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The Effect of Concentrated Salt Solutions on a Merocyanine Dye, a Vinylogous Amide¹

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Abstract: The spectral changes which occur in aqueous solutions of the merocyanine dye 1-methyl-4-[(oxocyclohexadienylidene)ethylidene]-1,4-dihydropyridine (MOED) in the presence of 13 salts exhibit isosbestic points. The increases and decreases in absorbance at the wavelengths at which maximum changes of absorption take place are linearly related to each other. This suggests that the spectral changes of this vinylogous amide are caused by the appearance of a new absorbing species in the presence of salt, rather than by a continuous, nonspecific solvent effect. The spectral changes show a leveling off with increasing salt concentration and, for most of the salts examined, follow closely the behavior expected for the formation of a 1:1 complex between the dye and a component of the salt. Equilibrium constants near $1.0 M^{-1}$ were calculated for a series of univalent salts of widely varying character. Complex formation can account for the salting in observed with sodium and potassium chloride and contributes significantly to the observed effects of other salts on the solubility of the dye. However, there remain very large salting in and salting out effects of some salts, such as sodium perchlorate, potassium iodide, sodium fluoride, and sodium sulfate, which are not accounted for by complex formation.

Denaturation and other changes in the physical state of proteins in the presence of concentrated salt solutions have been studied since the time of Hofmeister, but the detailed mechanism by which salts bring about these changes in state has not been established. It has been shown that many of these effects are correlated with the effects of salt solutions on the free energy of a model peptide, acetyltetraglycine ethyl ester (ATGEE).³ This suggests that the degree to which amide (peptide) groups of the protein are exposed to the solvent and, consequently, the physical state of the protein, may be in large part accounted for by the stability or free energy of these groups in the presence of salt, but the detailed mechanism of the interaction between salt solutions and amides has also not been

established. The proposed mechanisms for these interactions may be roughly divided into three classes, which are by no means mutually exclusive: (1) salting out of organic groups and molecules, for which a number of mechanisms including the "internal pressure" theory have been proposed;^{4,5} (2) a more or less direct interaction between ions and amides, which would increase the solubility and stability and decrease the free energy and activity coefficient of amide groups exposed to the salt;^{3,6} and (3) indirect effects on the solvent "structure."⁷ There is no simple correlation between the effects of salts on water "structure" and on ATGEE, and it was suggested previously on the basis of

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(2) Postdoctoral Fellow of the National Cancer Institute of the National Institutes of Health, Fellowship No. 6-F2-CA-17846-02, 1965-1967.

(3) D. R. Robinson and W. P. Jencks, *J. Am. Chem. Soc.*, **87**, 2470 (1965).

(4) (a) W. F. McDevit and F. A. Long, *ibid.*, **74**, 1773 (1952); (b) F. A. Long and W. F. McDevit, *Chem. Rev.*, **51**, 119 (1952).

(5) N. C. Deno and C. H. Spink, *J. Phys. Chem.*, **67**, 1347 (1963).

(6) A. R. Docking and E. Heymann, *ibid.*, **43**, 513 (1939); K. H. Meyer and O. Klemm, *Helv. Chim. Acta*, **23**, 25 (1940); J. Bello, H. C. A. Riese, and J. R. Vinograd, *J. Phys. Chem.*, **60**, 1299 (1956); L. Mandelkern and W. E. Stewart, *Biochemistry*, **3**, 1135 (1964); J. Kurtz and W. F. Harrington, *J. Mol. Biol.*, **17**, 440 (1966); A. Ciferri, R. Garmon, and D. Puett, *Biopolymers*, **5**, 439 (1967), and references therein.

(7) P. H. von Hippel and K.-Y. Wong, *Biochemistry*, **1**, 664 (1962); *Science*, **145**, 577 (1964).

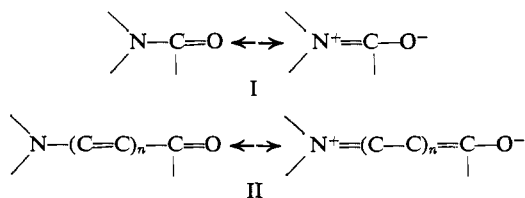
indirect evidence that the effects on ATGEE are caused by a combination of salting out and a direct interaction between salts and amide dipoles.⁸ Schrier and Schrier have extended this view and suggested that the effects of salts on amides may be treated quantitatively by a summation of salting out and salting in effects on the different parts of a given amide.⁸

It should be emphasized that the detailed nature of the interactions of amides with concentrated salt solutions is of secondary importance compared to the fact that such interactions exist as demonstrated, for example, by the greatly increased solubility of ATGEE in the presence of certain salts. Favorable interactions of this kind tend to change the physical state of proteins, decrease the free energy of amide groups, and lead to a clustering of ions around amide groups, regardless of their mechanism. Since salts decrease the activity coefficient and increase the concentration of peptide groups in their vicinity, it follows that peptide groups will decrease the activity coefficient and increase the concentration of ions in *their* vicinity, as described by eq 1, in which n and f are the amounts and activity

$$\delta \ln f_1 / \delta n_2 = \delta \ln f_2 / \delta n_1 \quad (1)$$

coefficients, respectively, of two solutes in an aqueous solution. Because of this reciprocal stabilization, ions which stabilize amides in water will tend to cluster around amide groups in peptides and proteins whether or not there is a specific site binding of the ion to the amide. Thus, the finding that denaturing salts are associated with proteins cannot be taken as evidence for a direct site binding.⁶ It is difficult to make a firm distinction between interactions with salts which result from changes in the "ionic atmosphere" of this kind and a direct site binding, but an experimental distinction has been made in a few instances, such as the binding of cations to polyphosphates.⁹

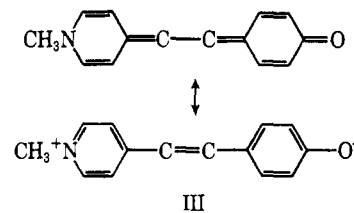
The experiments reported here were initiated in an attempt to obtain more direct evidence bearing on the possible importance of mechanisms 1 and 2 in the interactions of salt solutions with amides and proteins. A number of preliminary experiments with simple amides were inconclusive, because of the difficulty in measuring physical properties of simple amides that show changes in the presence of salts of sufficient magnitude to distinguish between an interaction of the amide with salt and with solvent water. In order to take advantage of their readily accessible electronic spectra, an examination was carried out of vinylogous amides, II, in which the carbonyl and amino groups of the amide, I, are separated by a conjugated system of



double bonds, through which the normal amide resonance can occur. The compound selected for detailed investigation is the merocyanine dye¹⁰ III

(8) E. E. Schrier and E. B. Schrier, *J. Phys. Chem.*, **71**, 1851 (1967).

(9) U. P. Strauss and Y. P. Leung, *J. Am. Chem. Soc.*, **87**, 1476 (1965); M. M. Crutchfield and R. R. Irani, *ibid.*, **87**, 2815 (1965).



which, although hardly identical with the usual peptide or amide, is a vinylogous amide with the characteristic partial positive charge on the nitrogen atom and partial negative charge on the oxygen atom. These partial charges give merocyanine dyes some of the highest dipole moments known for organic molecules which do not contain completely charged groups.¹¹ The results are of interest with respect to the general problem of the salting in and out of polar organic molecules, in addition to their significance with respect to solvent interactions with peptides and other amides.

Experimental Section

Materials. Organic reagents, except for trichloroacetic acid, were distilled or recrystallized before use. *p*-Hydroxybenzaldehyde was purified by sublimation. Inorganic salts and trichloroacetic acid were reagent grade except for lithium bromide which was Fisher Purified grade, a mixture of hydrates. Its concentration was determined by titration with silver nitrate. Sodium perchlorate and sodium trichloroacetate were prepared by neutralization of the corresponding acids with sodium hydroxide. Tetramethylammonium salts were recrystallized. Trichloroacetic acid was shown to be free of chloride ions.

The preparation of 1-methyl-4-[(4-oxocyclohexadienylidene)ethylidene]-1,4-dihydropyridine (MOED) from 4'-hydroxystilbazole methiodide¹² was carried out by a slight modification of published procedures.^{10,13} A suspension of the salt in cold water was neutralized with 1 equiv of 1 *M* sodium hydroxide and the dye was recrystallized several times from water. The absorption maxima are at 443 and 263 $m\mu$, and there are absorption minima at 330 and 239 $m\mu$ in water. The pure product gave the following absorbance ratios in 0.01 *M* sodium hydroxide: $A_{263}/A_{443} = 0.307$; $A_{239}/A_{443} = 0.088$. A sample dried under vacuum in the dark at 25° for 1 week gave the following analysis: Calcd for $\text{C}_{14}\text{H}_{13}\text{NO} \cdot 0.5\text{H}_2\text{O}$: C, 76.30; H, 6.41; N, 6.36. Found: C, 76.26; H, 6.72; N, 6.48. The absorption maxima in water, ethanol, and 20% aqueous pyridine agree with those reported by Brooker.¹⁰ The dye melted with decomposition at about 220° (lit.^{10,13} 195 and 208°) and exhibited a molar extinction coefficient in water¹⁴ at 443 $m\mu$ of 3.72×10^4 . Dye preparations in the crystalline state or in solution were found to undergo decomposition over a period of weeks, as indicated by an increase in the ratios of absorbancy at 239 and at 263 $m\mu$ to that at 443 $m\mu$; however, there was no significant decomposition over the period of time in which experiments with a given preparation were carried out. The dye used for experiments in aqueous solution consisted of well-formed hexagonal plates which had been crystallized several times from water and equilibrated with atmospheric moisture. The extinction coefficients were 1.50 or 1.40 $\text{cm}^2 \text{mg}^{-1}$, corresponding to the dihydrate and trihydrate, respectively. Analyses of two preparations corresponded approximately to those expected for the dihydrate. However, the solubility in water was the same (0.0063 *M*), by spectrophotometric measurement, for all preparations, indicating that the solid phase in the presence of water was constant.

The most rigorous test for purity of the dye was a measurement of the absorbance of an aqueous solution equilibrated with increasing amounts of added solid dye. A linear increase of ab-

(10) L. G. S. Brooker, G. H. Keyes, and D. W. Heseltine, *ibid.*, **73**, 5350 (1951).

(11) L. M. Kushner and C. P. Smyth, *ibid.*, **71**, 1401 (1949).

(12) A. P. Phillips, *J. Org. Chem.*, **14**, 302 (1949).

(13) S. Hünig and O. Rosenthal, *Ann. Chem.*, **592**, 161 (1954).

(14) Values of 2.7 and 5.5×10^4 have been reported previously.¹⁰ Dr. D. W. Heseltine has kindly informed us that the latter value represents a clerical error and that a thoroughly dried sample which analyzed as the hemihydrate gave an extinction coefficient of 3.76×10^4 at 443 $m\mu$, in good agreement with the value reported here.

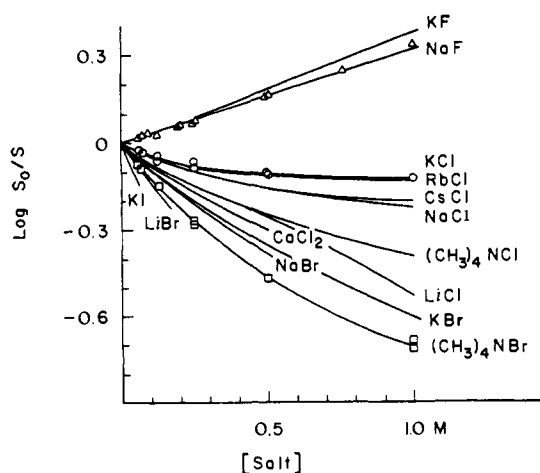


Figure 1. Effects of simple halides on the solubility of MOED in water at 24.9°.

sorbance was observed up to a sharp inflection at the saturating concentration of 0.0063 *M*. The increase in absorbance with higher dye concentrations corresponds to a maximum concentration of impurity of 0.03% for material absorbing at 443 $m\mu$ and 0.2% for material absorbing at 263 $m\mu$, assuming equal extinction coefficients of dye and impurity.

Solubility Determinations. The solubility of MOED was determined from the absorbance of saturated solutions at 443 $m\mu$. Solutions were equilibrated for at least 24 hr with solid dye in 1-ml ground-glass-stoppered tubes which were sealed with melted paraffin and continuously rotated end over end in a water bath at $24.9 \pm 0.1^\circ$. All solutions contained 0.01 *M* sodium hydroxide to ensure that the dye was completely in the uncharged form. After equilibration the tubes were allowed to stand upright in the water bath until solid material had settled and a sample of the supernatant solution was removed with a Pasteur pipet containing a plug of glass wool in the stem. After the sample had been aspirated through the plug into the body of the pipet, the plug and neck of the pipet were broken off and discarded and the clear supernatant solution was emptied into a test tube and diluted in 0.01 *M* sodium hydroxide before measurement of its absorbance. A number of spot checks with solutions which were equilibrated for 48 hr or which had been supersaturated with dye by previous incubation at a higher temperature gave results in agreement with those from the 24-hr experiments and showed that equilibrium was reached in this time.

Spectral Measurements. The effect of salt solutions on the spectrum of MOED was generally determined by difference spectroscopy of dye in a salt solution compared to the same concentration of dye in water. Spectra were obtained with a Cary 14 spectrophotometer with a 10X expanded scale slide wire and 1-cm cells held in jackets through which water at $22.3 \pm 0.2^\circ$ was circulated. In order to obtain satisfactory reproducibility of dye concentration (within 0.25%), a single 4- or 5-ml volumetric pipet and 25-ml volumetric flask were used for all of the dye solutions for a given experiment. All preparations of dye were examined in the presence of 0.01 *M* sodium hydroxide, and salt concentrations were generally in the range of 0–1 *M*. In most experiments difference spectra were measured in the range 390–500 $m\mu$; in some experiments measurements were confined to the region of the isosbestic point and the maxima and minima of the difference spectra at or near 477 and 403 $m\mu$. Difference absorbancy measurements were found to be reliable up to a base-line absorbance of at least 2.0, but most experiments were carried out with preparations exhibiting a maximal absorbance of 0.65–0.95 and correspondingly smaller absorbancies at the positions of the maxima and minima in the difference spectra. The maximum slit widths were 0.15 to 0.2 mm in most experiments; a slit width of 0.7 mm was reached in a few experiments at high dye concentrations with a 1-cm light path.

The apparent association constants and extinction coefficients of the dye-salt complexes were obtained from plots of $1/\Delta A$ against $1/[\text{salt}]$ and plots of $\Delta A/[\text{salt}]$ against ΔA , in which ΔA is the change in absorbancy, generally at 403 and 477 $m\mu$, caused by the addition of salt and measured by the method described above. The reported association constants represent the average of the abscissa intercepts of the double reciprocal plots at the two wavelengths. With

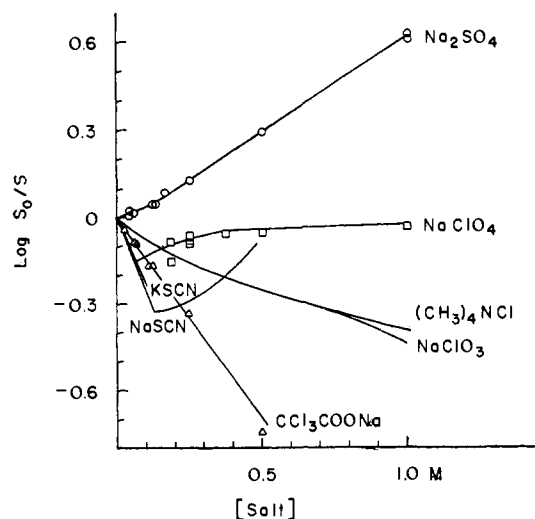


Figure 2. Effects of other salts on the solubility of MOED in water at 24.9°.

the exception of the plots based on dye concentration for divalent salts and in one experiment with lithium chloride, an experiment was rejected if a value agreeing with this association constant to within 0.15 M^{-1} was not obtained from the slope of plots of $\Delta A/[\text{salt}]$ against ΔA . The extinction coefficient of the dye-salt complex was obtained from the double reciprocal plots by dividing the reciprocal of the ordinate intercept by the dye concentration. The association constants based on salt activities¹⁵ were also calculated from double reciprocal plots.

Results

Solubility. The effects of a series of simple halides and of some more complex salts on the solubility of MOED are shown in Figures 1 and 2, respectively. The experimental points are shown for a few representative curves to illustrate the precision of the data. Salt-ing-out constants, k_s , were estimated from the initial slopes of these plots and are summarized in Table I.

Table I. Salting Out Constants for MOED at 24.9°

Salt	k_s^a	$k_s'^b$
1. NaClO ₄	-2.45	-2.07
2. NaSCN	-2.44	
3. KSCN	-2.36	
4. KI	-2.15	-1.53
5. CCl ₃ COO ⁻	-1.32	
6. (CH ₃) ₄ NBr	-1.30	
7. LiBr	-1.21	-0.80
8. KBr	-0.92	
9. NaBr	-0.90	-0.30
10. (CH ₃) ₄ NCl	-0.87	
11. NaClO ₃	-0.83	-0.02
12. CaCl ₂	-0.75	-0.16
13. LiCl	-0.61	
14. CsCl	-0.56	
15. RbCl	-0.49	
16. KCl	-0.45	+0.26
17. NaCl	-0.42	+0.12
18. KF	+0.30	
19. NaF	+0.30	+0.75
20. Na ₂ SO ₄	+0.78	+1.70

^a $\log S_0/S = k_s N$, where S_0 is the solubility of dye in water, S is the solubility in salt solution, and N is the concentration of salt in equivalents per liter. ^b Corrected for complex formation (Table II) according to eq 5.

(15) R. A. Robinson and R. H. Stokes, "Electrolyte Solutions," Academic Press, New York, N. Y., 1955, p 176. Molalities were converted to molarities by the use of tables of densities of aqueous salt solutions ("International Critical Tables," Vol. III, McGraw-Hill Book Co., Inc., New York, N. Y., 1929, p 79).

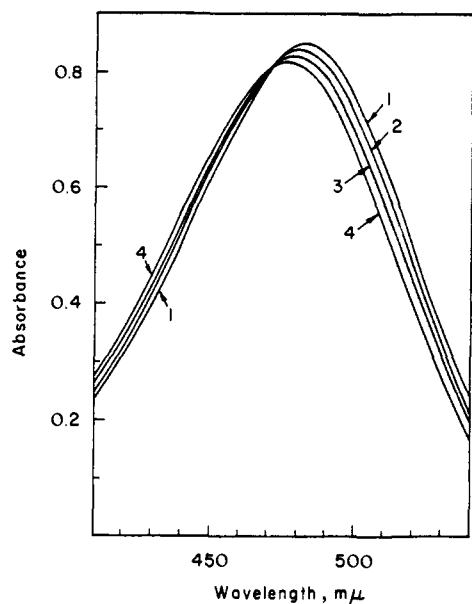


Figure 3. Effect of potassium iodide on the spectrum of MOED in methanol containing 0.01 *M* sodium hydroxide: (1) no added salt; (2) 0.05 *M*; (3) 0.15 *M*; (4) 0.48 *M* potassium iodide.

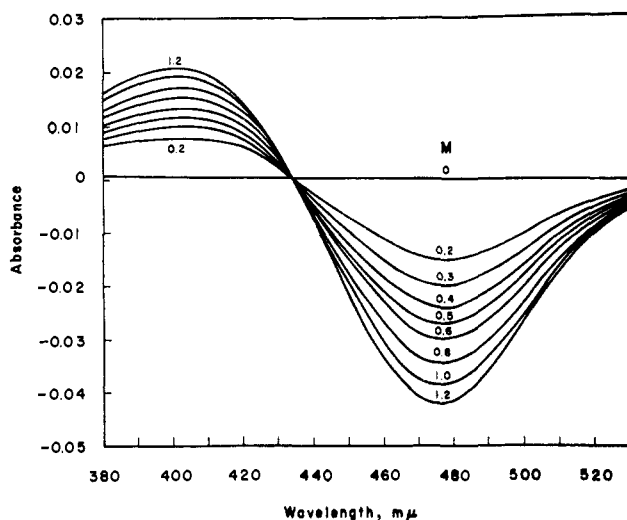


Figure 4. Difference spectra of 2.2×10^{-5} *M* MOED in water in the presence of sodium chlorate at the indicated concentrations.

These constants are not of high precision, because of the nonlinearity of many of the plots, but they do serve to indicate the order and approximate magnitude of the observed salt effects. The majority of salts cause salting in of MOED and exhibit large negative values of k_s ; for example, the solubility is doubled by 0.125 *M* sodium thiocyanate.

For a number of salts which cause a large increase in dye solubility at low salt concentrations, including KI, LiBr, NaSCN, and NaClO₄, there is a range of salt concentration in which the crystalline form and color of the solid phase changes. Solubility measurements were made at salt concentrations above this for NaSCN and NaClO₄ (Figure 2); the phase change corresponds to a break in the solubility curve and in more concentrated salt solutions the dye solubility decreases.

In one experiment, after a sample of crystalline dye had dissolved completely in 0.25 *M* potassium iodide, a new, red, solid phase precipitated from solution. These

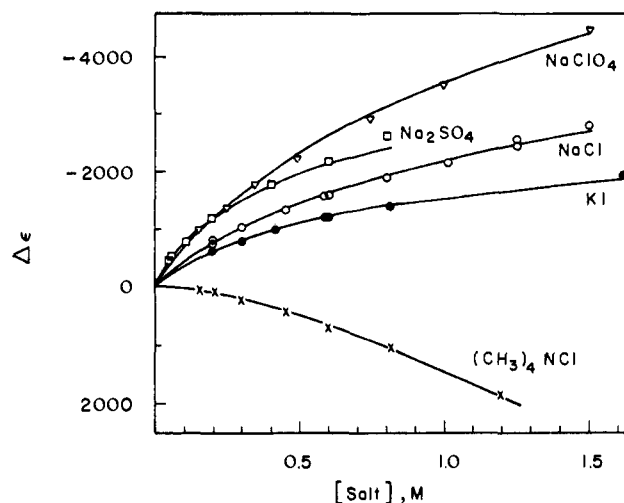
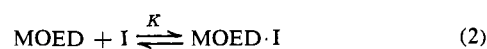


Figure 5. Effect of salts on the molar extinction coefficient of MOED at 477 $m\mu$ (potassium iodide at 474 $m\mu$ and tetramethylammonium chloride at 475 $m\mu$). The solid lines for the inorganic salts were calculated from the constants based on salt activity given in Table II.

results indicate that MOED forms solid complexes with certain salts and that these complexes determine the solubility behavior of the dye at high salt concentrations.

Effect of Salts on the Spectrum of MOED. The visible absorption maximum of MOED in methanol shows a shift to lower wavelengths in the presence of increasing concentrations of potassium iodide (Figure 3). Similar results were obtained with lithium chloride in ethanol. The important points regarding these spectral shifts are, first, that they occur with an isobestic point and, second, that the spectral changes exhibit a tendency toward a leveling off or saturation with increasing salt concentration. Although quantitative experiments were not attempted with methanol solutions, the data shown in Figure 3 could be explained by the binding of an ion to the dye with an association constant of approximately $8 M^{-1}$.

In water the observed spectral changes are smaller, but of more general significance. The effects of salts on the spectra of aqueous solutions of MOED are seen most clearly in difference spectra of the dye in the presence and absence of salt. A typical experiment in the presence of sodium chlorate at concentrations up to 1.2 *M* is shown in Figure 4. There is an isobestic point at 435 $m\mu$ and the maxima and minima in the difference spectrum are at approximately 403 and 477 $m\mu$, respectively. The effect of increasing concentrations of a series of salts on the absorbance change at the minimum of the difference spectrum is shown in Figure 5. All of the salts, with the exception of tetramethylammonium salts, exhibit saturation behavior with respect to the absorbance change with increasing salt concentration. On the assumption that this saturation behavior represents complex formation between the dye and a component of the salt, I (eq 2),



double reciprocal plots were made of the changes in absorbance at the maxima and minima of the difference spectra against salt concentration. Examples of such plots, for sodium chlorate, sodium bromide, and sodium

Table II. Apparent Association Constants and Extinction Coefficients for MOED·Salt Complexes at $22.3 \pm 0.2^\circ$

Salt	Dye concn, $M \times 10^6$	Based on salt concentrations			Based on salt activities			Isosbestic point, $\lambda, m\mu$
		K_{assoc}, M^{-1}	$\Delta\epsilon \times 10^{-3} a$		K_{assoc}, M^{-1}	$\Delta\epsilon \times 10^{-3} a$		
			477 $m\mu$	403 $m\mu$		477 $m\mu$	403 $m\mu$	
NaClO ₄	2.9	1.0	7.5	3.8	1.1	8.3	4.1	437
NaClO ₃	2.2	1.6	2.8	1.3	1.8	3.3	1.6	435
NaI	2.5	1.3	3.0 ^c		1.8	2.9 ^c		None
KI ^b	2.9	1.4 ^c	2.7 ^c		1.8 ^c	2.8 ^c		None
LiBr	2.0	1.1	4.8	2.2	1.4	5.0	2.1	434
NaBr	2.9	1.5	3.2	1.4	1.5	3.8	1.6	434
	6.0	1.5	3.2	1.3	1.7	3.5	1.5	434
	2.4 ^d	1.4	3.3	1.4	1.6	3.8	1.8	435
LiCl	2.8	0.9	5.3	2.7	1.2	5.6	2.8	437
	3.1	1.1	5.0	2.7	1.1	5.3	2.9	437
	30.6 ^e	0.9	5.0	2.6	1.0	5.7	2.8	437
NaCl	1.8	1.1	3.8	1.9	1.2	4.8	2.3	436
	4.0	1.1	3.7	1.8	1.3	4.3	2.1	436
	4.1	1.2	3.3	1.7	1.2	4.6	2.3	436
KCl	2.4	1.5	3.2	1.4	1.8	3.6	1.7	438
	2.9	1.5	3.5	1.7	1.6	3.9	1.9	438
CsCl	2.2	1.1	5.0	2.2	1.0	7.4	3.1	436
	2.5	1.1	5.0	2.5	0.9	6.8	3.4	438
NaF	3.0	1.3	2.4	1.3	1.2	3.5	1.6	438
CaCl ₂	2.5	(2.8)	5.7	3.0	4.4	6.4	3.4	436
Na ₂ SO ₄	2.6	(4.0)	2.4	1.5	2.2	9.0	4.8	437

^a Maximum change in molar absorptivity at the indicated wavelength; the values at 477 $m\mu$ are negative. The molar extinction coefficients of the dye in water at 477 and 403 $m\mu$ are 1.50×10^4 and $1.40 \times 10^4 M^{-1} \text{cm}^{-1}$, respectively. ^b Stock solutions of 2 to 4 M potassium iodide containing 0.025 M sodium thiosulfate. ^c From measurements at 474 $m\mu$ only. ^d At $29.3 \pm 0.1^\circ$. ^e The light path for this experiment was 1 mm.

perchlorate, are shown in Figures 6 and 7. The plots are linear at low salt concentrations, but frequently show negative deviations at higher salt concentrations near 1 M . Apparent association constants for a series

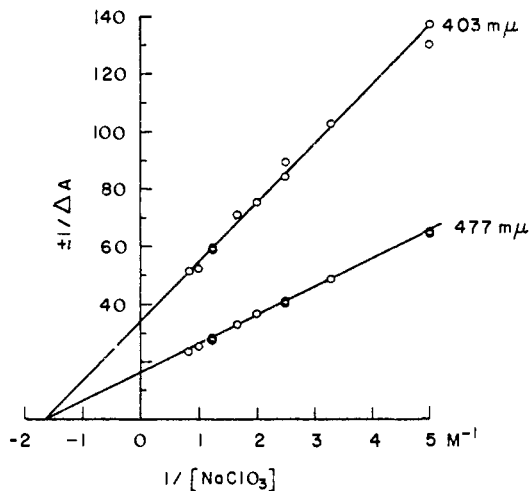


Figure 6. Double reciprocal plots of the absorbance changes for MOED in the presence of sodium chlorate. Note that ΔA values are negative at 477 $m\mu$ and positive at 403 $m\mu$. The mean association constant derived from these plots is $1.6 M^{-1}$.

of salts, K , obtained from the abscissa intercepts, and maximal absorption coefficient changes, $\Delta\epsilon$, obtained from the ordinate intercepts, are summarized in Table II. Very similar results were obtained with plots of $\Delta A/[\text{salt}]$ against ΔA . These constants are not of high precision (there are both theoretical and experimental difficulties in determining equilibrium constants from spectral data of this kind¹⁶ and the association con-

(16) P. J. Trotter and M. W. Hanna, *J. Am. Chem. Soc.*, **88**, 3724 (1966), and references therein.

stants are not large), but the data are adequate to indicate that a stoichiometric interaction takes place and that the equilibrium constants are not markedly different for salts of different monovalent ions. An isosbestic

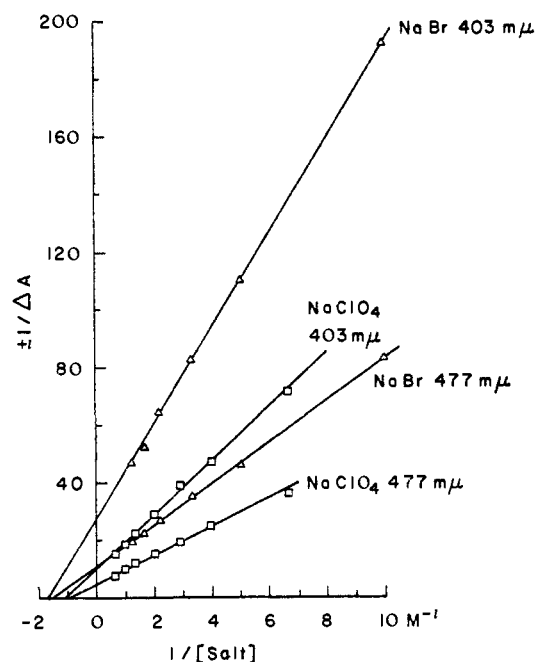


Figure 7. Double reciprocal plots of the absorbance changes for MOED in the presence of sodium bromide and sodium perchlorate.

point and saturation behavior with respect to the absorbance changes were found with all of the salts examined except iodides and tetramethylammonium salts. Sodium and potassium iodide do not give a sharp isosbestic point, but do show saturation behavior

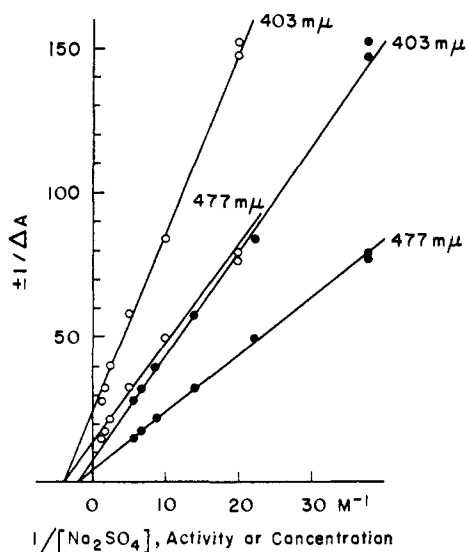


Figure 8. Double reciprocal plots of the absorbance changes for MOED in the presence of sodium sulfate, based on concentration (○) and activity (●) of the salt.

with respect to the change in absorbance at 474 $m\mu$ from which an apparent association constant could be calculated (potassium iodide does give an isosbestic point in methanol (Figure 3)). Tetramethylammonium salts show an isosbestic point, but do not exhibit saturation behavior (Figure 5) and do not give linear reciprocal plots.

The curvature which is frequently observed in double reciprocal plots and in plots of $\Delta A/[\text{salt}]$ against ΔA at high salt concentrations is generally reduced or eliminated if the plots are made as a function of salt activity rather than salt concentration. An extreme case of this behavior is seen in the case of sodium sulfate. Double reciprocal plots for this salt based on salt concentration are so curved as to be almost worthless for quantitative interpretation, but the corresponding plots based on mean ionic activity are essentially linear (Figure 8). The apparent equilibrium constants and maximal changes in absorbance based on salt activities are also summarized in Table II. The solid lines in Figure 5 for potassium iodide, sodium chloride, sodium sulfate, and sodium perchlorate were calculated from these values and show surprisingly good agreement with the observed changes in absorbance. The constants based on salt activities are somewhat more reliable than those based on salt concentration, but do not show a large or systematic difference compared to the latter results, except in the case of salts containing divalent ions.

In the presence of tetramethylammonium salts and in the presence of other salts at ionic strength greater than 1.0, the absorbance changes generally show deviations from the behavior expected for a simple association equilibrium. Under these conditions there is no longer an isosbestic point and there is frequently a progressive shift in the absorption maximum, as summarized in Table III. These shifts of the absorption maximum are similar to those observed by Gordon in the presence of a series of salts, which were ascribed to a solvent effect.¹⁷

(17) J. E. Gordon, *J. Phys. Chem.*, **70**, 2413 (1966).

Table III. Absorption Maximum of MOED in Concentrated Aqueous Salt Solutions^a

Salt	Concn, M	λ_{max} , $m\mu$	Salt	Concn, M	λ_{max} , $m\mu$
None		443	KF	4	439
NaClO ₄	1	439	KBr	4	440
	2	437	NaI	4	440
	4	433	KI	4	442
LiBr	1	442	CH ₃ COONa	2.5	444
	2	440	MgCl ₂	0.8	438
	4	437	BaCl ₂	0.8	439
	8	432	CaCl ₂	0.8	439
LiCl	11.4	424	(CH ₃) ₄ NCl	1.2	447
NaBr	4	436	(CH ₃) ₄ NBr	1.2	448
NaCl	4	438			
	4	438			

^a Each solution contained 0.01 M sodium hydroxide.

MOED follows Beer's law up to a concentration of $3 \times 10^{-4} M$ in 0.01 M sodium hydroxide. At higher concentrations it shows changes in absorption maximum and extinction coefficient suggestive of self-association. Spectrophotometric experiments were generally carried out with dye concentrations in the range of $2-4 \times 10^{-5} M$. In addition the dye was shown to follow Beer's law up to concentrations of $7-11 \times 10^{-5} M$ at 480 $m\mu$ in the presence of approximately 1.5 M sodium chloride, potassium iodide, sodium thiocyanate, lithium bromide, tetramethylammonium chloride, 0.7 M sodium fluoride, and 0.5 M sodium sulfate. Experiments with lithium chloride over a tenfold range of dye concentration gave very similar apparent association constants and changes in extinction coefficient at both concentrations (Table II). It is concluded that self-association does not perturb the spectrophotometric experiments carried out at the lower dye concentrations. Experiments with $2.5 \times 10^{-3} M$ dye in the presence of sodium bromide and potassium chloride at concentrations up to 1.5 M , using a cell with 0.1-mm light path, revealed spectral changes, including an isosbestic point, similar to those observed with the more dilute dye solutions. However, quantitative experiments were not carried out with these concentrated dye solutions.

Titration. The pK_a of protonated MOED was found to be 8.37 by titration under nitrogen of a $3 \times 10^{-3} M$ solution of the base with hydrochloric acid. The addition of sodium hydroxide to a more concentrated solution of the protonated dye gave a red precipitate at pH 8.3, presumably an insoluble complex of the dye and its hydrochloride. Upon the further addition of base the precipitate dissolved and in alkaline solution the characteristic dark red hexagonal crystals of neutral dye precipitated.

Discussion

Interpretation of Spectral Changes. The spectral shifts of MOED in methanol in the presence of potassium iodide and in water in the presence of a large number of salts exhibit an isosbestic point (Figures 3 and 4). Of the 15 salts examined in aqueous solution, all but sodium and potassium iodide give rise to isosbestic points. This means that the influence of these salts on the spectrum does not result in a continuous spectral shift of the sort that might be expected from a gradual change in solvent properties, but rather is manifested in a "quantized," all or none manner; *i.e.*,

the addition of salt appears to give rise to a new population of dye molecules with a spectrum different from that of the original population. The interpretation of isosbestic points is complicated,¹⁸ but the simplest and most probable interpretation of this result is that the population is a complex of the dye, one component of the salt, and an unknown number of solvent molecules. This interpretation is supported by the fact that there is a linear relationship between the absorption changes at 477 and 403 m μ in the presence of increasing concentrations of sodium perchlorate (Figure 9) and of all other salts examined, except for tetramethylammonium and iodide salts.

This interpretation is further supported by the fact that the formation of this new population follows the simple mass law behavior expected for complex formation with one of the ions of the salt, according to eq 2 (Figures 5–7). Of the 15 salts examined, all but tetramethylammonium chloride and bromide show the saturation behavior with increasing salt concentration that would be expected for such complex formation. Equilibrium constants were calculated for these salts and provide a satisfactory description of the dependence of the absorption changes upon salt concentration.

The equilibrium constant for complex formation is more accurately expressed in terms of activities according to eq 3. As a first approximation, the effects of

$$K = \frac{a_{\text{MOED}\cdot\text{I}}}{a_{\text{MOED}}a_{\text{I}}} = \frac{c_{\text{MOED}\cdot\text{I}}f_{\text{MOED}\cdot\text{I}}}{c_{\text{MOED}}c_{\text{I}}f_{\text{MOED}}f_{\text{I}}} \quad (3)$$

salts on the activity coefficient of the hydrocarbon portion of the dye would be expected to be similar for the free and complexed dye. To the extent that the ion interacts with the dye, it will be less exposed to the solvent, so that the effect of salts on the activity coefficient of the free ions would be expected to be somewhat greater than on ions in the ion-dye complex. This is consistent with the fact that the experimental data are fitted somewhat better by the use of mean ionic activities, rather than concentrations of salts. However, for salts of uniunivalent ions, there is a good fit of the data to theoretical curves based on either activity or concentration and only a small difference between the fit for the two scales.

Merocyanine dyes are highly sensitive to the charge-solvating ability of the solvent, because of the large difference between the polarities of the ground and excited states, and would be expected to show spectral shifts as a result of salt-induced changes in the properties of the bulk solvent, as well as from complex formation.^{10, 13, 19, 20} Such shifts are observed when the concentration of most salts is increased above 1 M and at lower concentrations with salts of tetramethylammonium and of iodide ions, which are known to have relatively large effects on the properties of water and organic solutes in water. These effects appear *in addition* to the effects ascribed to complex formation, except for the last-mentioned salts, with which they occur at sufficiently low concentration to prevent the appearance

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(19) L. G. S. Brooker, G. H. Keyes, R. H. Sprague, R. H. Van Dyke, E. Van Lare, G. Van Zanat, F. L. White, H. W. J. Cressman, and S. A. Dent, Jr., *J. Am. Chem. Soc.*, **73**, 5332 (1951).

(20) L. G. S. Brooker, A. C. Craig, D. W. Heseltine, P. W. Jenkins, and L. L. Lincoln, *ibid.*, **87**, 2443 (1965).

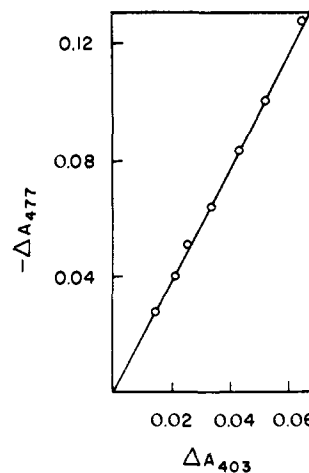


Figure 9. The linear relationship between the absorbance changes at 477 and 403 m μ of 2.9×10^{-5} M MOED in the presence of 0–1.5 M sodium perchlorate.

of an isosbestic point (iodides) or saturation behavior of the spectral change with increasing salt concentration (tetramethylammonium salts). There is some evidence that the absorption changes of merocyanines and related dyes in mixtures of hydroxylic and nonhydroxylic solvents involve complex formation by hydrogen bonding of the dye to the hydroxylic solvent.²¹ It is probable that MOED in water is already hydrogen bonded to the solvent, but it is possible that complex formation with ions involves hydrogen bonding to the hydration sheath of the ions. The observed absorption changes occur at a relatively low salt concentration, at which there is little change in the activity of water, and cannot be explained by a removal of hydrogen-bonded water from the dye by binding to salt.

The results do not permit a definite decision as to whether the spectral changes are caused by an interaction of cations with the negative end or of anions with the positive end of the merocyanine dipole, but they do suggest that both ions of the salt are not interacting with dye at the same time, because such an interaction would give rise to effects proportional to the square of the salt concentration (or activity). The most surprising result is the small variation in the equilibrium constants for different salts; with the exception of salts containing divalent ions, all of the formation constants based on salt activities are between 1.0 and 1.8 M⁻¹, and those based on salt concentration are between 0.9 and 1.6 M⁻¹. There is no correlation with ion size or polarizability. In an inert solvent, such as benzene, one would expect small ions to interact with the dye dipole much more strongly than large ions, with their smaller electrostatic field. However, in water the interaction must involve some type of competition between the dipoles of water and of the dye for the ions, so that a leveling effect of the solvent is to be expected. Eisenman has suggested that the strength of interaction of cations of varying radius with anions and dipoles in water depends on the anionic (or dipole) field strength in such a way that different interaction orders are observed with different field strengths; if the field strength is similar to that of water, there will

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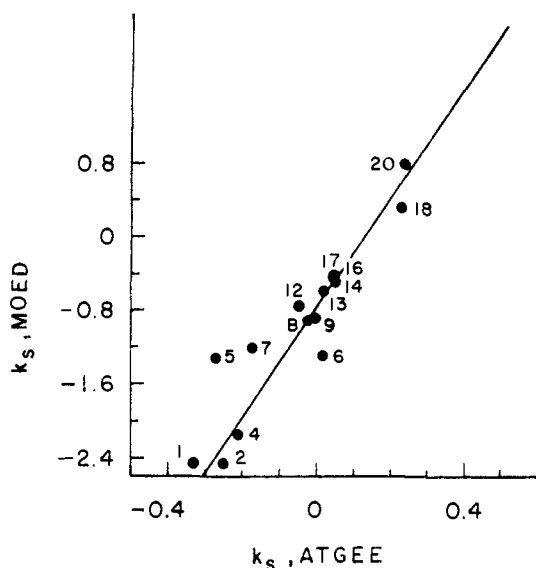


Figure 10. Values of k_s for MOED compared to those for ATGEE in water at 24.9° for a variety of salts. The numbers refer to Table I. Note the difference in scale between the ordinate and abscissa. All k_s values are based on equivalents per liter.

be little difference in the observed interaction strength for different ions.²² This corresponds to the situation observed with MOED. Another precedent is found in the stabilities of ion pairs of simple univalent ions in water, which show little sensitivity to the nature of the ions.²³

Salting In and Out of MOED. The effects of salts on the solubility of MOED are notable for their large magnitude and for the fact that most salts increase the solubility of the dye. The Setschenow constants are among the largest which have been reported. The high polarity of the dye certainly contributes to the strong salting in, but it is of interest that salts increase the solubility of MOED, with its partial charges at each end, more than they increase the solubilities of amino acids, such as cystine, with fully developed charges.²⁴

The Setschenow constants for the salting out of MOED are also unexpectedly large. Salting out constants increase with increasing size of the solute.^{4,5} The molecular volume of MOED is approximately 200–206 ml/mole, estimated from the parachor.^{5,25} The salting out constant for MOED of 1.56 in the presence of sodium sulfate (based on salt molarity) is larger than that for either diphenylmethane or 2,4-diphenyl-2-methyl-2-pentene, which have molar volumes of 177 and 251 ml/mole and k_s values of 0.88 and 1.05, respectively.⁵ This large salting out constant is particularly surprising in view of the large polarity of MOED, which would be expected to reduce or

eliminate the salting out effect with sodium sulfate, and, indeed, does so with most other salts. However, the absolute values of k_s for MOED should be regarded with reserve, because self-interaction effects^{4,26} are probably significant with this dye in spite of its low concentration in saturated solutions. Such self-interaction effects may increase the absolute magnitude but are unlikely to change the sign of k_s values.

The Setschenow constants for MOED are compared with those for the tetrapeptide, ATGEE, in Figure 10. The order of the constants is similar for the two compounds, but their magnitude is considerably larger for the dye and the values are generally displaced in a negative direction. It is as if the fan-shaped array of salting out curves for ATGEE were subjected to an opening and downward movement of the fan in the case of MOED. MOED shares the relative insensitivity toward alkali cations, compared to anions, exhibited by ATGEE³ and by basic solutes.⁴ The largest deviations from the correlation of Figure 10 are observed with sodium trichloroacetate, tetramethylammonium bromide, and lithium bromide. It is possible that these deviations reflect specific interactions of these salts with one or the other compound, such as chelation of cations by carbonyl groups of the peptide and a dispersion or "hydrophobic" interaction of tetramethylammonium ion with the hydrocarbon part of the dye.

The behavior of MOED provides an especially striking illustration of the fact that the salting in of a polar compound with a high dipole moment is usually most marked with large ions of low charge density, rather than with the small, highly charged ions that might have been expected to cause strong salting in from the simplest electrostatic considerations. The fan-shaped array of lines for the salting out of a nonpolar compound, such as benzene, is not inverted,²⁷ but is simply displaced downward in the case of a polar compound which is salted in. Any complete theory of the effects of salts on polar solutes in water must account for this very large divergence of the effects of different salts, ranging from strong salting out to strong salting in, which is not accounted for by the internal pressure theory, as well as for complex formation between the salt and the solute. Salting in of MOED is observed with salts, such as sodium perchlorate and potassium bromide, which cause electrostriction and an increase in the internal pressure of the solution.

Complex formation between ions and MOED will increase the observed salting in and decrease the observed salting out of this compound. The (linear) increase in total dye concentration in solution caused by complex formation is given by eq 4, in which S'

$$S/S' = 1 + K_{\text{assoc}}[\text{salt}] \quad (4)$$

and S are the concentrations of free and total dye, respectively, and K_{assoc} is an empirical association constant, based on the spectrophotometric evidence for complex formation. Assuming that the total dye in a

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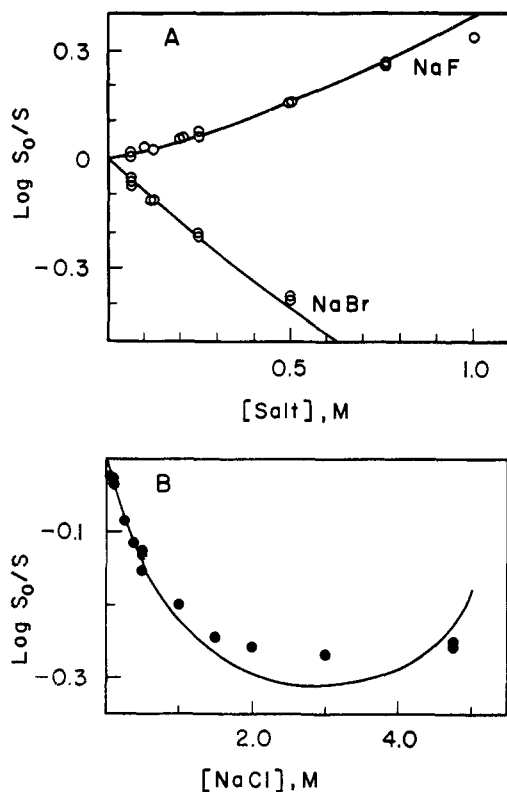


Figure 11. Effects of salts on the solubility of MOED, calculated according to eq 5.

saturated solution at any salt concentration consists of complexed dye and free dye, this equation may be combined with the Setschenow equation to give eq 5

$$\log (S/S_0) = k_s'[\text{salt}] - \log (1 + K_{\text{assoc}}[\text{salt}]) \quad (5)$$

in which the salting out constant k_s' and complex formation are treated as separate terms. Since the Setschenow equation predicts a linear increase in the *logarithm* of the solubility with increasing salt concentration, eq 5 does not predict a linear increase in solubility on either the linear or logarithmic scale. Equation 5 is certainly an oversimplification, because much of the dye is aggregated in saturated solutions and because the relationships of the activity coefficients of free, complexed, and aggregated dye are too complex to evaluate either theoretically or experimentally. Nevertheless, aggregated dye may be expected to behave in a qualitatively similar manner to free dye, and it is of interest to determine the extent to which eq 5 describes the behavior of saturated dye solutions. The calculated curves for the effects of sodium fluoride, bromide, and chloride on the solubility of MOED, based on eq 5 and the values of K_{assoc} in the third column of Table II, are shown in Figure 11. The values of k_s' for these and some other salts were calculated from K_{assoc} and the observed solubility in 0.25 M salt and are given in the third column of Table I. The calculated lines show reasonably good agreement with the experimental points up to salt concentrations of 0.5 to 1.0 M; in particular, they predict the observed nonlinearity of the data with increasing salt concentration. In the case of sodium chloride, the calculated line is not far from the experimental points up to a salt concentration of 4 M.

Another test of eq 5 is provided by Figure 12, which

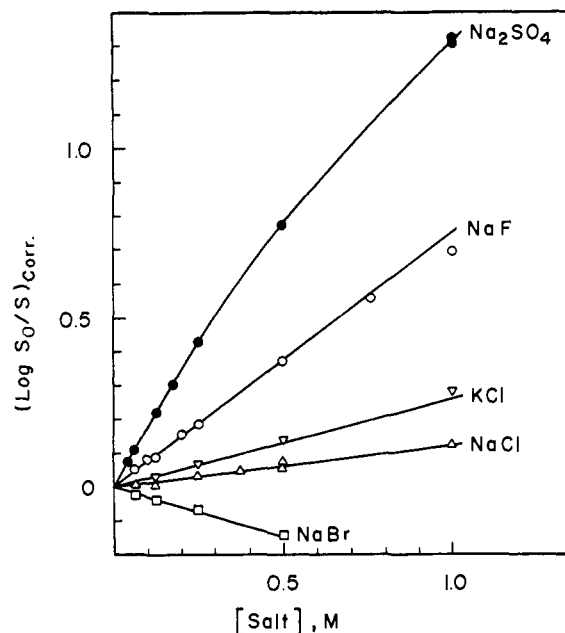


Figure 12. Salting out and salting in of MOED, corrected for association between the dye and salt.

shows the salting out curves for a representative series of salts after correction for the increase in solubility caused by complex formation, based on the equilibrium constants of Table II. The correction brings the curves for the alkali halides considerably closer to linearity, compared to the uncorrected curves shown in Figures 1 and 2. The negative observed k_s values for sodium and potassium chloride become positive after correction for complex formation; *i.e.*, all of the observed salting in by these salts may be accounted for by complex formation. The correction shifts all of the salting out constants in a positive direction, but it should be noted that not more than a small fraction of the strong salting in exhibited by sodium perchlorate, potassium iodide, and lithium bromide may be attributed to complex formation.

Thus, it appears that a significant fraction, but by no means all, of the observed salting in of this colored vinylogous amide may be accounted for by complex formation between the salt and the dye. It is not known what the relative contributions of complex formation and of other effects are to the salting in of more conventional amides and peptides, but it is reasonable to expect, by analogy, that complex formation also contributes to the observed salting in of peptides and the interaction of salts with proteins. In MOED, the partial positive charge of the nitrogen atom is partially shielded from the solvent by a methyl group, and if complex formation involves an interaction with this charge, it might occur more readily with the hydrogen-bearing nitrogen atom of a primary or secondary amide. Chelation of an ion between two peptide dipoles represents another possibility for complex formation with a peptide, which cannot take place with MOED. It has been postulated that the effects of certain antibiotics on the transport of cations into mitochondria involves a similar binding to peptide carbonyl groups.²⁸

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It is of interest that iodide and perchlorate ions (but not phosphate or sulfate) bind firmly to cyclodextrin, which contains a number of weak hydroxyl group dipoles; the dissociation constant for the cyclodextrin-perchlorate complex is $3.4 \times 10^{-2} M$.²⁹ It is known that salts bind to polypeptides and amides in the solid state.^{6,30} Such binding provides no direct information

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regarding the occurrence of binding in solution, but it does indicate that amide-ion interactions are of sufficient strength to compete effectively with ion-ion interactions. Finally, it has been shown that dimethylformamide forms a complex with Al^{3+} in an aqueous-organic solvent with a lifetime sufficient to give rise to a distinct nuclear magnetic resonance signal.³¹

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Hydrogen-Deuterium Exchange in Lactams^{1a}

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Contribution from the Department of Biochemistry, University of Iowa, Iowa City, Iowa 52240. Received August 5, 1968

Abstract: The kinetics of hydrogen-deuterium exchange have been measured for three *cis* lactams (butyrolactam, valerolactam, and caprolactam), the *cis-trans* lactam (caprylactam), and a *trans* amide (N-methylacetamide) in deuterium oxide solution. Exchange rates and activation energies (12–15 kcal/mole) are comparable for both the *cis* and *trans* configurations. Furthermore, activation energies are of a similar magnitude as those for other model peptides, polypeptides, and proteins.

Recent studies on the hydrogen-deuterium exchange reaction of model peptides and polypeptides^{2–9} have attempted to elucidate some of the various factors which affect the observed exchange rates. Even for the simple amide, N-methylacetamide (NMA), the exchange is not instantaneous and depends on the acidity and polarity of the solvent system.^{2–4} The acid activation energy in pure D_2O is of the same magnitude as those of other model peptides, polypeptides, and proteins either in the helical or the disordered form.^{7,9} Studies with small peptides⁶ and polypeptides^{7,9} further showed that the parameters for the exchange reaction reflected the inductive effect on the amide group and the local environmental and conformational factors.

A *cis* lactam, butyrolactam, was studied by Klotz and Feidelseit.⁵ They found its exchange rate and activation energy to be much higher than that of the *trans* amide, NMA. However, our spectral studies on a series of lactams¹⁰ showed that this lactam is not a good model for the *cis* configuration due to its ring strain. It is therefore interesting to study other *cis* lactams.

(1) (a) This research was supported by two grants from the Division of Molecular Biology, National Science Foundation, GB-4835 and GB-2049. (b) This paper includes portions of the thesis of C. Y. S. Chen, submitted in partial fulfillment of the requirements for the Ph.D. degree at the University of Iowa, Aug 1968. (c) To whom correspondence should be directed.

(2) A. Berger, A. Loewenstein, and S. Meiboom, *J. Am. Chem. Soc.*, **81**, 62 (1959).

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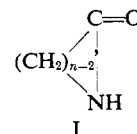
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Such studies will show more conclusively if the rates of exchange for the *cis* and *trans* forms of the amide group are significantly different.

The configuration of the peptide group in the lactams (I) depends on the ring size (n).^{11,12} The *cis* configura-



tion is the only form present for $n \leq 8$ and the *trans* configuration is the predominant form for $n > 9$. The $n = 9$ lactam is present in both the *cis* and *trans* configurations. The parameters for the exchange reaction of NMA and lactams with ring sizes of 5, 6, 7, and 9 in D_2O as a function of acidity and temperature are reported here.

Experimental Section

Materials. NMA of Eastman grade was further purified by vacuum distillation at 59–62° and 1 mm. Butyrolactam ($n = 5$) and valerolactam ($n = 6$) from K and K Laboratories were purified by vacuum distillation at ≤ 1 mm, 86–90° and 95–96°, respectively, with precautions to avoid moisture contamination. Caprolactam ($n = 7$) was obtained from Matheson Coleman and Bell and was recrystallized from benzene to give colorless crystals, mp 69–70°. Caprylactam, mp 72–73°, was synthesized in this laboratory.¹⁰

D_2O of 99.8% purity was purchased from Volk Radiochemical Co. and International Chemical and Nuclear Corp. A solution of 38% DCl in D_2O (99% deuterium) was obtained from Stohler Isotope Chemicals. The anhydrous sodium acetate was Baker analyzed reagent.

Preparation of Reaction Mixtures. Buffers of the desired pH were obtained by the addition of 1 M DCl to a solution of 0.02 M sodium acetate in D_2O . In order to minimize the time required for solu-

(11) R. Huisgen, H. Brade, H. Walz, and I. Glogger, *Chem. Ber.*, **90**, 1437 (1957).

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