

A Nuclear Magnetic Resonance Investigation of the Aggregation of Acridine Orange in Aqueous Solution¹

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Abstract: A nuclear magnetic resonance investigation has been made of the aggregation of the acridine orange cation in aqueous solution. Large downfield dilution shifts were observed for the acridine orange protons in the concentration range 10^{-2} to 5×10^{-4} M. These shifts have been interpreted in terms of aggregation-disaggregation equilibria for the acridine orange. The most favored configuration for the aggregates is one in which the planes of the acridine rings are stacked in a nearly parallel array. In such an association ring-current magnetic anisotropy effects cause high-field shifts for protons in the aggregates. From the differential shift changes it is concluded that protons in positions 1 and 9 are oriented more nearly over the center of adjacent rings than are the other ring protons. For solutions having the same acridine orange concentration but containing added NaCl, an upfield shift occurs for all protons. This shift change is due to an increased stacking induced by a "salting in" effect of the added NaCl. In acid solution all of the acridine orange protons are shifted to low field. This deshielding has been attributed to a combination of protonation and disaggregation of the acridine orange cation.

The optical properties of planar anionic and cationic dye molecules in aqueous solution have been extensively studied for a number of years.²⁻¹¹ Of particular interest have been the changes in optical density and wavelength of the absorption maximum which occur as the dye concentration is increased. For example, the absorption spectrum of the cationic dye acridine orange (AO) shows a decrease in optical density for the main absorption band at 492 m μ and an increase in OD at 464 m μ as the concentration of dye is increased from 10^{-6} to 10^{-4} mole/l.^{4,7} At still higher dye concentrations a further shift to lower wavelength occurs, and at 10^{-3} M the absorption maximum is located at approximately 450 m μ .^{4a} Similar changes have been observed for other dyes.^{2,3,7} These spectral changes have generally been attributed to an aggregation of the dye molecules into dimers, trimers, and higher oligomers as the concentration is increased.⁴ In the case of acridine orange, Zanker^{4a} obtained a quite satisfactory fit of the optical data by postulating a monomer \rightleftharpoons dimer equilibrium in the range 10^{-6} to 10^{-4} M. However, the validity of the dimer equilibrium constants obtained for the AO system and for other cationic dyes has been seriously questioned recently by Lamm and Neville¹⁰ who showed that the dimer spectrum of AO could be fitted equally well by two different dimerization equilibria, one of which involved anion participation. The possibility of interpreting the optical measurements by more than one type of dimer equilibrium raises a further question about the configuration of

the dimer as inferred from the optical spectra. Thus the spectral changes for the case where dimer formation involves anion participation, *i.e.*, to form, in effect, a complex ion of the type $A^+ \cdots A^+ \cdots X^-$, are consistent with a theoretical model in which the dye molecules are arranged parallel to each other,^{12,13} while for the case where anions are not included the changes are more in line with a model in which the planes of the dyes are tilted at a slight angle with respect to each other.¹⁴

In view of the importance of dyes such as acridine orange in a variety of biological phenomena,¹⁵⁻¹⁹ notably those in which the dye is complexed with a biopolymer,²⁰⁻²⁶ a more detailed knowledge of the configuration of the aggregates in solution is desirable. In this study an alternative technique, *i.e.*, high resolution nuclear magnetic resonance, has been employed to establish the most favored orientation of the rings in the aggregate. The nmr method is particularly advantageous in this case, compared to optical techniques, since the parameters involved (chemical shifts and coupling constants) are directly dependent upon the inter- and intramolecular electronic and magnetic environment of the molecules. In particular, it should be possible to establish the most favored orientation of adjacent rings in the aggregate from the chemical shift dependence upon the dye concentration. For example, if the rings are

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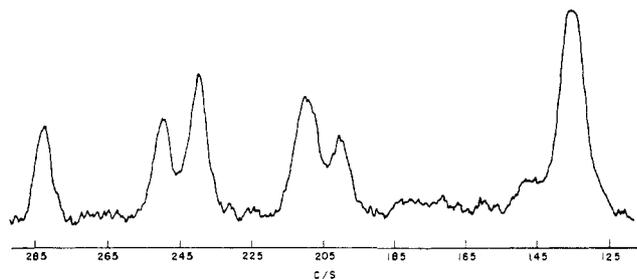


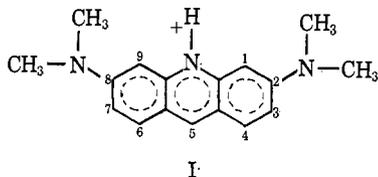
Figure 1. Spectrum of 0.01 *M* AO in D₂O after 120 scans. Shifts are given to low field relative to internal (CH₃)₄NBr.

stacked vertically, then a shift to low field would be observed for the ring protons upon dilution; if the rings are initially arranged in a coplanar configuration, then dilution would produce a high-field shift. Such dilution shifts have been used effectively recently to establish the mode of aggregation of purines,²⁷ nucleosides,²⁸ and nucleotides.²⁹

In the present work the proton spectra of AO have been measured as a function of concentration in aqueous solutions and in acidified and salt solutions. The experimental results confirm that the most favored orientation of the dye molecules in the AO aggregate is one in which the planes of the rings are stacked in a slightly skewed parallel array.

Experimental Section

Materials. Purified acridine orange salt (I) was kindly supplied



by Dr. L. G. Bunville of Argonne National Laboratory. The D₂O was supplied by the U. S. Atomic Energy Commission and was 99.84 atom % in deuterium. The sodium chloride and hydrochloric acid used were both reagent grade. Water was glass-distilled.

Preparation of Solutions. Dilute solutions of AO were made up in a solvent consisting of 90% by volume D₂O and 10% H₂O. The acid solution consisted of 90% D₂O and 10% 7 *N* HCl. The high D₂O concentration was used in order to minimize the effect of the relatively large intensity for the H₂O signal upon the base line during long scans.

Measurement of Spectra. All nmr spectra were recorded using a Varian DA 60 spectrometer equipped with a field-lock accessory and high-sensitivity insert. The probe temperature was 36 ± 1°. Protons in dilute solutions used in this work (1 × 10⁻² to 5 × 10⁻⁴ *M*) are normally unobservable by conventional methods, and a signal-to-noise enhancement was achieved by repetitively scanning the appropriate parts of the spectrum and storing the accumulated data in a Varian C1024 time-averaging computer. The C1024 was triggered from the H₂O peak of the solvent. At the termination of data accumulation spectra were calibrated by audiomodulation of the H₂O signal. A Hewlett-Packard 5253B counter was used to check the audiofrequency. The spectrometer resolution was optimized before recording a spectrum and checked after the final scan. Those spectra for which the resolution was not maintained to a line width of 0.5 ± 0.2 cps for TMS were discarded. Depending upon the AO concentration used, a satisfactory spectrum could be obtained over a wide range of scans (120 to 6700). A minute

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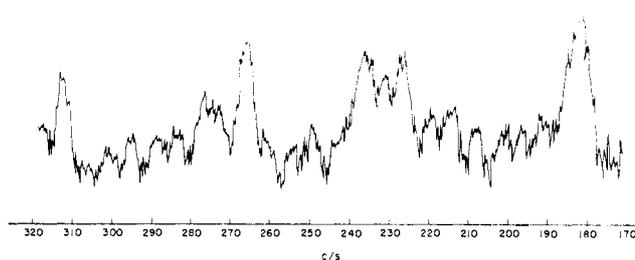


Figure 2. Spectrum of 5 × 10⁻⁴ *M* AO in D₂O after 6700 scans.

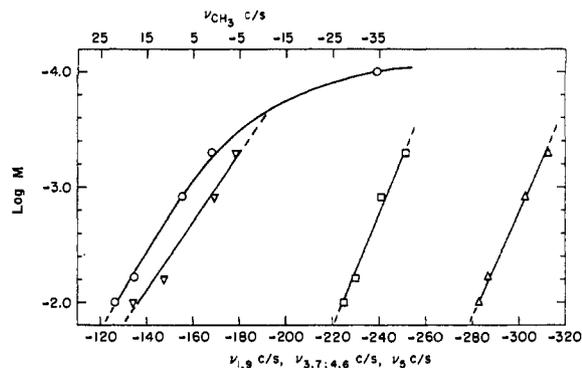


Figure 3. Chemical shifts of AO protons as a function of AO concentration.

trace of (CH₃)₄NBr was added to each solution to serve as an internal reference; this compound was selected since its chemical shift proved to be almost independent of AO concentration in both saline and acidic solutions. The constancy of the shift for (CH₃)₄NBr at different solvent and solute (AO) conditions was checked against trace amounts of methanol and acetone. In no case did the shifts for these signals alter by more than 1 cps, indicating that the reference selected was unaffected by the AO at the concentrations studied.

The chemical shifts are accurate to ±2 cps and the coupling constants to 0.5 cps. The errors in these parameters are larger than is usually the case in high resolution nmr and reflect an uncertainty in line position due to the relatively large (3–5 cps) line widths. These line widths arise from a combination of unresolved long-range couplings and small spectrometer instabilities. Also an additional line-broadening factor was introduced by the use of a relatively fast scan rate (approximately 20 cps) during accumulation of the spectrum. This scan rate was used since it gave a more efficient data collection than a slower scan rate.

Results

The complete nmr spectrum of AO consists, in order of decreasing field, of a high-field resonance due to the methyl protons, a singlet due to the 1,9 protons, an AB quartet due to the 7,6 and 3,4 protons, and a low-field singlet due to the 5 proton. The spectrum of the ring protons of a 0.01 *M* AO solution obtained after 120 scans is shown in Figure 1, while the spectrum for a 5 × 10⁻⁴ *M* solution obtained after 6700 scans (~90 hr) is shown in Figure 2; the methyl signals are located to high field of the internal reference (CH₃)₄NBr in each case and are not shown in the figures. No satisfactory ring proton signals could be observed for AO solutions whose concentration was less than 10⁻⁴ *M* because of spectrometer instabilities incurred over the long time periods required to accumulate the signals.

A summary of the chemical shifts and coupling constants obtained for the concentration range 10⁻²–10⁻⁴ *M* is given in Table I. Both the chemical shifts

and the magnitude of $J_{3,4}$ lie in the expected range. An unresolved long-range coupling is also noted for proton 3 and presumably arises from an interaction with proton 1. A strong concentration dependence is noted for all of the AO protons in aqueous solution. As the concentration of AO is decreased, the protons show progressive shifts to low field. This is illustrated in Figure 3 where the shifts for the different protons are plotted as a function of concentration. The largest dilution shift (observed over the concentration range 10^{-2} to 5×10^{-4} M), amounting to 44.6 cps, is observed for the protons in positions 1 and 9; somewhat smaller shifts are noted for the other protons with $\Delta\nu_5 = 30.5$; $\Delta\nu_{3,7;4,6}$ (shift of the midpoint of the AB spectrum) = 26.4, and $\Delta\nu_{\text{CH}_3} = 18.4$ cps. The shift of the methyl protons to low field increases by an additional 37.9 cps on further dilution of the AO to 10^{-4} M giving a total N-methyl dilution shift of 56.3 cps. No data were obtained for the ring protons for a 10^{-4} M solution, but it is apparent from Figure 3 that an additional shift to low field is very likely for these protons on dilution from 5×10^{-4} to 10^{-4} M. No detectable change in $J_{3,4}$ or $(\nu_3 - \nu_4)$ is noted with dilution.

Table I. Dilution Shifts for AO in Aqueous Solutions^a

Concn, M \times 10^{-2}	ν_{CH_3} ^b	$\nu_{1(9)}$	$\nu_{3,4(6,7)}$ ^c	ν_5	$J_{3,4}$	$\nu_3 - \nu_4$
1.00	21.9	-134.1	-224.9	-282.3	9.7	38.1
0.60	17.8	-147.6	-229.8	-286.6	9.3	37.8
0.12	7.4	-169.2	-240.9	-302.8	8.9	37.3
0.05	3.5	-178.7	-251.3	-312.8	9.3	38.1
0.01	-34.4					

^a 90% D₂O-10% H₂O solvent. ^b Chemical shifts in Tables I to III are in cps relative to internal (CH₃)₄NBr; coupling constants are also in cps. ^c Midpoint of AB spectrum for 3,4 and 6,7 protons.

Salt Solutions. The chemical shifts and coupling constants for several AO solutions containing in addition 0.29 mole/l. of NaCl are summarized in Table II. (No signals were observed for the 1,9 protons because the upfield shift which occurs in salt solution is such that the signal is obscured by the wings and/or side bands of the H₂O signal.) Comparison of Tables I and II shows that all of the AO protons

Table II. Dilution Shifts of AO in Salt Solution^a

Concn, M $\times 10^{-2}$	ν_{CH_3}	$\nu_{1(9)}$	$\nu_{3,4(6,7)}$	ν_5	$J_{3,4}$	$\nu_3 - \nu_4$
1.00	33.8	...	-189.3	-249.4	10.1	41.7
0.60	30.0	...	-206.7	-259.9	9.5	35.7
0.12	22.2	...	-217.8	-269.8	8.7	34.3

^a 90% D₂O-10% H₂O solvent containing 0.29 M NaCl.

are shifted upfield very markedly in the salt solution. For example at 10^{-2} M AO the shift differences ($\nu_{\text{salt}} - \nu_{\text{aq}}$) amount to +11.9 cps for CH₃, +35.6 cps for the midpoint of the AB quartet, and +32.9 cps for proton 5. Dilution of AO in the salt solution leads to a low-field shift for all of the protons. The magnitudes of the dilution shifts (cps), *i.e.*, 11.7 for N(CH₃)₂, 28.5

for the AB protons, and 20.4 for proton 5, are similar to those noted for aqueous solutions over the same concentration range. Small, but detectable, changes are noted for both $(\nu_3 - \nu_4)$ and $J_{3,4}$ in the salt solution.

Acid Solutions. A summary of the chemical shifts and coupling constants for AO at several concentrations in 0.70 N acid solution is given in Table III. All of the protons are shifted to low field in the acid solution as compared with aqueous and NaCl solutions. (The color of the AO solution changes from yellow-orange in H₂O to red-orange in 0.70 N HCl. Similar observations were noted by Zanker.^{4b}) The largest shift ($\nu_{\text{acid}} - \nu_{\text{aq}}$) = -41.3 cps is observed for the protons in the 1 and 9 positions; shift changes for the remaining protons are substantially less, amounting to 14 to 18 cps. As in the case of aqueous and NaCl solutions the AO chemical shifts of the N-methyl and 1, 3, and 4 protons are shifted to low field upon dilution in acid solution. Proton 5, however, is shifted upfield by a small amount, 3.6 cps. The magnitudes of the downfield shifts are somewhat less than those observed in aqueous and salt solutions. Again, the largest dilution shift change is noted for the 1 and 9 protons.

Table III. Dilution Shifts for AO in Acid Solution^a

Concn, M $\times 10^2$	ν_{CH_3}	$\nu_{1(9)}$	$\nu_{3,4(6,7)}$	ν_5	$J_{3,4}$	$\nu_3 - \nu_4$
1.00	13.2	-175.4	-238.4	-296.4	9.3	31.7
0.60	10.9	-185.8	-241.1	-296.5	8.9	31.3
0.12	0	-207.6	-251.0	-292.8	8.1	30.1

^a 90% D₂O-10% 7 N HCl solvent.

Discussion

Relative Shifts. The relative shifts of the AO protons increase in the order $\delta_5 < \delta_{4(6)} < \delta_{3(7)} < \delta_{1(9)} < \delta_{\text{NCH}_3}$, in agreement with the shifts reported by Kokko and Goldstein.³⁰ The relative shieldings of protons in a given molecule are determined mainly by intramolecular effects, although in some cases intermolecular interactions may also have a significant influence. Some of the more important intramolecular effects acting in the AO cation include the following: (a) inductive and resonance interaction of the substituent group, N(CH₃)₂, with the ring; (b) electric field effects due to the N-H⁺ and N(CH₃)₂ groups; (c) neighbor-ring current effects; (d) magnetic anisotropy of the N-H and C-N-CH₃ bonds. From chemical shift calculations which have been carried out on related nitrogen heterocycles,³¹⁻³³ it is likely that for AO the effects of contributions a and c are appreciably greater in magnitude than b and d. Inductive and/or resonance interaction of the dimethylamino group with the ring leads to an increase in electron density at the *ortho* and *para* positions in the ring; little change is expected at the *meta* position. An upfield shift would therefore be predicted for protons in positions 1 and 3 relative to the proton in position 4; the shift of proton

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5 would not change significantly. A quantitative estimate of the substituent shift is not feasible for AO although it can be noted that upfield shifts for protons *ortho* and *para* to amino substituents generally lie in the range 0.70–1.00 ppm, while *meta* protons are shifted upfield by about 0.10 to 0.20 ppm.^{34–36} The effect of neighbor-ring currents can be estimated semiquantitatively using the equations of Pople.^{37,38} If appropriate values of bond distances and bond angles are used, then the following deshielding values (ppm) are calculated for the ring protons: $\delta_{1,9} = -0.68$, $\delta_{3,7} = -0.29$, $\delta_{4,6} = -0.68$, $\delta_5 = -1.08$. The contributions from effects b and d are not expected to amount to more than -0.20 ppm and would act mainly on the 1 (9) and to a lesser extent upon the 3 (7) protons. Accordingly, based upon a simple consideration of contributions a–d, the shieldings are predicted to increase in the order $\delta_5 < \delta_4 < \delta_1 < \delta_3$ which, apart from δ_1 , is in agreement with the observed shifts. The chemical shift for the 1 and 9 protons is at much higher field than expected and cannot be interpreted in terms of any reasonable combination of intramolecular effects. It is likely that the shielding at these protons is due in part to an intermolecular contribution from the ring currents of adjacent AO molecules. Such a shielding is possible if the AO molecules are oriented with the planes of the rings parallel to each other and with the center of adjacent rings somewhat closer to the 1 (and 9) proton(s) than to the other ring protons. In such an arrangement the chemical shifts of the 1 and 9 protons would be expected to change much more markedly with concentration and pH than the other ring and N-methyl protons; a comparison of Tables I–III shows that this is in fact the case.

Dilution Shifts in Aqueous Solutions. Dilution of aromatic solutes in inert nonaromatic solvents generally causes a shift of the ring protons to low field. This dilution shift has been attributed³⁹ to the diminishing effect of the ring currents of disk-shaped aromatic molecules at low concentrations when the solute molecules are further apart on the average. No specific molecular interaction is assumed to be involved. Dilution shifts for aromatic solutes, defined as $(\delta - \delta_\infty)$, where δ is the shift for the pure solute and δ_∞ the shift at infinite dilution, vary over a wide range (0.3 to 2.00 ppm) and depend upon factors such as substituent groups, solvent, and number of aromatic rings.³⁹ For polycyclic compounds the dilution shifts are appreciably greater than for single-ring compounds because of the additional effect of neighboring rings. In cases where weak solute–solute or solute–solvent molecular complexes are formed, an additional effect due to the complex equilibrium is superimposed upon the dilution shift.^{40–42} Considering the dilution shifts of AO, it is apparent that the magnitude of the ring

proton shifts is at least as large in the concentration range studied, 10^{-2} to 10^{-4} M, as has been observed for aromatic hydrocarbons in inert solvents over the entire binary concentration range. It is quite unlikely that the deshielding is due to the same type of non-specific interactions which are assumed to affect the shifts of aromatic solutes in nonaromatic solvents. Nor can the results be interpreted in terms of more specific solute–solvent interactions, *i.e.*, hydrogen bonding between H₂O and the N(CH₃)₂ groups, since shift contributions due to such interactions would not change measurably at the very low solute:solvent ratios employed.

Of the various interactions involving the solute molecules alone, the ones which are possible in the concentration range 10^{-2} – 10^{-4} M are ion-pair dissociation, ion-dipole association, and formation of aggregates (dimers, trimers, and higher oligomers). In the case of the first two types of interaction, the shift change expected at very low solute concentration^{35,43} is at least an order of magnitude smaller than the observed changes.⁴⁴ Moreover, the electric field effects responsible for the shift changes would have little effect upon the shifts of the 3, 4, and 5 protons. Accordingly, the most plausible explanation of the dilution shifts is one which invokes aggregation \rightleftharpoons disaggregation equilibria for the dye cations in solution. As the concentration is decreased the aggregates of AO initially present at higher concentration dissociate into monomers, dimers, and lower oligomers. The low-field shift which accompanies the disaggregation shows clearly that the cations must be stacked in a vertical array in the aggregates at high concentration. This confirms the mode of aggregation indicated by the optical measurements.¹⁰ Vertical stacking is presumably favored because of an interaction between the π -electron clouds of adjacent cations.

Additional information about the relative orientation of adjacent AO molecules in the aggregate can be obtained from the magnitude of the dilution shifts for individual ring protons and N-methyl protons. Two alternative configurations can be considered;⁴⁵ the AO cations are arranged so that (i) the centers of the rings of adjacent molecules are exactly superimposed or (ii) the centers of adjacent rings are displaced laterally (the planes of the rings may also be tilted at a slight angle with respect to each other in this configuration); *cf.* Figure 4. The dilution shifts would vary in a different and characteristic manner for the two configurations. An estimate of the shielding due to the ring currents of adjacent rings can be made from the shielding values calculated by Johnson and Bovey⁴⁷ for the benzene ring.

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(45) Although the lifetime of the aggregates is of the order of molecular reorientation times, *i.e.*, 10^{-11} to 10^{-10} sec,⁴⁶ the most favored configurations on a time-average can be represented by either i or ii.

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Table IV. Estimated Intermolecular Ring Current Contributions in the Acridine Orange Cation Dimer^a

Proton	Adjacent ring	Adjacent neighbor-ring	Total shift ^b
Case i (A) $A = 0$ $B = 3.0 \text{ \AA}$			
1	0.35 ± 0.10	0.15 ± 0.05	0.50 ± 0.15
3	0.35 ± 0.10	-0.05 ± 0.05	0.30 ± 0.10
4	0.35 ± 0.10	0.15 ± 0.05	0.50 ± 0.10
5	0.35 ± 0.10	0.30 ± 0.10	0.65 ± 0.10
N(CH ₃) ₂ ^c	0.05 ± 0.05	0 0	0.05 ± 0.05
Case i (B) $A = 0$ $B = 3.5 \text{ \AA}$			
1	0.30 ± 0.10	0.17 ± 0.05	0.47 ± 0.10
3	0.30 ± 0.10	0	0.30 ± 0.10
4	0.30 ± 0.10	0.17 ± 0.05	0.47 ± 0.10
5	0.30 ± 0.10	0.34 ± 0.10	0.64 ± 0.10
N(CH ₃) ₂	0.10 ± 0.05	0	0.10 ± 0.05
Case ii (A) $A = 0.50 \text{ \AA}$ $B = 3.0 \text{ \AA}$			
1	0.75 ± 0.20	0.20 ± 0.10	0.95 ± 0.20
3	0.35 ± 0.10	0	0.35 ± 0.10
4	0.20 ± 0.10	0.05 ± 0.05	0.25 ± 0.10
5	0.20 ± 0.10	0.05 ± 0.05	0.25 ± 0.10
N(CH ₃) ₂	0.17 ± 0.05	0	0.17 ± 0.05
Case ii (B) $A = 1.50 \text{ \AA}$ $B = 3.0 \text{ \AA}$			
1	1.50 ± 0.30	0.25 ± 0.10	1.75 ± 0.30
3	0.17 ± 0.05	0	0.17 ± 0.05
4	0.10 ± 0.05	0	0.10 ± 0.05
5	0.10 ± 0.05	0	0.10 ± 0.05
N(CH ₃) ₂	0.30 ± 0.10	0.05 ± 0.05	0.35 ± 0.10
Case ii (C) $A = 0.50 \text{ \AA}$ $B = 3.5 \text{ \AA}$			
1	0.60 ± 0.20	0.20 ± 0.10	0.80 ± 0.20
3	0.35 ± 0.10	0	0.35 ± 0.10
4	0.30 ± 0.10	0.10 ± 0.05	0.40 ± 0.10
5	0.30 ± 0.10	0.20 ± 0.10	0.50 ± 0.10
N(CH ₃) ₂	0.20 ± 0.05	0	0.20 ± 0.05
Case ii (D) $A = 1.50 \text{ \AA}$ $B = 3.5 \text{ \AA}$			
1	1.10 ± 0.30	0.30 ± 0.10	1.40 ± 0.30
3	0.20 ± 0.05	0	0.20 ± 0.05
4	0.10 ± 0.05	0	0.10 ± 0.05
5	0.10 ± 0.05	0	0.10 ± 0.05
N(CH ₃) ₂	0.30 ± 0.10	0.05 ± 0.10	0.35 ± 0.10

^a A represents the distance by which adjacent rings are off-center with respect to each other. B represents the interplanar distance.
^b Shifts are in ppm. ^c Distances were measured from the midpoint of the triangle formed by the methyl protons.

A summary of the shifts estimated for the AO dimer, assuming reasonable values of the pertinent intermolecular distances (parameters A and B of Table IV), is given in Table IV. Because of the approximations involved, the magnitudes of the shifts may be in error by an appreciable amount; the relative shifts, however, can be estimated with somewhat more confidence. From consideration of the dimer with configuration i, it is clear from Table IV that although a diamagnetic shift is expected for all of the protons in the dimer the order of increasing shielding does not conform to the experimental results. In particular, the largest dilution shift is predicted for proton 5, and little change is expected for the N-methyl group. Both of these expectations are in disagreement with the observed concentration dependence. Since a change in the distance of closest approach does not alter the relative order of the shifts, the dimer must exist in a configuration other than i. It can therefore be concluded that the dilution shifts are more consistent with an aggrega-

Chem., 16, 43 (1965). This will affect the "absolute" magnitudes of the calculated shielding values but should not materially alter the relative shieldings.

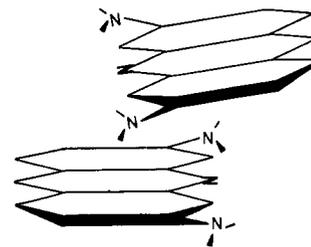


Figure 4. Dimer configuration ii.

tion of the AO cations into vertical stacks with the centers of adjacent rings slightly out of line. From the magnitudes of the shieldings calculated for configuration ii, it is likely that the horizontal displacement between the centers is not greater than 0.5 Å. The dimer configuration deduced from the nmr measurements is therefore in agreement with the structure postulated by the exciton theory of Levinson, Simpson, and Curtis¹⁴ for the case of strong coupling.

Although the stoichiometry and equilibrium constants for solute-solute and solute-solvent interactions have been evaluated in certain favorable cases from the concentration dependence of the chemical shifts of the solute and/or solvent molecules,^{48,49} this is not feasible for the present system because of the likelihood of simultaneous dimer, trimer, and higher oligomer equilibria. According to optical measurements,^{4a} the dimer form of AO predominates in the concentration range 10^{-4} to 10^{-6} M while trimers, tetramers, and oligomers are formed at progressively higher concentrations. Since the limiting infinite dilution shifts, δ_{∞} , could not be measured for the AO protons, a quantitative estimate of the dimer:trimer:oligomer ratio cannot be made. However, from the large dilution shifts observed in the range 10^{-2} to 10^{-4} (particularly for the N-methyl protons which shift by, -0.92 ppm), it is reasonable to conclude that the cations are aggregated at least into trimers at these concentrations. The arrangement of adjacent rings in the trimer would be the same as in the dimer.

Salt Effects. All of the protons in the cation show an upfield shift in 0.29 M salt solution relative to comparable salt-free solutions; cf. Table V. This effect is much more pronounced for the ring protons than for the methyl protons. Addition of salt to solutions of cationic dyes is known to favor aggregation of the dye molecules;⁵⁰ for example, the 492- $m\mu$ peak of AO is shifted to 467 $m\mu$ (dimer peak, $\lambda \sim 470$ $m\mu$) in a solution containing 0.9 M NaCl.⁵⁰ The upfield shifts observed for the AO protons can therefore be explained in terms of an increase in the stacking of the AO molecule in salt solution.⁵¹ The extent of the increase cannot be evaluated quantitatively with the present data; however, from the magnitudes of the shift changes, Tables V and VI, it is likely that aggregation in salt solutions is increased by more than

(48) D. M. Grant, *Ann. Rev. Phys. Chem.*, 15, 73 (1964).

(49) E. Gore and S. S. Danyluk, *J. Phys. Chem.*, 69, 89 (1965).

(50) G. Blauer, *ibid.*, 65, 1457 (1961).

(51) Alternatively the diamagnetic shifts could arise because of an increase in the lifetime of the dimer in salt solution. Since the ring-current effects could act over a longer period, the time-averaged chemical shifts would be shifted upfield (relative to aqueous solutions) at all concentrations.

twofold over that in comparable aqueous solutions.⁵² No change in the mode of stacking in salt solution is indicated by the shift data.

Table V. Chemical Shift Differences,^a $\nu - \nu_{\text{H}_2\text{O}}$, for the AO Cation in Acid and Salt Solutions

Concn, $M \times 10^2$	ν_1	$\nu_{3(7)}$	$\nu_{4(6)}$	ν_5	ν_{CH_3}
		$\nu_{\text{acid}} - \nu_{\text{H}_2\text{O}}$			
1.00	-41.3	-16.7	-10.3	-14.1	-8.7
0.60	-38.2	-14.6	-8.0	-9.9	-7.1
0.12	-38.4	-13.7	-6.5	-10.0	-7.4
		$\nu_{\text{salt}} - \nu_{\text{H}_2\text{O}}$			
1.00	...	37.4	33.8	32.9	11.9
0.60	...	22.0	24.2	26.7	12.2
0.12	...	21.7	24.5	33.0	14.8

^a Shift differences are in cps.

Table VI. Dilution Shifts over the Concentration Range 1.0×10^{-2} to $1.2 \times 10^{-3} M^a$

Solution	ν_1	$\nu_{3(7)}$	$\nu_{4(6)}$	ν_5	ν_{CH_3}
H ₂ O	-35.1	-16.4	-15.6	-20.5	-14.5
Acid	-32.2	-13.4	-11.8	+3.6	-13.2
Salt	...	-32.1	-24.9	-20.4	-11.6

^a Dilution shifts are in cps to low field with decreasing concentration.

The slight decrease ($\Delta\nu \sim 5$ cps) in the relative shifts of ring and N-methyl protons and the more pronounced dilution shift of the 3 and 7 protons in salt solution (Table VI) are probably due to the effect of ionic association between the large excess of Cl⁻ ions and the N-H⁺ groups of the aggregated cations.

Effects of Acid. The effect of pH upon the optical spectrum of AO has been studied by Zanker.⁴ At pH's lower than 1.5, the optical density of the monomer form of the cation⁵³ (maximum at 494 m μ) is decreased very sharply, and a new band appears at approximately 525 m μ . From the OD-concentration dependence of the new band, Zanker concluded that it was due

(52) Addition of the salt is not expected to change the relative shift by more than 3-5 cps.

(53) The optical measurements were obtained for a $10^{-5} M$ AO solution.

to a single species, *i.e.*, diprotonated acridine orange. In the present work, the low-field shifts which occur in 0.70 *N* HCl solution, relative to comparable aqueous solutions, and the dilution shifts in acid solution (Tables III and VI) can be attributed to a combination of protonation and disaggregation of the AO cation. Protonation is known to shift the protons in aromatic compounds⁵⁴⁻⁵⁶ and in N-heterocyclic compounds³¹⁻³³ to lower field by amounts ranging up to 2 ppm. In phenazine,³³ for example, protonation of the 9 and 10 ring nitrogen atoms results in shifts of -19.6 and -18.4 cps for protons in the 1 and 2 positions, respectively. Protonation of the amino nitrogen in aromatic amines and substituted anilines has also been shown to deshield the *ortho* and, to a lesser extent, *meta* ring protons.³⁵ For the AO cation, the most likely sites for proton addition are the two -N(CH₃)₂ groups to form doubly and triply charged cations (the latter would only be expected to form in 100% acid media). By analogy with the results for substituted N,N-dimethylanilines,³⁵ protonation of the AO would produce the largest deshielding at the 1 and 3 positions and lesser changes at other ring protons³⁷ as is observed (Table VI). The presence of an additional positive charge would also decrease the stacking tendency of the AO, thereby providing a further deshielding of all the protons. The relative contributions from disaggregation and protonation cannot be ascertained quantitatively from the present data. However, the smaller magnitudes of the dilution shifts in acid solution, compared with aqueous and salt solutions (Table VI), indicate that a much larger proportion of AO is disaggregated in acid solution even at $10^{-2} M$. The small shift to higher field with dilution, noted for proton 5 (Table VI), also suggests the possibility that the protonated AO cations aggregate in a slightly different manner in acid solution. However, the wide variety of interactions possible in these solutions complicates the interpretation of dilution shifts in terms of specific dimer configurations.

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(55) S. S. Danyluk and W. G. Schneider, *Can. J. Chem.*, **40**, 1777 (1962).

(56) C. McLean and E. L. Mackor, *Mol. Phys.*, **4**, 241 (1961).

(57) The decrease in ($\nu_3 - \nu_1$) of ~ 7 cps in acid solution, compared with aqueous solution, is an additional indication of protonation of the cation.