mμ. To determine $K_{pd(A)}$ at 0.200 *M* HCl, blank corrections were necessary to take into account the absorption due to the nonprotonated dye. The corrections were determined in 0.001 *M* HCl, in which protonation is negligible, as well as in 0.2 *M* KCl. The differences in optical density, ΔOD, between the blank and the values in 0.200 *M* HCl were ascribed to protonated MB species. The ΔOD values at all concentrations exhibited a peak in the 744–746-mμ region. The corrections reduced the absorbances obtained at 745 mμ in 0.200 *N* HCl by about 14% at the lowest concentration of MB, 2.53×10^{-5} *M*, and by about 65% at the highest concentration, 1.27×10^{-3} *M*. The blank values in 0.200 *M* KCl were somewhat higher and gave ΔOD values which were lower by 3–15% than those calculated by applying the blank corrections from 0.001 *M* HCl. We have considered the 0.200 *M* KCl blanks more appropriate. The choice of blank corrections does not affect the qualitative conclusions about the self-association equilibria. The derived as-

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(6) G. N. Lewis and J. Bigeleisen, *J. Amer. Chem. Soc.*, **65**, 1144 (1943).

(7) K. Bergmann and C. T. O'Konski, *J. Phys. Chem.*, **67**, 2169 (1963).

(8) E. Braswell, *ibid.*, **72**, 2477 (1968).

Table I. Apparent Proton Dissociation Constants for MBH^{2+} in 0.200 N HCl at 35.4°

$[dye]_{tot}$, equiv/l.	ΔOD[a]	$[MBH^{2+}]$,[b] mol/l. × 10^5	$K_{pd(A)}$,[c] mol/l.	$K_{pd(A)}$,[d] mol/l.	B,[g] equiv/l.
2.53×10^{-5}	0.196[e]	0.272	1.66	1.69	2.26×10^{-5}
5.09×10^{-5}	0.346[e]	0.481	1.92	1.97	4.60×10^{-5}
1.275×10^{-4}	0.610[e]	0.847	2.81	2.94	1.186×10^{-4}
2.567×10^{-4}	0.886[e]	1.231	3.97	4.25	2.44×10^{-4}
6.43×10^{-4}	0.575[f]	1.597	7.85	8.59	6.27×10^{-4}
1.294×10^{-3}	0.710[f]	1.972	12.88	14.37	1.27×10^{-3}

[a] Blank corrections obtained in 0.2 M KCl. [b] Apparent concentration = $\Delta OD/7.2 \times 10^4$, includes contributions from MB^+MBH^{2+} (ΔOD for 1-cm path length). [c] Not corrected for $MB^+MBH_2^+$. [d] Corrected for MB^+MBH^{2+}. [e] Measured in 1-cm cells. [f] Measured in 0.5-cm cells. [g] Concentration of all nonprotonated species.

sociation constants agree to within about 7%. Because of the size of the blank correction, the data at the highest concentrations are somewhat less reliable than those at the lower concentrations.

The data at 35.4° are reported in Table I. Less complete data at 25°, not included here, showed the same features except that the self-association was more marked.

Results

Preliminary calculations of $K_{pd(A)}$ values were made according to eq 3, neglecting any association of MBH^{2+}. The ϵ value of 7.2×10^4 for MBH^{2+}, obtained from the absorbance of MB in 3.2 M HCl (Figure 2), was used to convert ΔOD values to the concentration of MBH^{2+}. Any error in ϵ will, of course, be reflected in $K_{pd(A)}$ but will largely cancel in the B/b values (eq 4). The value of B was calculated by subtracting the estimated concentration of MBH^{2+} from the total dye concentration.

Participation of MBH^{2+} in the Association Equilibria. The self-association of MBH^{2+} is expected to be much less than that of MB^+ because of its higher charge and lower concentration. In our study of the mutual association of MB with quinoline and quinolinium ion,[9] it was found that the introduction of a localized charge reduces the association constant by a factor of 5. Because of the diffuse charge distribution in MB^+, the charge repulsion factor in the dimerization of MB^+ is probably lower.[4] MBH^{2+}, however, has a charge localized in the protonated nitrogen. Thus, the dimerization constant for MBH^{2+} is expected to be lower by a factor of about 10^2 than K_2, the dimerization constant of MB^+, because of charge repulsion alone. Moreover, the $K_{pd(0)}$ value of 1.40 mol/l. indicates that the ratio $[MBH^{2+}]/[MB^+]$ equals 0.143 at 0.200 M HCl. The self-association of MBH^{2+} should thus be negligible. This conclusion is supported by the observation that in 3.2 N HCl, MBH^{2+} obeys Beer's law,[3] unlike MB itself.

For the mutual association of MBH^{2+} with unprotonated MB^+

$$MB^+ + MBH^{2+} = MB^+MBH^{2+} \tag{5}$$

the association constant, K_2^+, is estimated to be about $K_2/5$ because of the extra localized charge in MBH^{2+}. As the ratio $[MBH^{2+}]/[MB^+]$ is 0.143, the calculated ratio $[MB^+MBH^{2+}]/[(MB)_2{}^{2+}]$ is only 0.0286, assuming $K_2^+ = K_2/5$. Neglecting the higher protonated multimers for which the ratio of the protonated to the unprotonated species is even less, the concentration of MB^+MBH^{2+} can be calculated from estimates of K_2 and b. It changes the estimates of B by only ~0.2% on the average, but does have appreciable effect on the estimated concentration of free MBH^{2+} in the calculation of $K_{pd(A)}$ (eq 3). The correction was, therefore, applied by a reiterative procedure by estimating K_2. It was assumed that MBH^{2+} in MB^+MBH^{2+} has the same absorption spectrum as MBH^{2+} itself.

Table I gives both the corrected and uncorrected $K_{pd(A)}$ values. The difference is small at low dye concentrations. The effect of the corrections on K_2 is about 10%. The corrections do not alter appreciably the conclusions about the qualitative nature of the self-association of MB^+ in 0.200 N HCl.

Figure 3 shows the $K_{pd(A)}$ values as a function of B. The upper curves show that $K_{pd(A)}$ varies linearly with B in dilute solutions.

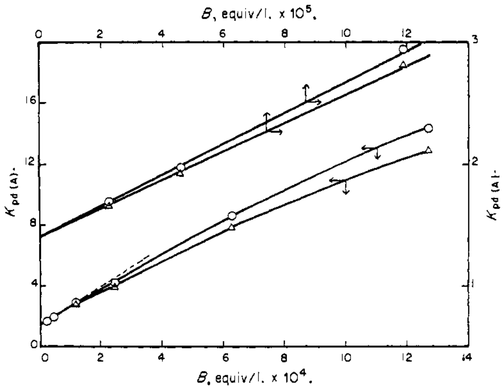

Figure 3. The variation of the apparent proton dissociation constant, $K_{pd(A)}$, of MBH^{2+} (equiv/l.) in 0.200 N HCl at 35.4° with the total concentration of all nonprotonated MB species, B (equiv/l.). Dilute solution data are represented by the straight lines in the upper portion of the figure. Δ, data not corrected for association of MBH^{2+}; ○, data corrected for association of MBH^{2+}.

Discussion

As the $K_{pd(A)}$ values are exactly analogous to the isoextraction K_A values[3] in that both reflect the change in b with B (eq 4), it is of considerable interest to note the close similarity of the $K_{pd(A)}$–B curve obtained at an ionic strength of 0.2 with the K_A–B curves[3,10] obtained at ionic strengths lower by a factor of about 100. All the curves exhibit an initial linear portion followed by a less rapid increase of B/b at higher B values. The linear portion allows precise determinations of the initial slope, from which K_2 can be cal-

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(9) P. Mukerjee and A. K. Ghosh, unpublished results; A. K. Ghosh, doctoral dissertation, Calcutta University, 1963.

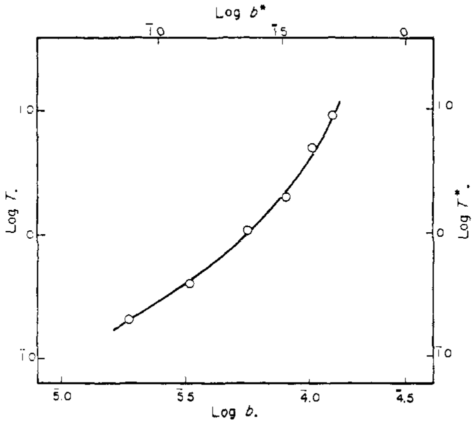

Figure 4. Description of the self-association behavior of MB in 0.200 *N* HCl at 35.4° by the two-parameter model IV.[4]

culated without reccourse to any model of self-association.[4]

The value of K_2 so derived at 35.4° in 0.200 *N* HCl from the corrected $K_{\mathrm{pd(A)}}$ data is 4.6×10^3 l./mol. This is to be compared with the value of 1.63×10^3 l./mol at very low μ at 34.9°.[10] The increase in K_2 at the higher μ is in line with similar increases found in our ionic strength studies using NaF.[5] These values extend to an ionic strength of about 0.1. A rough extrapolation suggests a K_2 value of about 3×10^3 l./mol at an ionic strength of 0.2. The present result is somewhat higher. The difference is not large, however, and may well be due to activity coefficient effects not properly taken into account in our rough and long extrapolation.

On the other hand, the difference may be due to participation of Cl^- in the association equilibria. If the equilibrium constant for the ion pairing of the MB^+ dimer, b_2, in the reaction $b_2 + Cl^- = b_2Cl^-$ is denoted by K_C, eq 6 gives the relation between the experimental values of K_2 and K_C. If the value of $[b_2]/b^2$, the true

$$K_2 = \frac{[b_2 + b_2Cl^-]}{b^2} = \frac{[b_2]}{b^2}(1 + K_C[Cl^-]) \qquad (6)$$

dimerization constant, is assumed to be 3×10^3 l./mol according to the previous rough extrapolation, the value of K_C is 2.6 l./mol. Considering that ion pairing with a doubly charged ion is involved, the magnitude of K_C is not unreasonable. It is low enough, however, to give strong support to the assumption of no counterion participation in our isoextraction studies at ionic strengths of 0.0012–0.004.

When the higher multimers are considered, the protonation data show the same features of association as the isoextraction data.[3,10] In particular, the self-association model IV,[4] found to be appropriate for MB at low ionic strengths[3,10] and Orange II,[4] fits the protonation data at 0.200 *N* HCl quite well, as shown in Figure 4. This model gives K_2 and K_3[3] values of 4.4×10^3 and 8.2×10^3 l./mol, respectively. Thus, K_3 is again found to be higher than K_2, as observed previously.[4] It seems, therefore, that even at this high ionic strength, counterion participation is not extensive enough, at least for the smaller multimers, to alter greatly the characteristics of sequential self-association previously derived from the isoextraction data.[4]

The present study, using a completely different experimental approach, provides strong independent support for our previous findings about the nature of the self-association of ionic dyes.[3–5]

Acknowledgment. The author is grateful to Professor P. Mukerjee for help and advice during this investigation, which had financial support from the Council of Scientific and Industrial Research, New Delhi, India.

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(10) P. Mukerjee and A. K. Ghosh, *J. Amer. Chem. Soc.*, **92**, 6419 (1970).