Spectra of Molecular Complexes of Porphyrins in Aqueous Solution[*]

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ABSTRACT: The changes in the absorption and emission spectra of porphyrins in various molecular complexes in aqueous solution have been studied. These complexes are formed with a variety of molecules including large organic cations and planar neutral heterocyclic molecules. Spectral analysis by the method of continuous variation, the lack of isosbestic points, and a new analysis based on band widths all point to the formation of a series of complexes with several addends clustered about the porphyrin ring. Ionic and dispersion forces contribute to the stability of the complexes, but the water structure about these large hydrophobic molecules is an equally important factor. An estimate of the latter interaction based on interfacial tension is given.

The spectral changes, particularly the broadening of the Soret band and not of the red band I, are interpreted as a change in the nearly degenerate x and y components of the energy levels, and a model of the complex is derived.

Studies at extreme dilution (10^{-8} M), an analysis of the optical effects of aggregation on the spectra, and the absence of polarization of fluorescence all favor the view that the uroporphyrin is monomolecularly dispersed. A suggestion is made that such complexes occur in heme proteins as part of the phosphorylating mechanism and that the widespread occurrence of heterocyclic molecules in biological systems is related to the formation of complexes.

The unique absorption spectra of the porphyrin pigments has allowed their identification and study throughout the biological realm. The large over-all variation of spectra with changes of structure has been used to identify these pigments. However, much information concerning the nonbonded or weakly bonded environment of these pigments remains untapped in minor spectral variations.

In the course of experiments on the photoreactions of porphyrins, a marked effect of pyridinium salts (NAD[1] models) on the photochemistry was traced to the formation of complexes with the porphyrin. These complexes were found to occur with a wide class of compounds, many of biological importance. Their description is relevant to the problem of deciphering the effect of environment on porphyrin pigments *in vivo*. Moreover, some insight is gained into both the theory of porphyrin spectra and the binding of molecular complexes in aqueous solution.

Experimental

The porphyrins used in this work were chromatographed, crystalline samples which have been described in previous publications (Mauzerall, 1962a). Unless otherwise stated, uroporphyrin isomer III or a mixture of isomers, mostly isomer III, was used in this work. Most of the quaternary salts were synthesized in the laboratory and their melting points, analysis, and absorption spectra agreed with previously published data. Other compounds were the purest commercially available and were recrystallized when necessary, e.g., hexadecyltrimethylammonium bromide. The water was glass redistilled, and quantitative technique was used throughout. Absorption spectra were measured on a Cary Model 14 MR spectrophotometer with a modified cell compartment. A channeled brass block surrounding the glass absorption cell allowed the temperature to be controlled to $<0.1°$ from an outside regulated bath. Cells with light paths from 0.01 to 10 cm were used. The spectral band width was kept below 0.05 of the absorption band width. Fluorescence spectra were measured by a simple but efficient apparatus. An EMI 9558B photomultiplier (S-20 response) was used as detector and an interference wedge (Leitz Versil-200) and slit were used as monochromator. The effective band width was ~ 12 mμ. A low-pressure discharge tube was used for excitation at right angles to the detector. The exciting light was a band at 355 mμ, half-width 35 mμ. Exact peak wavelengths and band shapes of the fluorescence as well as excitation spectra were determined using the Cary monochromator and beam chopper. Modified detectors and a phase-sensitive amplifier with long time constant were used to recover the very weak signal from noise. The arrangement to measure fluorescence polarization was similar to that described by Gouterman and Stryer (1962). Quantum intensities were measured with an Epply thermopile.

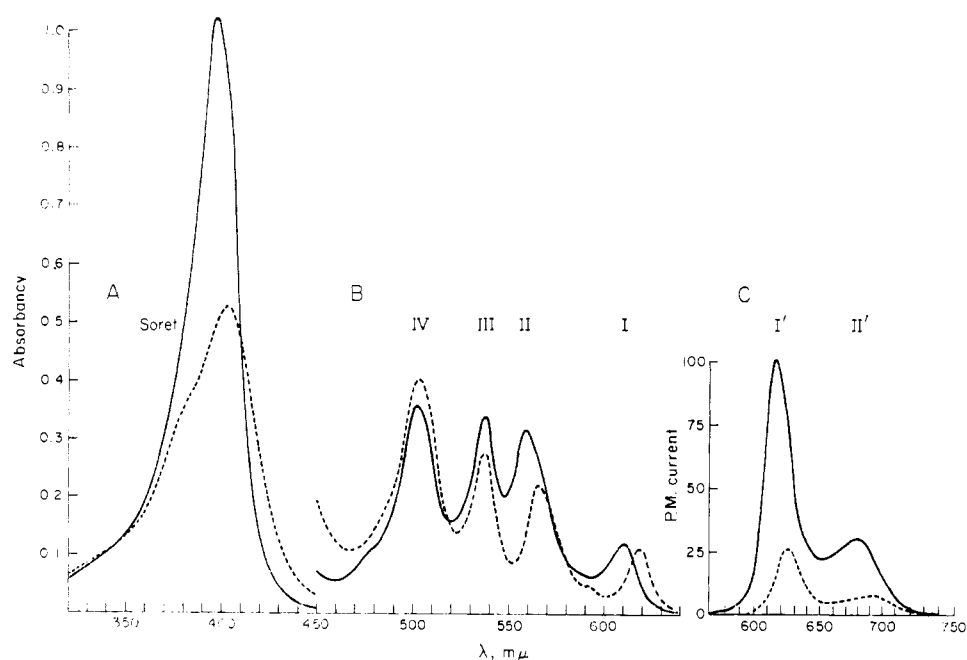[1] Abbreviation used in this work: NAD, nicotinamide–adenine dinucleotide.

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FIGURE 1: Complex between uroporphyrin (URO) and 1-(carbamidomethyl)-3-carbamylpyridinium chloride (CAMNACl). (A) Solid line: URO, 5 × 10⁻⁶ M in 0.1 M citrate, pH 5.9; dotted line: plus 0.037 M CAMNACl. (B) Solid line: URO, 3.5 × 10⁻⁵ M in 0.1 M EDTA, pH 6.0; dotted line: plus 0.095 M CAMNACl. (C) Solid line: fluorescence emission of URO, as in B; dotted line: fluorescence of complex, as in B. Correction of photomultiplier-wedge response to equal quanta per bandpass would increase the long wavelength peak intensity by a factor of 4.4.

TABLE I: Absorption and Fluorescence of Uroporphyrin and Complex with 1-(2-Hydroxyethyl)-3-carbamylpyridinium Chloride.[a]

| Absorption Band | URO | | Complex | | Band Shift |
	max (mμ)	D.S.[b] (A²)	max (mμ)	D.S.[b] (A²)	U–C (cm⁻¹)
Soret	397.5	6.2	403.7	5.8	380 ± 10
IV	502	0.26	503.5	0.27	70
III	538.5	0.12	537.5	0.13	−30
II	560	0.13	566.5	0.12	200
I	611.5	0.046	619.5	0.035	220
Fluorescence					
I′	615		622	. . .	180 ± 30
II′	680		690	. . .	210

[a] Solution of uroporphyrin (4.3 × 10⁻⁵ M) in 0.1 M NH₃, pH 9.5, with and without 0.05 M 1-(2-hydroxyethyl)-3-carbamylpyridinium chloride. [b] D.S. = Dipole strength = $w\epsilon/2510\lambda$, where w is full band width at half-amplitude, λ is wavelength of band maximum, ϵ is the molar absorbancy, and the constant converts units to square angstroms (Gouterman, 1961).

Results

Spectra and Binding. The absorption and emission spectra of uroporphyrin and of a typical complex with a pyridinium salt at pH 6 are shown in Figure 1. Quantitative data for a similar complex in alkaline solution are shown in Table I. The two longest wavelength bands (I and II) of the porphyrin shift to the red. A cor-

responding shift of 200 cm⁻¹ in energy units occurs in the two fluorescence bands. The spacing between bands I and II in the absorption spectra of both uroporphyrin and the complex is 1510 ± 10 cm⁻¹. Similarly in the fluorescence spectra the spacing is 1550 ± 40 cm⁻¹. These data show the intimate connection between the two longest wavelength absorption bands and

the two strong emission bands of porphyrins. A third very much weaker band is in fact present between these, at $\sim$590 mμ in absorption and $\sim$650 mμ in fluorescence. In Figure 1, the total intensity of the emission spectra decreases 4–5 times, but the average for similar complexes such as those listed in Tables I and III is closer to 2–3 times. Although the wavelength of the exciting light is in a region of nearly equivalent absorption of free and complexed porphyrin (360 mμ, see Figure 1), a factor of two in fluorescence intensity may be caused by variation in the number of quanta absorbed. The excitation spectra of the fluorescence corresponds closely to the absorption of the free and complexed porphyrin in each case (Table II). The absorption bands

TABLE II: Identity of Absorption and Fluorescence Excitation Band Maxima for Uroporphyrin and Complexes.[a]

| Addend | | Bands | | |
		I	II	Soret
None	Absorption	611	560	398
	Fluorescence	612	562	398
(2-Hydroxyethyl)-trimethylammo-nium chloride, 1 M	Absorption	614	562	398
	Fluorescence	615	563	398
1-(2-Hydroxyethyl)-3-carbamylpyri-dinium chloride, 0.06 M	Absorption	619	566	404
	Fluorescence	619	567	405
Caffeine, 0.1 M	Absorption	622	568	404
	Fluorescence	622	569	405

[a] Uroporphyrin, 1–4 $\times$ 10^{-6} M in 0.1 M citrate, pH 6.

III and IV in the visible are far less affected by complexing. The Soret band shifts to longer wavelengths, broadens, and decreases in intensity. During these spectral shifts no large change in dipole (or oscillator) strengths occur. No significant shifts occur in the ultraviolet spectra of either the porphyrin or of the pyridinium salt, and no new absorption bands with $\epsilon \geq 10^2$ occur at longer wavelengths, 0.65–1.35 μ.

A series of substituted pyridinium salts and various nitrogen heterocyclic compounds were found to give similar spectral shifts on mixing with uroporphyrin (Table III). To simplify terminology, the compounds forming complexes with porphyrins will be referred to as addends. In the first group, A, changing the over-all charge from positive through zwitterion to negative causes a 100-fold decrease in stability. Moreover, the binding of uroporphyrin to a positively charged addend increases with decreasing ionic strength (group B, Table III).

The next group (C, Table III) shows that more than electrostatics is involved in the formation of the com-

TABLE III: Spectral Properties of Complexes between Uroporphyrin and Various Compounds.

Compound[a]	Soret[c] (mμ)	$^1/_2W^d$ (mμ)	pC[e]	Fluorescence[c]
A None	398	27	...	1
1-Carbamidomethyl-3-carbamylpyridinium chloride	404	50	2.7	0.2
1-Carbamidomethyl-pyridinium-3-carboxylate betaine	...	..	1	0.2
1-Carboxymethylpyri-dinium-3-carboxylate, sodium	...	..	0.5	1
B				
1-(2-Hydroxyethyl)-3-carbamylpyridinium chloride, pH 10[b]				
μ = 0.01	404	50	3.6	...
μ = 0.003	404	50	3.9	...
μ = 0.001	404	50	4.5	...
C				
(2-Hydroxyethyl)-tri-methylammonium chloride	398	31	$\sim$0	0.4
1-(2-Hydroxyethyl)pyri-dinium chloride	404.5	44	2	0.5
Hexadecyltrimethyl-ammonium bromide	403.5	38	2.8	0.4
1-Hexadecylpyridinium bromide	405	45	3.5	0.5
D				
Nicotinamide	403	35	1	...
Caffeine	404	30	2	0.6
Adenine	404	35	3	...
E				
α,α'-Dipyridyl	402	36	3	0.4
γ,γ'-Dipyridyl	402	39	3	0.03
o-Phenanthroline	411	50	4.3	0.01
Methyl viologen	406	37	...	<0.002

[a] Complexes determined in 0.1 M citrate, EDTA, or hydroxylamine buffer, pH 6, 23 $\pm$ 1°. [b] Ionic strength μ, varied with NaCl. [c] Soret shifts and fluorescence (relative to free porphyrin at 620 mμ) determined at 0.01–0.1 M addend except adenine, 3 $\times$ 10^{-3} M; α,α'-dipyridyl, 6 $\times$ 10^{-3} M; γ,γ'-dipyridyl, 6 $\times$ 10^{-4} M; and o-phenanthroline, 4 $\times$ 10^{-3} M. [d] $^1/_2W$ is the full width of the Soret band at half-maximum height. [e] pC = $-$log (addend) at 50% complex (see text).

plexes. Choline chloride forms only a very weak complex, but replacing the trimethylammonium group by the pyridinium group produces a good complexing agent. The aromatic ring is not essential since replacing the hydroxyethyl group of choline by a hexadecyl chain also results in a good complexing agent. The increased polarizability leads to a tight complex. Replacing the

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trimethylamine group by a pyridine ring further increases the strength of the complex.

Some compounds (group D, Table II) do not involve ionic forces: nicotinamide, caffeine, and adenine. The last group, E, shows an interesting effect of geometry. Both α,α'-dipyridyl and the γ,γ' isomer form good complexes, but the complex with the rigid, planar o-phenanthroline is much tighter. The larger than usual shift of the Soret band with phenanthroline is a pH effect, since the band of the complex is at 404 mμ in alkaline solution. The pK of phenanthroline, 4.9, allows the protonated form to be preferentially bound at pH 6. γ,γ'-Dipyridyl is also bound in the protonated form at this pH. The last group of addends shows an increased electronic interaction with the porphyrin as seen by the increasing quenching of the fluorescence and increasing changes in the visible spectra: no addend, 502, 539, 560, 611 mμ; α,α'-dipyridyl, 505, 540, 563, 617 mμ; γ,γ'-dipyridyl, 507, 539, 567, 622 mμ; o-phenanthroline, 508, 541, 571, 623 mμ; and methyl viologen, 502, 534, 569, 623 mμ; all at pH 6.

Complexes were also obtained with a variety of 1-substituted nicotinamide cations, with nicotinamide 1-oxide, pyridine, protonated pyridine, Girard reagent P, adenosine, and histidine. No observable complex was obtained with sodium nicotinate or adenylate. Uroporphyrin isomer I gave quantitatively the same complex as isomer III with 1-(2-hydroxyethyl)-3-carbamylpyridinium chloride. Small cations in concentrations up to 1 m have no effect on the porphyrin spectra. A salting-out effect leading to pigment aggregation occurred at higher concentration. However, an interaction

TABLE IV: The Effect of Size of Cation on the Spectra of Uroporphyrin.

Addend[a]	Concentration (M)	Soret (mμ)	$\frac{1}{2}W$ (mμ)
None	. . .	397.5	26.5
LiBr, NaBr, KBr	1	397.5	27
CsCl	1	397.5	30
(Methyl)$_4$NBr	0.1	397.5	29
	1	397.5	34
(Ethyl)$_4$NBr	0.1	398	32
	1	398	37
(n-Butyl)$_4$NBr	0.1	399	36
	0.5	400	35

[a] Uroporphyrin, 3.2×10^{-7} m; NH$_3$, 3×10^{-3} m, EDTA, 10^{-4} m, pH 9.6, 15°.

is clearly evident with large organic cations (Table IV). The Soret band broadens symmetrically with a decrease in maximum absorptivity, and shifts slightly to longer wavelengths.

The importance of water as solvent was noted by looking for such complexes in other polar solvents. The tetramethyl ester of coproporphyrin was used to avoid charge effects, and the broadening of the Soret band was used as the criteria of a complex, since solvent red shifts were already present. With an alkyl nicotinamide cation, the complex was found in the highly structured solvent formamide, but not in dimethylformamide or in dimethyl sulfoxide.

As expected, no complex is formed between the positively charged pyridinium salts and the doubly positively charged porphyrin cation in acid solution. However, the neutral molecule caffeine does form a complex with uroporphyrin in 1 m HCl. The wavelength shift of the Soret band is very small, 6 A, but the band is doubled in width and halved in height (constant dipole strength).

These complexes are not restricted to uroporphyrin. This porphyrin was studied since all available evidence (Mauzerall, 1962b; below) indicates that it is monomolecularly dispersed in the dilute solutions used in the work. All other porphyrins with carboxylated side chains are aggregated even in alkaline, dilute solution. Thus, both a higher concentration of addends and an increase in alkalinity were required to form the same degree of complexes as with uroporphyrin. The wavelength shifts in the aggregated forms involve too many variables to be easily interpretable, but the shifts of the farthest red band I of the complexes with 1-methyl-3-carbamylpyridinium iodide, relative to uroporphyrin at 16,155 cm^{-1}, are: coproporphyrin III, $+80$ cm^{-1}; protoporphyrin IX, -155 cm^{-1}; deuteroporphyrin IX-2,4-diacrylic acid, -330 cm^{-1}. These shifts are in agreement with the expected order of electron delocalization caused by the side chains at the 2,4 positions, respectively: methyl < vinyl < trans-acrylate which replace the carboxymethyl group of uroporphyrin.

These results indicate that similar complexes should be formed with the metalloporphyrins. The case of quaternary ammonium derivatives are particularly interesting since these compounds cannot interact with the metal in the usual ligand unshared pair–empty metal orbital fashion. In fact, both copper and zinc coproporphyrin form tight complexes (pC $\sim$ 3) with 1-(2-hydroxyethyl)-3-carbamylpyridinium chloride. The spectrum of the zinc chelate shifts about 2 mμ to longer wavelengths. The Soret band broadens by 40% with a corresponding decrease in maximum absorptivity, again conserving dipole strength. The spectral change of the copper chelate shows two distinct steps: a shift of the visible spectrum to longer wavelengths (2–3 mμ) at low concentrations of pyridinium salt, followed by a return to even shorter wavelengths at higher concentration. The Soret band shifts from 379 to 398 mμ in two steps, retaining its band-width and absorptivity.

The effects of various heterocyclic compounds on the spectra of hemes have been well described by Keilin (1943). She has pointed out the particularly marked effect of caffeine and chlorocaffeine. The self-aggregation of the metallocopro- and -protoporphyrins com-

plicates a quantitative study, but the uroporphyrin derivatives would be interesting to study.

Composition. The spectral data obtained by the formation of these complexes can be roughly fitted by a 1:1 complex titration curve, i.e., a plot of absorbancy change versus log concentration of addend at very low porphyrin concentrations. However, analysis shows a continual drift of the calculated binding constant over the range of addend concentration. Some dependence in wavelength was also noted. Most important, no good isosbestic points were obtained. This was particularly noticeable above room temperature and at low ionic strength. In a wavelength region where the addend does not absorb, a single complex (or several having identical functional dependence on the variable parameter) will have isosbestic points (Cohen and Fischer, 1962) wherever the molar absorptivity curves of the free molecule and of the complexed form cross.

Most methods designed to determine the number of absorbing species in a solution depend on analysis of the absorbancy at different wavelengths. Possibly the most general method is to determine the rank of a matrix relating the absorbancies of solutions at different wavelengths to the differing concentrations (Briegleb, 1961). However, practical use of this method is severely limited by experimental error. In the case of the porphyrin complexes, calculations by this method give evidence for at least two complexes. A simpler scheme can be used to detect the presence of more than one complex and should be particularly useful where the total pigment concentration is variable, e.g., because of side reactions or for *in vivo* systems. Thus, when a single complex is formed and a band shift occurs with only limited band broadening, the observed band width (full width at half-height) will reach a maximum at the equivalence point and then will decline again. This maximum band width can be calculated by summing molar equivalent spectra of the free porphyrin and of the complexed form present with excess complexing agent. If, however, a sequential series of complexes of increasing band displacement are formed, the band will never broaden to the extent expected from observations at the extremes. It is not necessary to have a "pure" spectrum of the complex, but only that the band of the complex be not more broadened than shifted in wavelength. The longest wavelength red band (I) fulfills these criteria nicely. For the complex of uroporphyrin with the alkylated nicotinamide salts, the observed free and complexed band widths are 16.2 and 15.5 mμ, with a band shift of 6.8 mμ. The maximum width observed, near a 1:1 mole ratio, is 17.2 mμ, whereas the width calculated for a two-species system is 21.0 mμ. The difference, 3.8 mμ, is well outside the measurement error of ± 0.2 mμ. The result argues strongly that a sequential series of complexes are formed. As expected, the largest spectral change occurs with the first addend, leading to an observed maximum width near the 1:1 complex. The same result was obtained with caffeine; maximum width observed: 18.0 mμ; calculated: 21.0 mμ. The case of phenanthroline is complicated by a definite broadening in the complex to 19 mμ. The

width increased from 16 to 19 mμ at a ratio of about 4 phenanthrolines/porphyrin, then remained constant. The calculated maximum width for a two-component system is 20.4 mμ. Again, this is evidence for the presence of a series of complexes. Corresponding effects occurred with the 560 mμ band II. Bands III and IV simply broadened without appreciable shift in peak wavelength. The Soret band broadened far too much to allow this simple analysis to be useful.

The method of continuous variation (Rossotti and Rossotti, 1961) was used to obtain further evidence that several complexes do indeed exist. The extrapolation of this method to obtain quantitative data on a series of complexes has been justly criticized (Woldbye, 1955). However, the qualitative detection of more than one complex is feasible. Since the spectral changes are small, the complexity of the porphyrin spectra were put to good use. Because of the many narrow bands, two or more wavelengths of the free porphyrin could be found that had the same absorptivity. The differences of absorbancy between such a pair of wavelengths is less susceptible to random errors because the absorbance of the free porphyrin and of any small base-line variation cancels out, leaving only the absorbance of the complex. The asymmetry of the curve for the complex between an alkylated nicotinamide salt and uroporphyrin (Figure 2) shows that at this concentration definitely more than one, possibly an average of two, molecules of the organic cation are bound to the porphyrin. Curves similar to the above were obtained with phenanthroline and caffeine as complexing agents under identical experimental conditions. The peaks occur somewhat closer to the 1:1 complex. The average composition varied with the experimental conditions. At lower ionic strength, the complex with alkylated nicotinamide salts averaged close to 4 cations/porphyrin ring. At ten times the above caffeine concentration (4 $\times$ 10^{-2} M) and pH 6, the spectral data indicate a large complex, of the order of 10 caffeine molecules/porphyrin. Suggestive evidence of a 2 porphyrin:1 adduct complex was also obtained. A quantitative treatment of this obviously complex system is deferred. The binding constants pC, quoted in Table III and elsewhere, were calculated from data points near the beginning of complex formation and so favor the 1:1 complex.

The above experiments define the ratios of addend to porphyrin in the complexes. The number of porphyrin molecules/complex was investigated by the method of extreme dilution. The spectra of both free uroporphyrin and the complex with excess (0.01 M) 1-(2-hydroxyethyl)-3-carbamylpyridinium chloride are constant ($\pm 5\%$ in band width and absorptivity) over a 10^4-fold range (10^{-4} to 10^{-8} M) of concentration of the porphyrin. Moreover, the same constancy of the spectrum of the complex is observed between 1 and 49° at a porphyrin concentration of 4 $\times$ 10^{-6} M. These data strongly indicate that the porphyrin is monomeric in the complexes.

If the complexes were very large or contained several porphyrin units in an ordered array, a polarization of
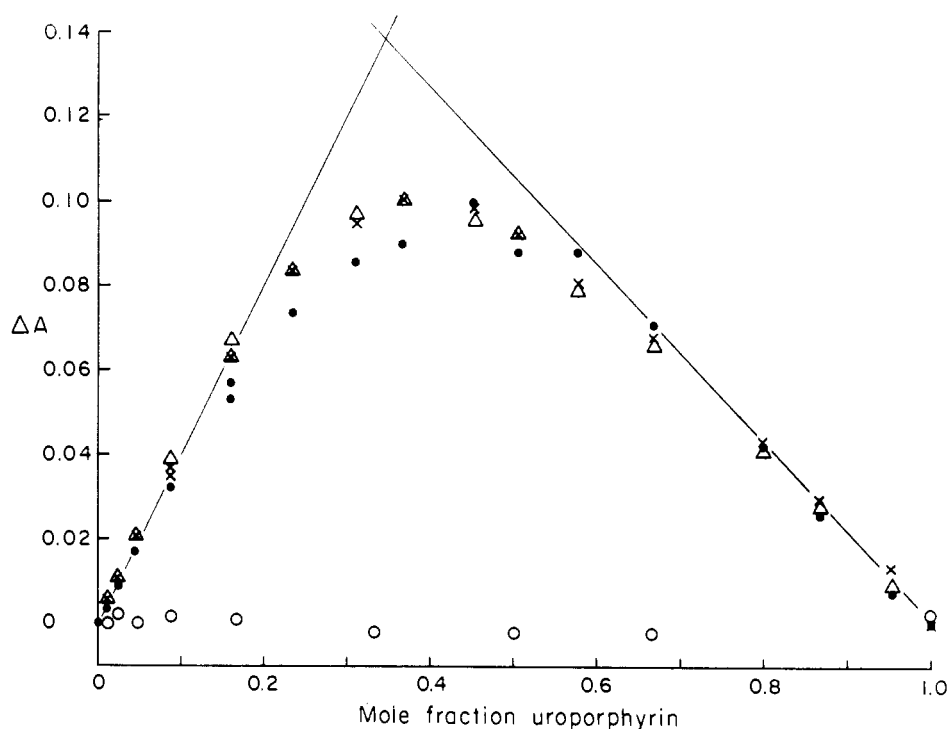
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FIGURE 2: Differences in the absorbancy, ΔA, plotted against mole fraction of porphyrin. Varying ratios of uroporphyrin and 1-(2-hydroxyethyl)-3-carbamylpyridinium chloride, total concentration 4.3×10^{-3} M in 0.1 M NH_3, pH 9.6, 24°; ●, $A_{620} - A_{600}$; ×, $0.19(A_{434} - A_{610})$; △, $0.74(A_{445} - A_{465})$; ○, $A_{620} - A_{600}$, uroporphyrin alone; error = $\pm 0.002A$.

the fluorescence would be observed when the rotational relaxation time of the aggregate exceeded the fluorescent lifetime. The complexes of uroporphyrin with 1-(2-hydroxyethyl)-3-carbamylpyridinium chloride, α,α'-dipyridyl, and caffeine showed no detectable polarization of fluorescence ($p < 0.01$). Under the experimental conditions used the polarization of fluorescence of the free porphyrin in 95% glycerin is readily observed ($p = +0.14$).

Discussion

The complexes between porphyrins and the variety of compounds considered in this paper may be described as induced micelles. One to several addends cluster about the porphyrin plane and thus transfer the chromatophore from an aqueous to a polar organic environment. Analysis of the spectral changes by various methods leads to the conclusion that a series of complexes are formed of increasing addend:porphyrin ratio. The largest changes in absorption and emission spectra often occur on forming the 1:1 complex, and so it tends to dominate the observed changes (Figure 2). The strength of the interaction between porphyrin and addend in the ground state can be judged by the concentration of addend required to form the complex, while the shift and broadening of the spectra measure the difference of interaction between ground and excited states (Tables I, III, IV). A discussion of the forces in-

volved in the stability of these complexes will be followed by an analysis of the causes of the spectral perturbations.

Stability. The forces holding the complex together can be separated into a definite ionic component and a less definite van der Waal's component: a sum of dispersion, dipole, and hydrogen bond interactions. Because of the eight negative charges clustered about the uroporphyrin ring, cations, zwitterions, and anions having a similar structure are bound in that order (group A, Table III). Moreover, the increase in binding of a cation with decreasing ionic strength (group B) is such that the monopositive addend "sees" a charge of -10 ± 3, in fair agreement with the expected value of -8 for uroporphyrin. Note that the opposite effect is expected if the porphyrin is dissolving in a preformed micelle of pyridinium salt. The critical micelle concentration increases with decreasing ionic strength (McBain and Hutchinson, 1955; Osipow, 1962).

The nonionic forces contributing to the stability of the complex can be seen not only in the neutral addends (group D, Table III), but also in the cations. Increasing the size of the alkyl chains (group C, Table III and Table IV) or adding a more polarizable pyridine ring (group C, Table III) causes a large increase in binding. It had been assumed that the binding of caffeine and similar heterocyclic compounds occurs at the carboxyl groups of the porphyrin (Lemberg and Legge, 1949). However, the complexes are readily formed even in

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the presence of a >10⁵-fold excess of carboxylate groups (citrate ion). Clearly the carboxyl groups of a porphyrin are very special, viz., adjacent to the large polarizable plane of the aromatic macrocycle. A complex also forms between uroporphyrin and caffeine in acid solution where the carboxyl groups are not ionized. The data, particularly the good correlation of molecular (van der Waal's) volume of the addend with binding (Table III, groups C and D; Table IV), suggest a more general interaction involving the solvent, water. In support of this hypothesis, similar complexes are observed in the highly structured solvent formamide, but not in the polar solvents dimethylformamide or dimethyl sulfoxide. The delicate balance among the forces involved in the interaction of nonpolar groups in polar, structured solvents has been termed hydrophobic bonding (Némethy and Scheraga, 1962). Diamond (1963) has discussed the increased interaction of large organic ions in water. The distorted water structure around the large ions, or neutral organic molecules in the present case, favors their coalescence. A simple estimate of the energies involved can be made by calculating the decrease in surface area on forming a complex and using the *interfacial* tension of, e.g., benzene-water as a measure of the force. With parallelopipeds or flat cylinder models to estimate the interfacial area lost on forming a complex, a binding energy (ΔF) at 25° of $\sim$3 kcal/mole of porphyrin is obtained if both sides of the porphyrin plane are covered. This corresponds to a binding constant of $\sim$10² and could account for a large part of the nonionic interaction that stabilizes the observed complexes. A spherical model, less appropriate for the porphyrin, requires correction for decreased surface energy with decreasing curvature, and gives an increased binding energy.

Spectra. The spectral shift to the red is the qualitative basis for the model of the complex: the porphyrin is transferred from an aqueous to a polar organic environment. The theory of solvent spectral shifts is based on interaction of the transition moment of the chromatophore with its environment (Bayliss and McRae, 1954; McRae, 1957; Basu, 1964) and predicts a wavelength shift to the red for an increasing refractive index. The spectrum of uroporphyrin in formamide is similar to that of the complex given in Table I: 619, 566, 537, 502, and 401.5 mμ. The band widths of the visible bands also agree to within 1 mμ, but the width of the Soret band in formamide is 25 mμ, in contrast to 50 mμ in the complex. The form of the theories, restricted to nondegenerate electronic transitions, predicts a shift in wavelength of the absorption spectra, but is silent about band-width and intensity. Experimentally, the allowed, nondegenerate transitions of various dyes show a wavelength shift but no broadening (Sheppard, 1942; West and Geddes, 1964). Specific changes in vibronic interactions by the solvent lead to changes in vibrational fine structure, but not in the over-all envelope of the absorption band. The broadening of the Soret band in the porphyrin complexes is ascribable to a change in the close-lying energy levels causing this

transition. Before presenting arguments in favor of this hypothesis, the spectral shifts will be discussed, and charge-transfer interactions and aggregation effects will be examined as alternative explanations.

The usual formulae for solvent red shifts on increasing the refractive index (Bayliss and McRae, 1954; McRae, 1957) always contain the dipole strength of the transition. It is evident that this could not possibly account for the spectra of the porphyrin complexes: the shift of the allowed Soret band is only about three times that of the 100-fold weaker red band I. Even if all of the increase in width of the Soret band is caused by such a shift, it would still be less than ten times the shift of band I. This discrepancy has also been noted in other pigments (see, e.g., West and Geddes, 1964). More complete theoretical discussions of solvent effects on spectra include just such terms independent of the transition moment but depending on the excitation and ionization energies of the pigment and solvent (Basu, 1964; Liptay, 1964). This term dominates for very weak bands, and since it produces to a first approximation a shift proportional to the excitation energy of the band, the result is compatible with observations.

The effect of charge-transfer interactions (see Briegleb, 1961) on these spectra is also small. No new bands are detectable and only small changes in dipole strength of the bands are observed. This effect, however, may contribute to the spectral shifts in the case of the dipyridyl derivatives to be discussed later.

Aggregation (or disaggregation) of the porphyrin is a possible cause of the observed spectral changes. The theory of the spectra of pigment aggregates predicts a variety of wavelength shifts and band broadening or narrowing depending on the strength of interaction (DeVoe, 1964) and orientation of transition moments (McRae and Kasha, 1964). The spectral changes observed in the porphyrin complexes are definitely not related to those observed on aggregating the porphyrin by protonating the carboxylate side chains in mild acid. Not only are the spectral changes on aggregation different, causing large broadenings of both Soret and visible bands, but the fluorescence is completely quenched. This is presumably caused by the close contact of the porphyrin planes, which results in a strong interaction of the transition moments. In contrast, the fluorescence of most of the complexes is only slightly quenched. The wavelength shift of the fluorescence shows that it is the excited state of the complex that emits light. Moreover, the excitation spectra for this fluorescence (Table II) corresponds to that of the complex, not to that of the free porphyrin. No appreciable change in structure occurs during the lifetime of the excited state. The similarity of the changes of the fluorescence and absorption bands (I and II) indicate, moreover, that no large change of polarizability or dipole moment occurs in the fluorescing excited state.

The question of aggregates in the present porphyrin-addend system was investigated by the method of extreme dilution. The data presented in the experimental section show that if any dimer or aggregate of uroporphyrin is present at all, it has a very strong inter-
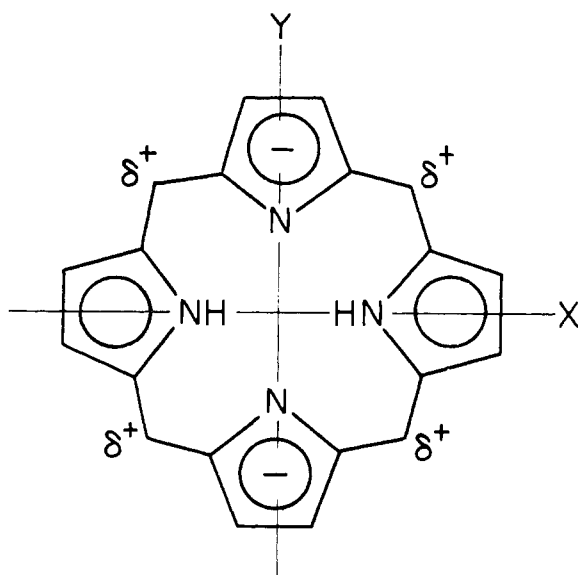
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FIGURE 3: Free base porphyrin, showing well-defined x- and y-axes.

action energy indeed. The association constant would have to be greater than 10^9 M^{-1}. By comparison, the association constant for the "dimer" of phthalocyanine-tetrasulfonate (Bernauer and Fallab, 1962) in water at 25° is about 10^8 M^{-1}. The heat of dimerization calculated from their data is -12 kcal/mole of dimer. Since the area for increased dispersion (or hydrophobic) interactions is less in uroporphyrin, and the repulsive charge density is higher, the association should be less pronounced. In fact, for copper uroporphyrin, the dimer association constant can be estimated to be $\sim 10^4$ M^{-1} from the data of Blumberg and Peisach (1965). Their analysis of the electron spin resonance spectrum of copper uroporphyrin shows that it exists as a sandwich-type dimer in alkaline solution at relatively high concentrations (10^{-2} M). The Cu-Cu "bond" of 3.5 A would contribute very little to the binding constant. This dimer is dissociated by dilution (10^{-4} M) and by amines and heterocyclic compounds. The "monomer" in the latter case is undoubtedly a complex of the type described here. The absorption band maxima quoted are in agreement with this supposition. All evidence thus points to a monomeric state for uroporphyrin in dilute solution.

An interesting variation of the subject of aggregates is that the addends, particularly if cations, allow the formation of an alternating chain of porphyrin-addend units. This kind of structure localizes the pigment in groups in the solution and can lead to an optical heterogeneity effect: the strongly absorbed regions in a strong band are washed out by "stray" light. The difference of 20-fold in the absorptivities of the Soret and visible bands would discriminate between the two regions. For example, in a cluster of 1000 units, light would be largely absorbed in the Soret region, but only slightly in the visible region. The theory of light

absorption in a heterogeneously distributed pigment system (Duysens, 1956; Gledhill and Julian, 1963; see also Butler, 1962) shows that the absorption efficiency decreases markedly only at wavelengths of high absorption in the cluster, thus leading to a flattening of the spectra. This effect predicts a loss in observed dipole strength, since the absorption "saturates" at high absorptivities. A calculation shows that the observed halving of intensity of the Soret band requires at least a 25% loss in dipole strength, but a maximum loss of only 6% is observed. A possible example of this heterogeneous light absorption effect, aside from the in vivo example given by Duysens (1956), is the spectrum of the acid aggregate of copper uroporphyrin (Blumberg and Peisach, 1965). About one-third of the dipole strength is apparently lost relative to the spectrum in dilute alkaline solution. The acid aggregates of uroporphyrin itself also show this washed-out spectra. The "sitting-atop" complexes of $\alpha,\beta,\gamma,\delta$-tetrapyridylporphyrin with cations in acid media (Fleischer et al., 1964) show characteristics of such aggregation, and the tetrapositive porphyrin may be held together by the nitrate anions.

An independent confirmation of this point was obtained by observing the fluorescence depolarization. Long chains of porphyrin-adduct units would be expected to cause polarization by prolonging at least the out-of-plane rotational relaxation time of the porphyrins beyond the fluorescence lifetime. Even for the axially symmetric case a polarization of $+^1/_7$ is expected in the limit, whereas none ($p < 0.01$) was observed. By the Stokes-Einstein equation, the radius of the complex must be less than ~ 30 A (lifetime $10^{-7}s$). A two- or three-unit chain, or a very loose aggregate with random orientation of porphyrin planes to give depolarization by energy transfer, would be missed by these purely optical studies. A thorough study by sedimentation or similar size-dependent method of analysis could be used to confirm this point.

Having shown that various alternative hypotheses cannot explain the spectra of these porphyrin complexes, a qualitative explanation in terms of the nearly degenerate levels of the porphyrin spectra will be attempted. The best available description of the spectra of porphyrins of high symmetry (D_{4h}) such as the dications, the dianions, or the nontransition metal chelates is that the narrow (10–15 mμ), intense ($\epsilon \sim 5 \times 10^5$) Soret band is a doubly degenerate allowed transition and the two weaker ($\epsilon \sim 10^4$) visible bands are symmetry-forbidden counterparts (Gouterman, 1961; Solov'ev, 1961). In the free base porphyrin, well-defined x- and y-axes exist (Figure 3) and the resulting decrease in symmetry (D_{2h}) removes the degeneracy, causing the Soret band to broaden (25 mμ) and the visible bands to double in number. Measurements of the polarization of fluorescence (Gurinovich et al., 1961; Gouterman and Stryer, 1962) and of reflection (Anex and Umans, 1964) indicate that only the farthest red band (I) is highly polarized along one axis, and that this axis (x) contains the N-H bonds. The other visible bands have mixed polarizations. In the Soret band the two polarizations overlap, and it is the long wavelength

side that is polarized as band I. If the broadening of the Soret band in the porphyrin complexes is caused by a change in energy separation of the X and Y components, band I may show a spectral shift but should not broaden. This is what is observed. In contrast to the doubling of the width of the Soret band (1500 cm^{-1} increase) in the substituted pyridinium complexes, band I narrows slightly ($\sim$60 cm^{-1}). Moreover, as expected, the visible bands of mixed polarization broaden slightly and increasingly: II, 50 cm^{-1}; III, 160 cm^{-1}; IV, 180 cm^{-1}. Further confirmation of this interpretation comes from the effect of these complexes on the spectra of porphyrins with high symmetry, where the energy levels leading to the Soret band are closely degenerate. The Soret band of both uroporphyrin dication in acid with caffeine and zinc coproporphyrin with 1-(2-hydroxyethyl)-3-carbamylpyridinium chloride are almost doubled in width, but have only very slight shifts in wavelength.

In detail, the spectral changes may be conveniently discussed in two parts. In the first, the arrangement of the highly polar and hydrogen-bonded water molecules about the porphyrin plane is changed. The resulting symmetrical splitting of x and y components of the energy levels is most clearly seen in the complexes of the porphyrin with alkylammonium cations, and possibly with such addends as caffeine. The Soret band broadens symmetrically ($\sim$500 cm^{-1}) and even the visible bands I and II move to longer wavelengths ($\sim$200 cm^{-1}), while the bands III and IV move to shorter wavelengths ($\sim$100 cm^{-1}). In the second part of the spectral changes a more specific interaction of the addend and the porphyrin occurs. The spectra shift to the red, but the components along the x-axis are much more affected than those along the y-axis. This leads to a large, asymmetrical broadening of the Soret band, red shifts of bands I and II, and no shifts of bands III and IV. A tempting model is to localize the addend over one of the axes of the porphyrin plane. For the cations the charge distribution of Figure 3, adapted from Woodward and Skaric (1961), would favor the y-axis.

With certain addends such as protonated phenanthroline and γ,γ'-dipyridyl or the viologens, an additional red shift of all of the bands is observed. It is just these addends that severely quench the fluorescence of the porphyrin. A direct electron overlap may make a contribution to the interaction energy. In fact, the extent of this interaction measured by the red shift of band I increases in the order caffeine < pyridine, dipyridyls, o-phenanthroline < 1-alkyl-3-carbamylpyridinium cations < protonated γ,γ'-dipyridyl < viologens, protonated o-phenanthroline. This is just the order of the ability of these molecules and their protonated forms to act as electron acceptors as measured by their polarographic reduction potential (Volke, 1963).

Caffeine has the least broadening effect on the Soret band. This may be caused by the complex distribution of its polar groups and possibly by the differing orientation of several molecules about the porphyrin, leading to an averaging out of its interactions. It is possible that at high caffeine concentration the porphyrin dissolves in preformed caffeine micelles or helices. This could explain the specificity found by Keilin (1943) for the "caffeine effect," as very few uncharged molecules have the delicate balance of intermolecular forces necessary to form such aggregates in aqueous solution. Studies by light scattering and with the ultracentrifuge would serve to solve this problem.

Applications. This class of "solvation complexes" may explain many pecularities of porphyrin and phthalocyanine spectra and reactions. Solutions of these pigments in some solvents may show spectral changes in addition to those expected from general media effects. Pyridine, a very common solvent, is particularly troublesome because both pyridine and pyridinium ion form complexes and with different spectra. This causes measurements to be dependent on traces of acids or bases. Such an effect may contribute the spectral changes caused by adding ammonia to a pyridine solution of magnesium etioporphyrin (Allison and Becker, 1963). The 650-mμ band explained as a charge-transfer band is most likely an oxidation product of the porphyrin. The "pyridinium salts" of the tetraazaporphyrins and phthalocyanines described by Whalley (1961) are very likely the kind of complex under discussion. The "seventh" addend of the ferri-coproporphyrin-hydroxy-dipyridinate postulated by Clark and Perkins (1940) may be pyridine bound to the ring, not to the iron atom. Similarly, the complexes of hematin with hindered heterocyclic bases such as 2,6-lutidine (Scheler and Jung, 1957) may involve binding to the porphyrin ring as much as to the iron atom. The shifts and "doublings" of the Soret band of transition metal porphyrin chelates in various solvents and freely ascribed to d-orbital interactions (Caughey *et al.*, 1962; Whitten *et al.*, 1963) and to π-electron "steric" effects (Corwin *et al.*, 1963) may contain a considerable contribution from this type of solvation complex. The temperature sensitivity of these spectral changes agrees with this supposition. In some of these studies a change in the state of aggregation of the pigment when a complex is formed will contribute to the observed spectral changes.

Some of the variation in the hemochromogen spectra of the heme proteins may be caused by having imidazole or similar groups more parallel to the heme plane than in the usual perpendicular position on the iron atom. Such a variation offers intriguing possibilities for transforming redox energy to phosphate bond energy by transferring the phosphate group from the iron to the imidazole following a redox reaction.

The superiority of aqueous pyridine as a solvent for the photoreduction of chlorophyll (Krasnovskii reaction) has been well substantiated (Krasnovskii, 1960; Bannister, 1959). The study by Seely and Folkmanis (1964) ascribes some of the subtleties of the reaction to chlorophyllide-ethanol-pyridine solvates, and to pyridinium-ascorbate ion pairs. A marked simplification of the data is achieved if the most reactive species is the pyridinium-chlorophyllide complex. As pointed out in the introduction, these porphyrin complexes were found by their effect on the photochemistry of the porphyrins.

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Possibly one can move a step closer to the goal of the efficient energy utilization of photosynthesis by a study of these effects.

It is interesting that so many molecules of biological occurrence, e.g., purines and pyrimidines, aromatic amino acids, riboflavin, and porphyrins, have such a tendency to form complexes in aqueous solution. It would be of obvious value to collect the molecules of prebiological origin into clusters to favor their inter-reactions. Such a selection imposed on their ready formation (cf. Beilstein's Handbuch) could account for the wide range of heterocyclic molecules found in biological systems.

Acknowledgment

References

Allison, J. B., and Becker, R. S. (1963), *J. Phys. Chem.* 67, 2675.

Anex, B. G., and Umans, R. S. (1964), *J. Am. Chem. Soc.* 86, 5026.

Bannister, T. T. (1959), *Plant Physiol.* 34, 246.

Basu, S. (1964), *Advan. Quantum Chem.* 1, 145.

Bayliss, N. S., and McRae, E. G. (1954), *J. Phys. Chem.* 58, 1002, 1006.

Bernauer, K., and Fallab, S. (1962), *Helv. Chim. Acta* 45, 2487.

Blumberg, W. E., and Peisach, J. (1965), *J. Biol. Chem.* 240, 870.

Briegleb, G. (1961), Elektronen-Donator-Acceptor-Komplexe, Berlin, Springer-Verlag, p. 196.

Butler, W. L. (1962), *J. Opt. Soc. Am.* 52, 292.

Caughey, W. S., Deal, R. M., McLees, B. D., and Alben, J. O. (1962), *J. Am. Chem. Soc.* 84, 1735.

Clark, W. M., and Perkins, M. E. (1940), *J. Biol. Chem.* 135, 643.

Cohen, M. D., and Fischer, E. (1962), *J. Chem. Soc.*, 3044.

Corwin, A. H., Whitten, D. G., Baker, E. W., and Kleinspehn, G. G. (1963), *J. Am. Chem. Soc.* 85, 3621.

DeVoe, H. (1964), *J. Chem. Phys.* 41, 393.

Diamond, R. M. (1963), *J. Phys. Chem.* 67, 2513.

Duysens, L. N. M., (1956), *Biochim. Biophys. Acta* 19, 1.

Fleischer, E. B., Choi, E. I., Hambright, P., and Stone, A. (1964), *Inorg. Chem.* 3, 1284.

Gledhill, R. J., and Julian, D. B. (1963), *J. Opt. Soc. Am.* 53, 239.

Gouterman, M. (1961), *J. Mol. Spectry.* 6, 138.

Gouterman, M., and Stryer, L. (1962), *J. Chem. Phys.* 37, 2260.

Gurinovich, G. P., Sevchenko, A. N., and Solev'ev, K. N. (1961), *Opt. Spectry. (USSR)* 10, 396.

Keilin, J. (1943), *Biochem. J.* 37, 281.

Krasnovskii, A. A. (1960), *Ann. Rev. Plant Physiol.* 11, 363.

Lemberg, R., and Legge, J. W. (1949), Hematin Compounds and Bile Pigments, New York, Interscience, p. 241.

Liptay, W. (1964), *Angew. Chem. Intern. Ed. Engl.* 3, 756.

McBain, M. E. L., and Hutchinson, E. (1955), Solubilization, New York, Academic, p. 85.

McRae, E. G. (1957), *J. Phys. Chem.* 61, 562.

McRae, E. G., and Kasha, M. (1964), in Physical Processes in Radiation Biology, Augenstein, L., Mason, R., and Rosenberg, B., eds., New York, Academic, p. 23.

Mauzerall, D. (1962a), *J. Am. Chem. Soc.* 84, 2437.

Mauzerall, D. (1962b), *J. Phys. Chem.* 66, 2531.

Némethy, G., and Scheraga, H. A. (1962), *J. Phys. Chem.* 66, 1773.

Osipow, L. I. (1962), Surface Chemistry, New York, Reinhold, p. 167.

Rossotti, F. J. C., and Rossotti, H. (1961), The Determination of Stability Constants, New York, McGraw-Hill, p. 47.

Scheler, W., and Jung, F. (1957), *Biochem. Z.* 329, 222.

Seely, G. R., and Folkmanis, A. (1964), *J. Am. Chem. Soc.* 86, 2763.

Sheppard, S. E. (1942), *Rev. Mod. Phys.* 14, 303.

Solov'ev, K. N. (1961), *Opt. Spectry. (USSR)* 10, 389.

Volke, J. (1963), Physical Methods in Heterocyclic Chemistry, Vol. I, A. R. Katritzky, ed., New York, Academic, p. 288.

West, W., and Geddes, A. L. (1964), *J. Phys. Chem.* 68, 837.

Whalley, M. (1961), *J. Chem. Soc.*, 866.

Whitten, D. G., Baker, E. W., and Corwin, A. H. (1963), *J. Org. Chem.* 28, 2363.

Woldbye, F. (1955), *Acta Chem. Scand.* 9, 299.

Woodward, R. B., and Skaric, V. (1961), *J. Am. Chem. Soc.* 83, 4676.