

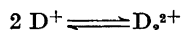
## On the Dimerisation of the Acridine Orange Cation

### A Potentiometric and a Spectrophotometric Proof that the Dimerisation Does Not Involve Counterions

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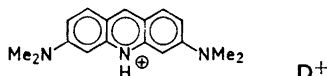
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The question of whether the dimerisation of the acridine orange hydrochloride involves only the cation ( $D^+$ ) or if the anion participates in the association as a "spacer" has been investigated. The concentration of free chloride ions has been measured in solutions with different degrees of dimerisation and the dimer/monomer distribution has been studied spectrophotometrically. It is shown that the dominating equilibrium is



A change of the absorbance with higher chloride concentration has been interpreted in terms of a formation of  $D_2Cl^+$ , the formation constant of which has been estimated to be of the order of  $2 M^{-1}$  at an ion-strength of 1 M.

The ability of amino acridines to form complexes with poly-peptides was early established from the change of the absorption spectra of the dyes.<sup>1</sup> Recently it was discovered by Blout and Stryer<sup>2,3</sup> that Cotton effects were induced at the visible absorption bands of certain dyes when bound to poly-L-glutamic acid in  $\alpha$ -helical conformation. The circular dichroism (CD) of acridine orange-poly- $\alpha$ ,L-glutamic acid complexes has been studied in this laboratory. By observing the polarized CD spectra of the streaming solutions, it has been possible to draw certain conclusions about the configuration of the complex.<sup>4</sup> The lack of more detailed knowledge of the solution chemistry of the dye itself, however, has been apparent. Thus for instance Blout and Stryer report an ionic strength dependence of the Cotton effects which can most certainly be explained<sup>4</sup> by a redistribution between a monomer and a dimer form of the dye. The dimerisation of the acridine orange



cation ( $D^+$ ) has actually been examined spectrophotometrically by Lamm and Neville.<sup>5</sup> From the concentration dependence of the absorption spectrum they have assigned the two absorbance maxima at 465 nm and 490 nm to the dimer and the monomer form, respectively. Depending on which model they use for the dimerisation reaction



or



they have obtained the equilibrium association constants  $K_A = 4.7 \times 10^8 \text{ M}^{-2}$  and  $K_B = 1.05 \times 10^4 \text{ M}^{-1}$ , respectively. The authors claim that the experimental data satisfy both models.

The main purpose of our investigation has been to establish according to which model (A or B) the dimerisation occurs. This has been done by a series of measurements of the free chloride concentration, in solutions with varying concentration of acridine orange hydrochloride (DCl) complemented by a study of the absorption spectra of solutions with constant acridine orange concentration but varying chloride proportion.

#### POTENTIOMETRIC INVESTIGATION

The emf ( $E$ ) of the element

- Ag, AgCl	Reference (2)	Sample (1)	AgCl, Ag +
	1.00 mM NaCl	5 mM NaNO <sub>3</sub>	v ml 1.00 mM DCl 30 ml 1.00 mM NaCl

was measured and was considered dependent on the chloride ion concentration in the sample cell (1),  $[Cl^-]_1$  in the following way:

$$E = 0.0592 \left( \log \frac{0.001}{[Cl^-]_1} + K_{D\&H} \right) \quad (1)$$

where  $E$  is expressed in volts and  $K_{D\&H}$  is a term given by a Debye-Hückel approximation according to

$$-\log f_1 + \log f_2 = K_{D\&H} = 0.509 \left( \frac{\sqrt{I_1}}{1 + \sqrt{I_1}} - \frac{\sqrt{I_2}}{1 + \sqrt{I_2}} \right)$$

where  $f_1$  and  $f_2$  are activity coefficients, and  $I_1$  and  $I_2$  are ionic strengths in the appropriate half-cells.

However, depending on the model for the dimerisation reaction, one obtains somewhat different expressions for  $I_1$ . According to model A

$$I_1^A = [Na^+] + [D^+] + [D_2Cl^+]$$

and introducing the quantity

$$F = [D_2Cl^+]/[D^+], \text{ there results } I_1^A = [Na^+] + C_D \cdot (1 + F)/(1 + 2 F)$$

$C_D$  = total concentration (M) of the dye. With model B

$$(F = [D_2^{2+}]/[D^+]) \text{ we obtain } I_1^B = [Na^+] + C_D \cdot (1 + 3 F)/(1 + 2 F).$$

The free concentration of chloride ion has been calculated from eqn. (1) where  $K_{D\&H}$  was estimated for model B. As the  $K_{D\&H}$  terms appear to be negligible we have used the same values for the other model as well. The ratio  $F$  according to model B, has been computed using Lamm's value of the dimerisation constant ( $K_B$  see above,

$$F = -0.25 + \sqrt{0.0625 + C_D \times 5.25}, C_D \text{ in mM}$$

*Experimental.* Acridine orange hydrochloride was prepared from Merck's "Acridinorange für Mikroskopie", which has the composition  $DCl \cdot ZnCl_2$  ( $D^+$  see Introduction). A 10 g portion of this substance was dissolved in 600 ml distilled water, hydrogensulphide was lead into the solution for 1 h, and pH was adjusted with  $Na_2S$  to 6.8. After stirring for 5 h a further pH-adjustment was made. The zinc sulphide was filtered off and 0.5 M NaOH was added to the filtrate slowly and with vigorous stirring until pH=10.5. The acridine orange base was filtered off and was washed with  $2 \times 30$  ml ice-cold water. It was dried over night at 60°. The base was dissolved in a minimum amount of hot methanol, a solution of 1 part conc. hydrochloric acid and 9 parts methanol was added slowly with vigorous stirring to pH=5.5. The acridine orange hydrochloride was then precipitated with ethyl ether (10 volumes), sucked off and dried. The procedure, from precipitation of the base, was then repeated once starting from a concentrated solution of acridine orange hydrochloride in water. (Analysis of DCl: Found: C 63.2; H 6.86; N 12.9; Cl 10.6. Calc. C 67.6; H 6.68; N 13.9; Cl 11.7). The molar absorptivity measured at 470 nm,  $\epsilon = 4.3 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ , was the same as reported in literature.<sup>4</sup> A 1.00 mM DCl solution was prepared from the solid compound by dissolving it in water (pH of the solution was 6.9). The solution was stored in polyethylene bottles in the dark. The dye concentration was checked spectrophotometrically before the titration.

Silver-silver chloride electrodes were prepared according to the method of Brown.<sup>6</sup> The silver electrodes were cleaned by anodic oxidation in conc. nitric acid (1 mA/cm<sup>2</sup> for 30 min), washed with water and coated with silver in a 1 %  $KAg(CN)_2$  solution (0.4 mA/cm<sup>2</sup> for 8 h). After washing with water and conc. ammonia, the electrodes were stored for 10 h in distilled water and were then electrolytically coated with AgCl in 0.1 M HCl (0.4 mA/cm<sup>2</sup>, 1 h). The electrodes were then used after 10 h soaking in distilled water. Although acridine orange is known to be extremely adhesive (*e.g.* to glass) and therefore should be expected to poison the electrodes by adsorption, the electrodes responded very well to the chloride concentration (this was checked before as well as after the titration).

The titration was performed with a concentration element consisting of two thermostated (25.0°) cells, each containing 30.00 ml 1.000 mM NaCl and two Ag-AgCl electrodes and connected *via* an agar-agar bridge. This consisted of a 15 cm long PVC tubing (diam. 5 mm) filled with agar-agar gel with 5 mM  $NaNO_3$ .<sup>\*</sup> The element was very sensitive to diffusion: With 1 mM HCl in one of the cells (+) and 1 mM NaCl in the other (-) an emf of -38 mV was measured. The two pairs of electrodes were cross-tested by comparing the four emf values obtained with the various pairings of the electrodes. The titration was carried out by adding portions of about 1 ml of 1.00 mM DCl in one cell (1) and the reading was taken after 1 min of stirring. The corresponding  $C_D$  and  $E$  values are reproduced in Table 1. The calculated values of the  $Cl^-$  concentration has been plotted against the calculated  $F$ -values (Fig. 1).

\* As we found later that *precipitates* were obtained with  $NaNO_3$  and  $NaClO_4$  added to a DCl solution a test titration with an NaF bridge was performed to rule out the possible interference from the  $NaNO_3$  salt bridge. The result agreed very well with the one reported here so we found no reason to suspect any diffusion from the bridge.

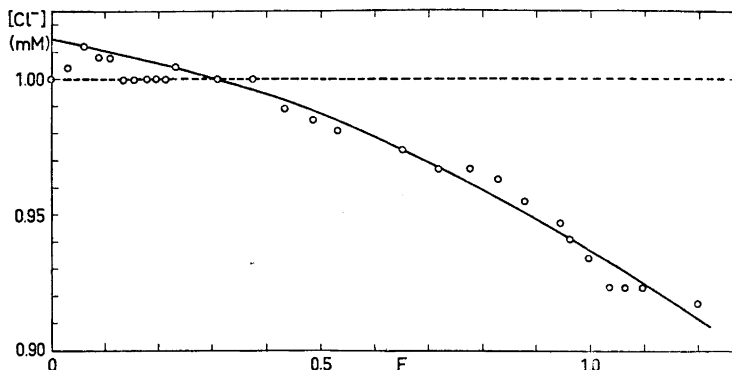


Fig. 1. The free chloride concentration (calculated from the measured emf) plotted against  $F = [D_2^{2+}]/[D^+]$ .

**Result of the potentiometric investigation.** From Fig. 1 we see that the dimerisation must proceed according to  $2 D^+ \rightleftharpoons D_2^{2+}$  (Model B), because if chloride ions are involved stoichiometrically, the decrease in free chloride ion concentration with increasing  $F$  should be greater than the observed. *E.g.*, for  $F=1$  one should expect  $[Cl^-] = 0.87$  mM or correcting for the actual chloride content of the preparation (see above)  $[Cl^-] = 0.84$  mM.

The titration showed a decrease only to 0.94 mM (at  $F=1$ ). Approximately half of the difference between the expected and observed decrease can be accounted for by the fact that the formally 1.00 mM acridine orange hydrochloride only contained about 0.90 mM chloride (*cf.* the result of analysis).

The  $[Cl^-]$  decrease, remaining after this correction, *i.e.* 0.03 mM, might very well be caused by an association,  $D_2^{2+} + Cl^- \rightleftharpoons D_2Cl^+$ . The corresponding equilibrium constant should then be of the order of  $5 \times 10^2 M^{-1}$  ( $I=0.001$ )\*.

Table 1. Corresponding  $C_D$  and  $E$  values at the titration.

$C_D$ (mM)	$F$ (model B)	$E$ (mV)	$C_D$ (mM)	$F$ (model B)	$E$ (mV)
0.0033	0.033	-0.1	0.1176	0.574	0.5
0.0066	0.061	-0.3	0.1304	0.614	0.5
0.0099	0.089	-0.2	0.1428	0.651	0.7
0.0132	0.112	-0.2	0.1667	0.719	0.9
0.0164	0.135	0.0	0.1892	0.777	0.9
0.0196	0.156	0.0	0.2105	0.830	1.0
0.0228	0.177	0.0	0.2308	0.879	1.2
0.0260	0.196	0.0	0.2500	0.945	1.3
0.0291	0.214	0.0	0.2683	0.962	1.6
0.0323	0.231	-0.1	0.2857	0.998	1.8
0.0476	0.308	0.0	0.3023	1.034	2.1
0.0625	0.374	0.0	0.3182	1.066	2.1
0.0769	0.433	0.3	0.3330	1.096	2.1
0.0909	0.485	0.4	0.4000	1.201	2.3
0.1045	0.531	0.5			

\*  $F=1$  corresponds to  $C_D=0.29$  mM and  $C_{Cl}=0.97$  mM. This gives, with  $[D_2Cl^+]=0.03$  mM,  $[D_2^{2+}]=(0.29/3-0.03)$  mM=0.07 mM and  $[Cl^-]=0.94$  mM, *i.e.*  $\beta=(5 \pm 2) \times 10^2 M^{-1}$ .

## SPECTROPHOTOMETRIC INVESTIGATION

In order to complete and elucidate the result of the potentiometric titration a number of spectrophotometrical measurements were performed on acridine orange solutions with constant concentration of dye but with varying chloride concentration. From the absorption spectra, computed by Lamm *et al.*, for the two species, D and D<sub>2</sub> (or D<sub>2</sub>Cl), one can calculate a hypothetical dependence on the chloride concentration according to Model A. The intention has been, to compare the calculated chloride dependence with the observed one, and thus, if possible, decide to which model the dimerisation can be assigned.

*Experimental.* Absorbance of DCl solutions were measured with a Hitachi EPS-3T recording spectrophotometer, in 1 mm quartz cells. Strong variations in absorbance of one and the same solution if the cell was not carefully cleaned between refillings were observed. This phenomenon which has also been observed by others,<sup>5</sup> can be attributed to a strong adsorption of acridine orange on the walls of the cell. Good reproducibility was, however, obtained if the cell was cleaned with dichromate-sulphuric acid solution and then ethanol immediately before measurement. The compositions and the measured absorbances of the solutions are given in Table 2 (*cf.* also Fig 2).

Table 2. Measured absorbances of solutions with  $C_{D^+} = 0.100$  mM and varying  $C_{Cl^-}$  (0.1–2.1 mM).

$C_{Cl^-}$ (mM)	log $C_{Cl^-}$	$d = 0.100$ cm	
		$A_{470}$	$A_{490}$
2.10	0.322	0.389	0.355
1.90	0.279	0.385	0.346
1.70	0.230	0.392	0.350
1.50	0.176	0.397	0.360
1.30	0.114	0.398	0.358
1.10	0.041	0.408	0.367
0.90	-0.046	0.400	0.370
0.70	-0.155	0.395	0.363
0.50	-0.301	0.398	0.367
0.30	-0.523	0.399	0.367
0.10	-1.000	0.399	0.370

*Result of the spectrophotometric investigation.* If the dimerisation occurred according to Model A, *i.e.*,  $2 D^+ + Cl^- \rightleftharpoons D_2Cl^+$ , the absorbances (A) at the maxima (*cf.* Fig. 2) should be expressed,

$$\begin{aligned} A_{470}/d &= 43 [D^+] + 2 \times 43 [D_2Cl^+] \text{ and} \\ A_{490}/d &= 62 [D^+] + 2 \times 28 [D_2Cl^+] \end{aligned} \quad (2)$$

(according to Lamm and Neville<sup>5</sup>).

From these expressions and from the stability constant of D<sub>2</sub>Cl<sup>+</sup> (470 mM<sup>-2</sup>, according to Lamm and Neville<sup>5</sup>) a hypothetical chloride ion dependence has been calculated for  $A_{470}$  and  $A_{490}$  (Table 3, Fig. 3.). In Fig. 3 it appears

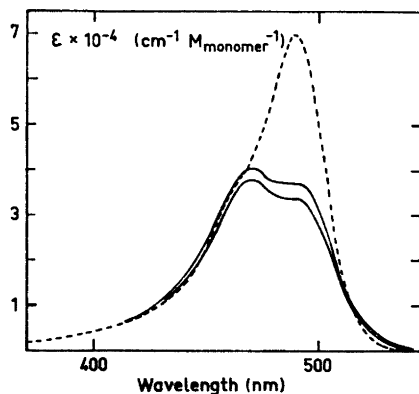


Fig. 2. Absorptivity of a 0.1 mM acridine orange hydrochloride solution: The solid upper curve,  $C_{Cl^-} = 0.1$  mM, the lower solid curve,  $C_{Cl^-} = 2.1$  mM, the dotted curve, in ethanol (0.1 mM); cf. Table 2.

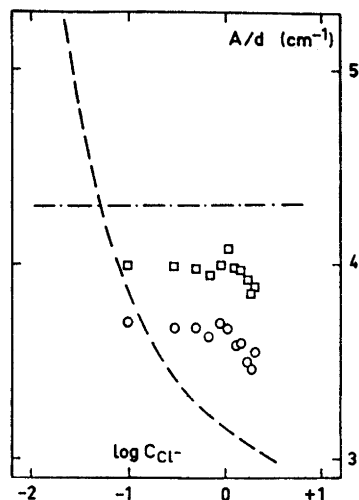


Fig. 3. Relation between calculated absorbances, using Model A ( $A_{470}$  — —,  $A_{490}$  — —) and the empirically found values ( $A_{470}$  □,  $A_{490}$  ○).

Table 3. Calculated absorbances ( $A_{470}$ ,  $A_{490}$ ) if  $[D_2Cl^+]/[D^+][Cl^-] = 470$  mM $^{-2}$ .  $C_D = 0.1$  mM,  $C_{Cl^-}$  varying between 0.1 mM and 2.1 mM in the experiment.

$C_{Cl^-}$ (mM)	$\log C_{Cl^-}$	$[D^+]$ (mM)	$[D_2Cl^+]$ (mM)	$A_{470}$ (cm $^{-1}$ )	$A_{490}$ (cm $^{-1}$ )
2.824	0.450	0.006	0.047	4.30	3.00
1.002	0.009	0.010	0.045	4.30	3.14
0.3284	-0.484	0.016	0.042	4.30	3.34
0.2168	-0.664	0.020	0.040	4.30	3.48
0.0930	-1.032	0.033	0.033	4.30	3.93
0.0318	-1.498	0.060	0.020	4.30	4.82
0.0173	-1.762	0.080	0.010	4.30	5.52

that  $A_{490}$  is supposed to decrease strongly with increasing chloride concentration. As the empirical result shows that  $A_{490}$  is practically constant it is possible to rule out the suggested Model A. The chloride ions do not stoichiometrically take part in the dimerisation.

As was mentioned in the introduction, strong ionic strength dependence of the long wavelength transition of acridine orange has been reported.<sup>1,2</sup> The absorbance maximum that, according to Lamm *et al.*, corresponds to the monomer, decreases with increasing salt concentration. A Debye-Hückel approach<sup>7</sup>

Table 4. Measured absorbances at higher variations in  $C_{Cl^-}$ .  $C_D = 0.100$  mM.

$$\epsilon_{D_2Cl(470)} = 1.50 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1} \text{ }^a$$

$$\epsilon_{D(470)} = 3.97 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$$

$C_{Cl^-}$ (M)	$1/C_{Cl^-}$ (M <sup>-1</sup> )	Absorbance $d = 0.201$ cm		$G = \frac{C_{D_2}}{[D^+]}$	$A_{470}/d - \epsilon_D[D^+]$ (cm <sup>-1</sup> )	$[D_2^{2+}]$ (mM)	$\beta$ (M <sup>-1</sup> )
		$A_{470}$	$A_{490}$				
2.00 <sup>b</sup>	0.50	0.511	0.311	1.782	1.67	0.0101	1.43
1.00	1.00	0.562	0.384	1.273	1.68	0.0126	1.94
0.80	1.25	0.611	0.422	1.234	1.89	0.0167	1.41
0.50	2.00	0.637	0.458	1.101	1.93	0.0182	1.78
0.30	3.33	0.668	0.493	1.023	2.02	0.0204	2.16
0.20	5.00	0.698	0.537	0.910	2.07	0.0222	2.27
0.10	10.0	0.728	0.587	0.796	2.09	0.0236	3.01
0.00	—	0.798	0.747	0.510	2.00	0.025	—

<sup>a</sup> Obtained by extrapolation in Fig. 5.

<sup>b</sup> At higher chloride concentrations precipitation occurs.

shows that a medium change should be expected to give rise to such an effect.\* However, the measurements reveal no *isosbestic point* at 470 nm (Fig. 2), and it is, in fact, difficult to attribute this effect to medium effects.\*\* It could, however, be ascribed to a third species, e.g.,  $D_2Cl^+$ . In order to test this possibility the following study has been made.

*Additional absorbance measurements.* Absorbance spectra were recorded for solutions with constant acridine orange hydrochloride concentration and with chloride concentrations between 0 and 2 M (Fig. 4, Table 4).

From the expression for the absorbance at 470 nm

$$A_{470}/d = \epsilon_D[D^+] + \epsilon_{D_2}2[D_2^{2+}] + \epsilon_{DCl}[DCl] + \epsilon_{D_2Cl}2[D_2Cl] + \epsilon_{D_2Cl_2}2[D_2Cl_2] \quad (3)$$

where all absorptivities are in units of (M monomer)<sup>-1</sup> cm<sup>-1</sup>, one obtains, neglecting [DCl] and [D<sub>2</sub>Cl<sub>2</sub>]

$$A_{470}/d = \epsilon_D([D^+] + 2[D_2^{2+}]) + \epsilon_{D_2Cl}2[D_2Cl^+] \quad (4)$$

In this equation it is assumed that  $\epsilon_D = \epsilon_{D_2}$ , i.e., the *isosbestic point* is considered to be preserved.

\* If for the equilibrium  $2 D^+ \rightleftharpoons D_2^{2+}$  the stoichiometric and thermodynamic equilibrium constants are  $K$  and  $K_a$ , respectively we have

$$K_a = \frac{\{D_2^{2+}\}}{\{D^+\}^2} = \frac{f_{2+}}{f_+^2} \times K$$

i.e. according to the limiting law of D&H

$$\log K_a = \log K - 2^2 \times A\sqrt{I} + 2A\sqrt{I} \quad (A > 0)$$

From  $\log K = \log K_a + 2A\sqrt{I}$  we see that an increase in  $I$  will cause an increase in  $K$ .

\*\* A 0.1 mM DCl solution in ethanol was found to have about the same absorptivity at 470 nm (Fig. 2) as an aqueous solution.

With

$$\beta = [\text{D}_2\text{Cl}^+]/[\text{D}_2^{2+}][\text{Cl}^-] \quad (5)$$

$$\text{and } C_{\text{D}} = [\text{D}^+] + 2[\text{D}_2^{2+}] + 2[\text{D}_2\text{Cl}^+] \quad (6)$$

one obtains

$$A_{470}/d = \varepsilon_{\text{D}}C_{\text{D}} - 2\beta[\text{D}_2^{2+}][\text{Cl}^-] (\varepsilon_{\text{D}} - \varepsilon_{\text{D}_2\text{Cl}})$$

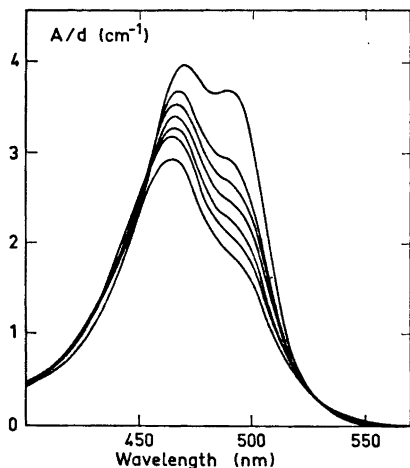


Fig. 4. Spectral change using higher chloride concentration (cf. Table 4.  $0 \leftarrow 2 \text{ M Cl}^- = 2 \text{ M} = \text{lowest curve}$ ).

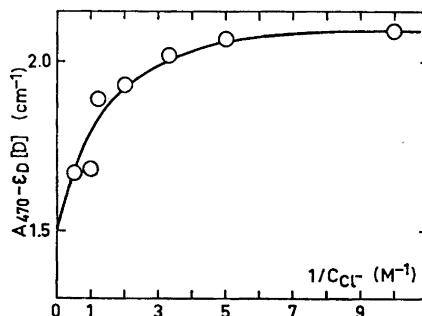


Fig. 5. Extrapolation of  $A_{470} - \varepsilon_{\text{D}}[\text{D}]$  to  $1/C_{\text{Cl}^-} = 0$ , giving  $\varepsilon_{\text{D}_2\text{Cl}}$ .

and

$$\beta = \frac{\varepsilon_{\text{D}}C_{\text{D}} - A_{470}/d}{2[\text{D}_2^{2+}][\text{Cl}^-](\varepsilon_{\text{D}} - \varepsilon_{\text{D}_2\text{Cl}})} \quad \text{i.e., } [\text{D}_2\text{Cl}^+] = \frac{\varepsilon_{\text{D}}C_{\text{D}} - A_{470}/d}{2(\varepsilon_{\text{D}} - \varepsilon_{\text{D}_2\text{Cl}})} \quad (7)$$

$\beta$ -values calculated from these expressions (5–7) are given in Table 4. In these calculations,  $C_{\text{Cl}^-}$  has been accepted as an approximation to  $[\text{Cl}^-]$ , and  $\varepsilon_{\text{D}}$  has been obtained from pure 0.1 mM DCl-solution (assuming no chloride association).  $\varepsilon_{\text{D}_2\text{Cl}}$  was obtained by extrapolation:

$$\lim(1/C_{\text{Cl}^-} \rightarrow 0)(A_{470}/d - \varepsilon_{\text{D}}[\text{D}])$$

$A_{470}/d - \varepsilon_{\text{D}}[\text{D}]$  constitutes the dimer contribution to  $A_{470}$  (see eqn. (4), Fig 5).  $C_{\text{D}_2}$  ( $= [\text{D}_2^{2+}] + [\text{D}_2\text{Cl}^+]$ ) and  $[\text{D}^+]$  were calculated from  $G = C_{\text{D}_2}/[\text{D}^+]$  which was obtained from the ratio

$$\begin{aligned} \frac{A_{470}}{A_{490}} &= \frac{\varepsilon_{\text{D}(470)}[\text{D}^+] + \varepsilon_{\text{D}_2(470)}2[\text{D}_2^{2+}] + \varepsilon_{\text{D}_2\text{Cl}(470)}2[\text{D}_2\text{Cl}^+]}{\varepsilon_{\text{D}(490)}[\text{D}^+] + \varepsilon_{\text{D}_2(490)}2[\text{D}_2^{2+}] + \varepsilon_{\text{D}_2\text{Cl}(490)}2[\text{D}_2\text{Cl}^+]} \\ &\approx \frac{\varepsilon_{\text{D}(470)}[\text{D}^+] + \varepsilon_{\text{D}_2(470)}2C_{\text{D}_2}}{\varepsilon_{\text{D}(490)}[\text{D}^+] + \varepsilon_{\text{D}_2(490)}2C_{\text{D}_2}} \end{aligned}$$

Here it has been assumed that  $\varepsilon_{\text{D}_2}/\varepsilon_{\text{D}_2\text{Cl}}$  is relatively invariant with the wavelength and that the error of approximation in the numerator is mainly compensated by that in the denominator.



## DISCUSSION

The results reported above show, that the dimerisation of the acridine orange cation does not involve the chloride ion. However, the interpretation of the spectral change, with increasing chloride concentration might appear somewhat dubious. A seemingly more obvious explanation might be that the "new species" is the trimer. However, the fact that the wavelengths of the two absorption maxima remain unaffected, is evidence against the trimer hypothesis. One could expect to find a new band at shorter wavelength or some other major spectral change with the formation of the trimer. On the other hand, as has been suggested by Sheppard,<sup>8</sup> there may be for the monomer one pure electronic transition (490 nm) and one "latent" band arising from a vibronic transition (470 nm), which should be favoured in the dimer. In that case an additional transition is not necessarily expected for the trimer. It is, however, very likely that the cations with higher charge (*e.g.*  $D_2^{2+}$ ) have a cover of associated chloride ions, and it is not unreasonable to believe that the electrostatic field from a closely approached chloride ion (*e.g.* in the neighbourhood of a positive nitrogen) might influence the aromatic  $\pi$ -electron system causing a diminished  $\pi \rightarrow \pi^*$  transition probability.

The increase of  $\beta$  with decreasing chloride concentration (Fig. 6) may be attributed to an ionic strength effect.  $\text{Log } \beta = \text{log } \beta_A - 4A\sqrt{I}$  for the equilibrium  $D_2^{2+} + \text{Cl}^- \rightarrow D_2\text{Cl}^+$ , *i.e.*, if  $I$  decreases  $\beta$  should increase. This apparent agreement is, however, only qualitatively and actually most dyes are found not to obey the common laws of electrolyte solutions.<sup>9,10</sup> Thus Barone *et al.*<sup>11</sup> have reported a reversed influence of tetramethylammonium halides on the dimerisation of acridine orange, *i.e.*, they cause dissociation.

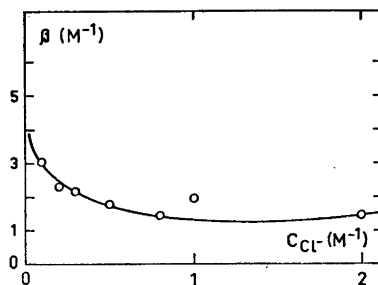
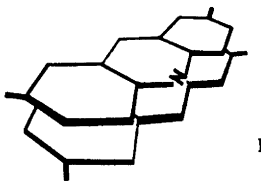


Fig. 6. Plot of  $\beta$  versus  $C_{\text{Cl}^-}$ .

Though  $\beta$  might be expected to depend exponentially on the ionic strength (chloride concentration), the value of  $\beta = 5 \times 10^2 \text{ M}^{-1}$  ( $C_{\text{Cl}^-} = 10^{-3} \text{ M}$ ) which was estimated to explain the small decrease in free chloride concentration; determined potentiometrically, does not seem to fit the values of Fig. 6. This

is not unreasonable, however, in light of the experimental uncertainty of the emf measurements.\*

The results obtained in this investigation suggest that in  $D_2^{2+}$  the aromatic rings are associated, by van der Waals forces, to a sandwich structure (I). This is well in accord with the recently published report on the electron structures of proflavine, acridine orange, *etc.*, by Ito *et al.*<sup>12</sup> who, using the exciton theory, estimate from the band shifts due to the formation of dimers, that the intermolecular distances should be in the order of 5 Å.



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Received January 31, 1970.

\* A recently performed potentiometrical experiment of the type described above is worth mentioning. In the cell,  $-Hg, Hg_2Cl_2 | KCl | \text{sample} | AgCl, Ag^+$ , a Radiometer K 401 electrode, containing a saturated KCl solution and a sintered glass connection, was used as a reference electrode. The relation between emf and chloride concentration was determined experimentally. When performing an experiment analogous to that described above ( $F=1$ , also checked spectrophotometrically), we did not find any decrease in  $[Cl^-]$  but an increase of about 10 % judging from the decrease in  $E$ . This observation which at first seems to further plead for Model B, however, is confusing and suggests that the presence of the dye may have changed the cell function. When adding water or chloride, anyhow,  $E$  still responded to  $[Cl^-]$  in a satisfactory manner.