BIOORGANIC CHEMISTRY 8, 69–81 (1979)

Dimerization Constants of Water-Soluble Porphyrins in Aqueous Alkali[1]

GAIL ANNE KARNS,[2] WAYNE A. GALLAGHER,[3] AND WILLARD B. ELLIOTT*

*Department of Chemistry, Niagara University, Niagara University, New York 14109, and *Department of Biochemistry, State University of New York at Buffalo, Buffalo, New York 14214*

Received April 5, 1978

Spectrophotometric analysis of changes in absorption spectra on dilution of different 2,4-disubstituted derivatives of deuteroporphyrin yielded dimerization constants (K_D) for each porphyrin in aqueous alkali. The K_D values appear to be related to the hydrophobic/hydrophilic interactions of the system such that K_D for proto- > meso- > deutero- > hemato- > coproporphyrin. The effects of alcohol, temperature, and ionic strength on the K_D were examined. A simple approach to the graphic analysis of the dilution curves is presented for use when absorbance readings at A_{100} and A_0 cannot be reliably determined, and the use of soluble porphyrins as model systems for studying hydrophobic/hydrophilic interactions in aqueous media is discussed.

INTRODUCTION

While it has long been known that most porphyrins and their derivatives tend to aggregate under certain conditions in aqueous solutions, quantitative work on the water-soluble porphyrins seems to have been limited to the meso-tetra-substituted porphyrins which are relatively easy to synthesize and purify (*1*). More recently, White and Plane (*2*) have reported dimerization constants for an homologous series of synthetic water-soluble diamine derivatives of protoporphyrin under varying conditions of ionic strength, pH, and temperature. Brown *et al.* (*3*) have carried out equilibrium and kinetic studies on the aggregation of deuteroporphyrin derivatives in aqueous solution and found that at concentrations below 4 μm the aggregation of the porphyrins is probably limited to dimerization, and at higher concentrations of porphyrin a more complicated "micellization" occurs which is affected by changes in temperature and pH.

In a review of some earlier work on the aggregation of porphyrin derivatives, we reported changes in the absorption spectra upon dilution of certain iron-free porphyrins in aqueous alkali, which seemed to represent a monomer–dimer equilibrium with hydrophobic/hydrophilic interactions playing a major role (*4*). In the present paper we have extended these ideas and applied them to several deuteroporphyrin derivatives, determined the dimerization constants of the porphyrins, and studied some of the factors which influence the monomer–dimer equilibria.

[1] This investigation was supported by USPHS Grant GM06241.

[2] Present address: Department of Biochemistry, Purdue University, West Lafayette, Ind. 47907.

[3] To whom correspondence regarding this paper should be sent.

0045-2068/79/010069-13$02.00/0
Copyright © 1979 by Academic Press, Inc.
All rights of reproduction in any form reserved.
Printed in Great Britain

The distinctive spectral properties of this system and the parameters which affect the absorption spectra suggest that it might be an excellent model for studying hydrophobic/hydrophilic interactions in aqueous solutions.

EXPERIMENTAL

Porphyrins

The absorption band positions and extinction coefficients of several commercially available samples of porphyrins were determined and compared with standard reference values (5). On the basis of their agreement with the standard values, the following samples were chosen and used without further purification. Deuteroporphyrin, coproporphyrin, and protoporphyrin were purchased from Porphyrin Products, Logan, Utah. Hematoporphyrin dihydrochloride (Lot No. 3111) was purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio, and mesoporphyrin was purchased from Pfaltz & Bauer, Inc., Flushing, New York.

Solutions

Stock porphyrins were made fresh each day in 0.02 *M* NaOH and used within a 6-hr period. Concentrations of porphyrin solutions are given in terms of all the porphyrin existing in dimeric form.

Spectrophotometry

Absorption spectra were recorded on a Cary 14 or a Bausch & Lomb 505 recording spectrophotometer using quartz cuvettes and spacers to vary the light paths as noted. In experiments designed to show isosbestic points associated with a simple monomer–dimer equilibrium (e.g., Fig. 1), the concentration of the porphyrin in each of the superimposed spectra is inversely proportional to the light path so that the amount of chromophore in the light path remains constant.

The exceptional tendency for meso- and protoporphyrin to adhere to glass surfaces necessitated scrupulous cleansing of cuvettes and spacers with detergent followed by a methanol rinse between sample readings.

Graphical Analysis

In spectrophotometric titrations where superimposed absorption spectra yield isosbestic points, the results can usually be expressed by the equation

$$\alpha = \frac{A_x - A_0}{A_{100} - A_0}, \qquad [1]$$

where α is the fraction of the porphyrin existing as monomer, A_0 is the absorbance of the porphyrin when it is 100% dimer, A_{100} is the absorbance when the porphyrin is 100% monomer, and A_x is the absorbance of the mixture of monomer and dimer at intermediate concentrations of the porphyrin. All of the above readings of absorbance must be taken at a wavelength other than one of the isosbestic wavelengths.

Porphyrins such as protoporphyrin and mesoporphyrin do not yield reliable readings of A_{100} and A_0 in aqueous media. Consequently, we used a fixed ratio of concentrations

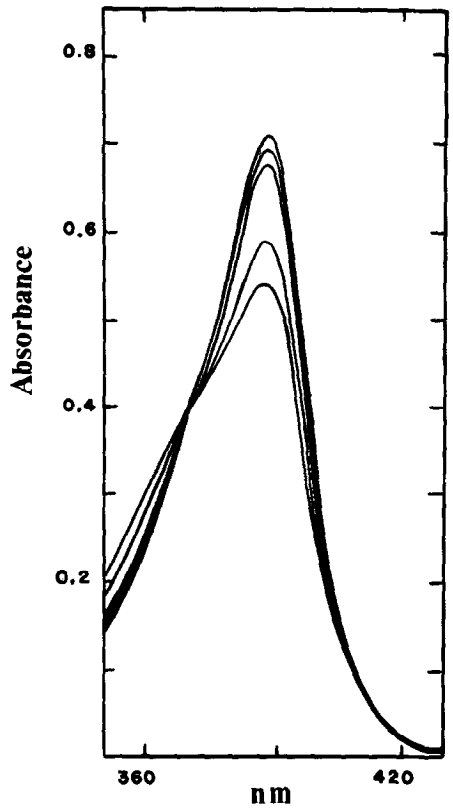


FIG. 1. Superimposed absorption spectra of a dilution experiment in 0.02 *M* NaOH of coproporphyrin. Concentration ratios are $x{:}2x{:}10x{:}50x{:}100x$, where $100x = 18 \times 10^{-6}$ *M* dimeric porphyrin. Light paths are 10 cm, 5 cm, 1 cm, 2 mm, and 1 mm, respectively. The highest Soret peak is x. The absorption spectra were recorded on a Bausch & Lomb 505 recording spectrophotometer.

$(1x{:}2x{:}10x{:}50x{:}100x)$ in order to construct a table of α values for each concentration based on the equation

$$K_D = \frac{M(1-\alpha)}{(2M\alpha)^2}, \qquad [2]$$

where K_D is the dimerization constant, M is the molar concentration of porphyrin dimer, and α is as in Eq. [1]. Thus for an ideal solution where concentration $1x$ corresponds to $\alpha_1 = 0.50$, it can be calculated that for concentration $2x$, $\alpha_2 = 0.39$, for $10x$, $\alpha_3 = 0.20$, for $50x$, $\alpha_4 = 0.095$, and for $100x$, $\alpha_5 = 0.068$. Taking the differences between successive values of α and dividing each difference by the difference between α_1 and α_2, the following ratios are obtained: 1:1.73:0.95:0.25.

Once a reference table is constructed for concentration $1x$ having values of α_1 from 0.99 down to 0.01 and the corresponding values for α_2, α_3, α_4, and α_5 are calculated in each case, the differences between successive values of α divided by $\alpha_1 - \alpha_2$ for each

group of five concentrations yields a table of ratios which allow the spectrophotometric data to be analyzed according to the following relationship derived from Eq. [1]:

$$\frac{\alpha_1-\alpha_2}{\alpha_1-\alpha_2}:\frac{\alpha_2-\alpha_3}{\alpha_1-\alpha_2}:\frac{\alpha_3-\alpha_4}{\alpha_1-\alpha_2}:\frac{\alpha_4-\alpha_5}{\alpha_1-\alpha_2}=\frac{A_1-A_2}{A_1-A_2}:\frac{A_2-A_3}{A_1-A_2}:\frac{A_3-A_4}{A_1-A_2}:\frac{A_4-A_5}{A_1-A_2}. \quad [3]$$

As long as the concentrations used in determining A_1, A_2, A_3, A_4, and A_5 are in the same ratio as the concentrations used in the construction of the table of ratios of α, simple calculations directly from absorbance readings as in Eq. [3] and comparison to the calculated table of ratios determine whether the correct model curve has been chosen and, if so, precisely where the experimental data fall on this theoretical curve.

RESULTS

Typical porphyrin dilution experiments with superimposed absorption spectra showing isosbestic points are given for coproporphyrin and hematoporphyrin in Figs. 1 and 2, respectively. In both experiments, the light path varies inversely to the concentration of the porphyrin so that the amount of chromophore in the light path remains constant. Although only the Soret region of the spectra is shown here, isosbestic points are present in the visible region as well. The presence of isosbestic points in this type of experiment permits analysis of the spectral changes according to Eq. [1].

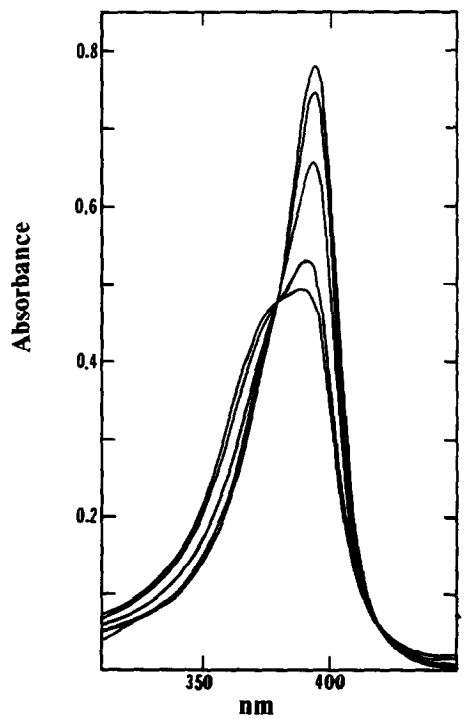


FIG. 2. Dilution experiment in 0.02 M NaOH of hematoporphyrin. Details are same as in Fig. 1. $100x = 22.5 \times 10^{-6}$ M dimeric porphyrin. A Cary 14 recording spectrophotometer was used.

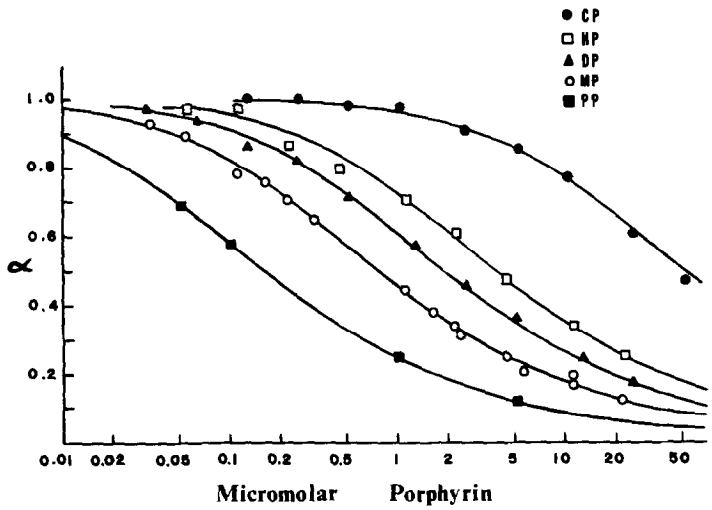


FIG. 3. Data from dilution experiments for all five porphyrins plotted as α vs micromolar dimeric porphyrin concentration. Solid lines are best fit of theoretical curves for monomer–dimer equilibrium.

Figure 3 is a plot of experimentally determined values of α vs log dimeric porphyrin concentration for the five porphyrins studied. The solid lines are theoretical curves for the dissociation of dimeric porphyrin into monomeric porphyrin, upon dilution, according to Eq. [2].

Although all the curves have the same shape, their positions along the abscissa are seen from Eq. [2] to be a function of the dimerization constant of the particular porphyrin. Each of the curves represents a best fit of the theoretical curve to the experimental data. The values for K_D of the individual porphyrins determined in this manner are listed in Table 1.

In Fig. 4, a dilution experiment of deuteroporphyrin in 0.02 *M* NaOH is superimposed on an identical deuteroporphyrin dilution experiment from the same stock solution. The medium in the second experiment is still 0.02 *M* NaOH but contains 10% ethanol (v/v) as well. Note that both sets of experimental curves go through the same isosbestic points. The titration of deuteroporphyrin with ethanol, again keeping the alkali concentration constant, is shown in Fig. 5. The superimposed spectra pass through the isosbestic points up to an alcohol concentration of about 20% (v/v). Above that concentration, the spectra do not go through the isosbestic point and the

TABLE 1

Porphyrin	2- and 4- Substituents	Dimerization constant, 25°C (M^{-1})
Protoporphyrin	Vinyl	3.1×10^6
Mesoporphyrin	Ethyl	6.3×10^5
Deuteroporphyrin	Hydrogen	2.6×10^5
Hematoporphyrin	α-Hydroxyethyl	1.7×10^5
Coproporphyrin	Propanoic acid	9.8×10^3

data are no longer amenable to simple graphical analysis. The relationship between the dimerization constants of the porphyrins and the alcohol concentration is depicted in Fig. 6. Similar experiments using urea instead of alcohol appear to be completely analogous.

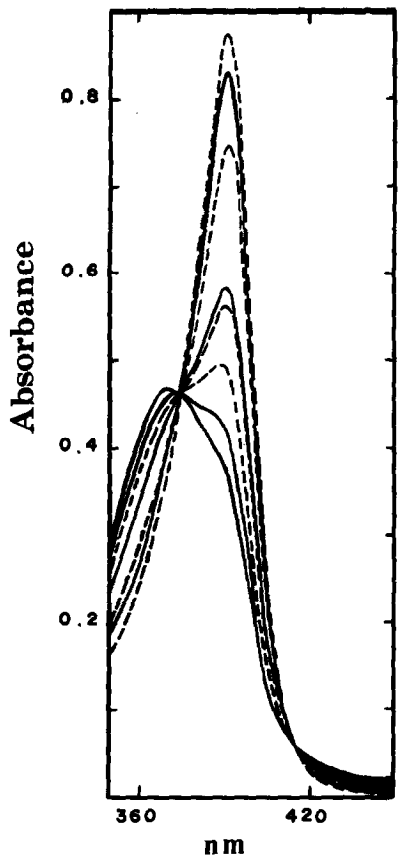


FIG. 4. Two superimposed dilution experiments run on deuteroporphyrin. Solid lines represent experiment in 0.02 M NaOH, dashed lines represent identical experiment in 10% ethanol (v/v). Concentration ratios shown are x:10x:50x:100x. 100x = 25.9 × 10^{-6} M dimeric porphyrin. The Cary 14 was used.

The effect of temperature on the porphyrin monomer–dimer equilibrium is shown in Fig. 7. Identical samples of hematoporphyrin were equilibrated for at least 30 min at the indicated temperatures before recording the differences in absorption spectra. When log K_D at the different temperatures is plotted against $1/T$, the slope of this Arrhenius plot allows calculation of the ΔH^0 of dimerization as −6.1 kcal/mol. From the relationship

$$\Delta G^0 = -RT \ln K_D$$

the free energy of dimerization at 25°C is found to be −6.9 kcal/mol, and the entropy of dimerization is calculated at +2.7 cal/mol-degree.

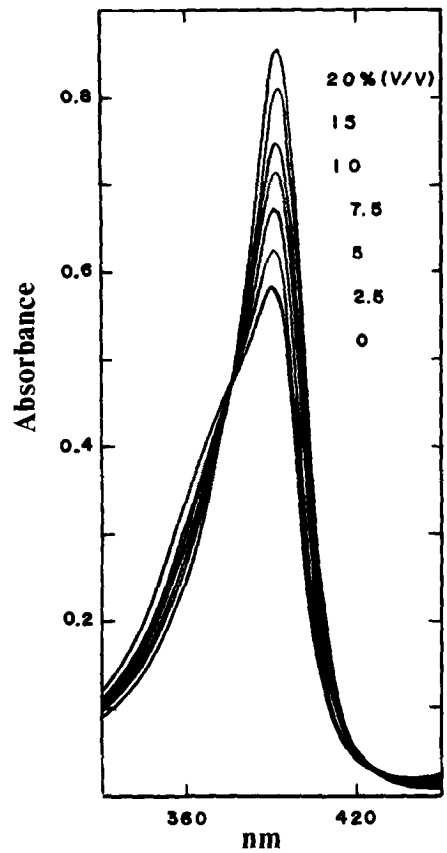


FIG. 5. Superimposed spectra of 2.50×10^{-6} M dimeric deuteroporphyrin showing the effect of increasing concentration of alcohol. Light path is 1 cm. The Cary 14 was used.

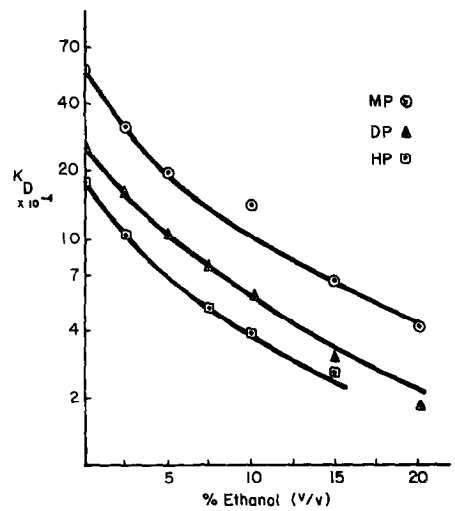


FIG. 6. Data showing the effect of increasing alcohol concentration on the dimerization constants of three different porphyrins. Light path is 1 cm. The Cary 14 was used.

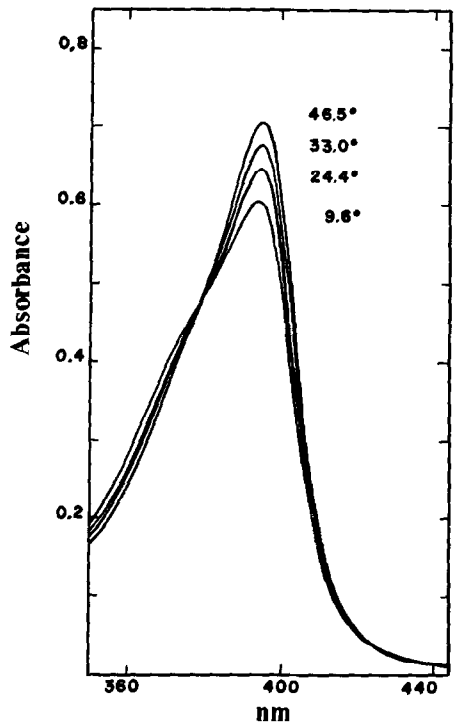


FIG. 7. Superimposed spectra of 2.25×10^{-6} *M* dimeric hematoporphyrin in 0.02 *M* NaOH equilibrated and run at the indicated temperatures. Light path is 1 cm. The Bausch & Lomb 505 was used.

Within the limits of the methodology, it appears that the isosbestic points in all the above experiments (i.e., the simple dilution, the alcohol titration, and the temperature variation) occurred at the same wavelength for a given porphyrin as long as the conditions did not vary appreciably from those of an aqueous medium.

Changes in the ionic strength of the medium do not have a strong effect on the K_D values of these porphyrins, although when dilution experiments were run on protoporphyrin and mesoporphyrin the changes in absorption spectra were much more amenable to graphic analysis when the dilution was done in 0.01 *M* NaOH rather than the usual 0.02 *M* NaOH. Further, the effect of increasing the ionic strength of the medium, even though slight, was to shift the equilibrium in favor of the dimer.

DISCUSSION

Porphyrin Sample

The wide variance in the absorption spectra of supposedly the same porphyrin purchased from different supply houses necessitates considerable caution on the part of the worker using commercial porphyrin products. The positions of the absorption bands as well as the extinction coefficients showed wide variance in most cases. The samples listed above were chosen because they conformed to the reference standards (*5*) and were used without further purification because they gave excellent isosbestic

points on dilution. As these commercial samples were listed at "purity greater than 95%" the values reported for K_D in Table 1 may be slightly low.

Graphical Analysis

In the dilution studies on hematoporphyrin, coproporphyrin, and deuteroporphyrin, their relatively high solubilities in aqueous alkali and their comparatively low dimerization constants permitted sufficient dilution of the samples to the extent that essentially all of the porphyrin was in the monomeric state. This direct determination of A_{100} facilitates the graphical analysis, and the experimental data were shown to fit the theoretical curves which were calculated from Eq. [2] and which indicated that the changes in the absorption spectra of the porphyrins on dilution were due to a monomer–dimer equilibrium. Use of our "fixed ratio of concentrations technique" involving the matching of theoretical ratios of $\Delta\alpha/(\alpha_1 - \alpha_2)$ with the experimentally determined ratios of $\Delta A/(A_1 - A_2)$ also confirmed that the spectral changes were due to a monomer–dimer equilibrium.

In the cases of protoporphyrin and mesoporphyrin dilution experiments, the tendency of these porphyrins to stick to glass surfaces, their high K_D values, their relatively low solubilities, and their strong tendencies to aggregate beyond the simple dimeric state made it impossible to determine reliable values for either A_{100} or A_0. Only by washing the cuvettes most thoroughly between measurements on individual samples to remove adhering porphyrin and by working over as short a time interval as possible in order to minimize changes in the stock solutions was it possible to show that these porphyrins also exhibited isosbestic points on dilution similar to those shown by coproporphyrin in Fig. 1. The application of the "fixed ratio of concentrations technique" to these data gave an excellent fit of the experimental data to the monomer–dimer equilibrium curves as shown in Fig. 3.

Our K_D values are in good agreement with K_D values for other water-soluble porphyrins reported by White and Plane (*2*) and generally agree with the earlier estimates of K_D values for deuteroporphyrin derivatives by Brown *et al.* (*3*) with the exception of the K_D value for coproporphyrin which we find to be an order of magnitude lower. It should be noted that while our work was done in aqueous alkali, these other workers used different buffer systems.

Alcohols and K_D

The effectiveness of alcohols in dispersing organic dye aggregates into monomers is well known (*6*). Apparently, the same dispersing effect occurs in our porphyrin systems because the monomer–dimer equilibrium of each of the five porphyrins is influenced in the same manner, as is shown for deuteroporphyrin in Fig. 4. The observation that the superimposed dilution spectra, one with alcohol and one without, have the same isosbestic points indicates that while the alcohol causes sufficient change in the dielectric constant of the medium to affect the porphyrin monomer–dimer equilibrium, it is not giving rise, at least at low concentrations, to what are generally termed solvent effects.

Plotting log K_D vs ethanol concentration (Fig. 6) shows the same type of relationship as that found by Moulik *et al.* (*7*) in their work on the dimerization of acridine orange. The addition of ethanol to the medium not only causes some disruption of the water

structure but it also introduces a hydrophobic component into the system. Both of these effects of the alcohol contribute to a more favorable environment for the monomeric porphyrin, resulting in a decrease in K_D for the porphyrin with increasing concentrations of alcohol. As noted above, the effects of changing the ionic strength on K_D in aqueous alkali were quite small, but in solutions where the ethanol concentration was 20% (v/v) they were virtually nonexistent.

Ionic Strength

Although White and Plane (*2*) were able to show a marked effect of ionic strength on the K_D values for their diamine porphyrins, Brown *et al.* (*3*) did not find a significant effect on the dimerization of porphyrins in their system when the phosphate buffer strength was varied between 0.1 and 0.4 *M*. In working with mesoporphyrin and protoporphyrin we found that to get good isosbestic points on dilution of these porphyrins, it was necessary to dilute them in 0.01 *M* rather than the usual 0.02 *M* alkali. We found that the addition of salts such as NaCl to porphyrin solutions had little effect on the absorption spectra while salts such as Li_2SO_4 and Na_2HPO_4 appeared to shift the equilibrium in favor of the dimer. This indicates that the relationship between dimerization and ionic strength must be augmented by an understanding of the chaotropic and antichaotropic potencies of ions demonstrated by Hanstein *et al.* (*8*) and exploited by Nair and Elliott (*9*) in their studies on the aggregation of hemochromogens. When we changed the ionic strength of our porphyrin solutions with Na_2HPO_4 there was not only a change in absorption spectra similar to that produced by the addition of ethanol but a marked shift in the isosbestic point which suggested a more complex phenomenon than a simple monomer–dimer transition.

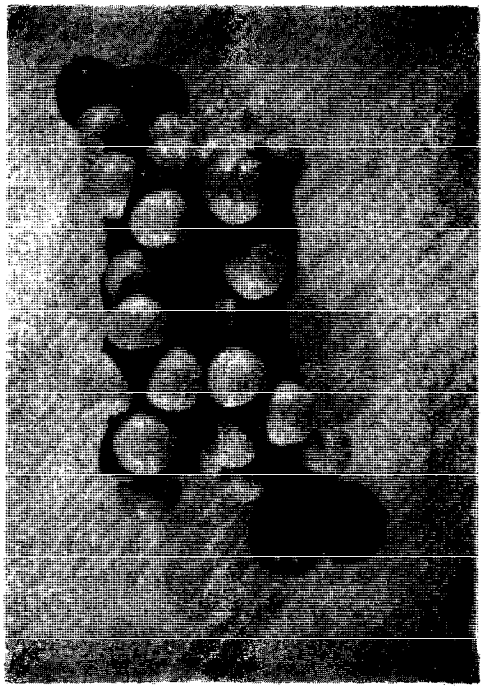
Temperature

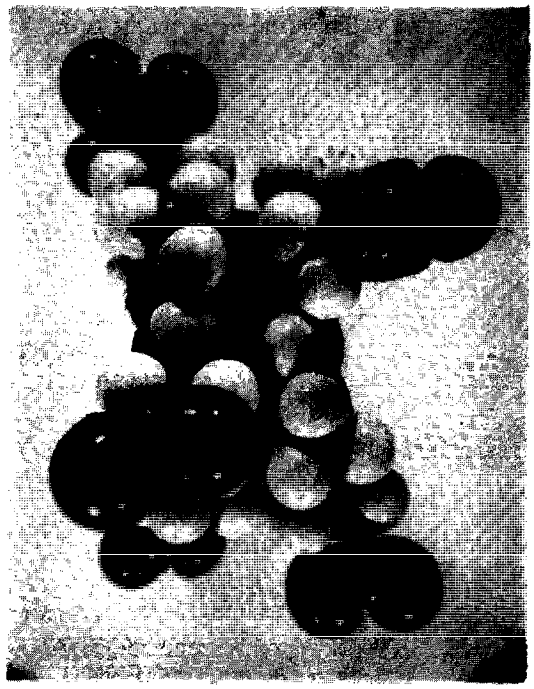
The thermodynamic parameters determined from the changes in the absorption spectra of hematoporphyrin with changes in temperature are similar to those reported by White and Plane (*2*) for their water-soluble porphyrins and fall within the range of values expected for reactions involving hydrophobic/hydrophilic interactions (*10*). Although our data were taken on hematoporphyrin alone and were originally designed to be merely supportive of our main hypothesis that the usual factors involved in hydrophobic/hydrophilic interactions were operative in these porphyrin systems, we have since undertaken a more extensive study of the thermodynamics of this family of porphyrins with particular regard to the influence of ionic strength which will be presented in a later paper.

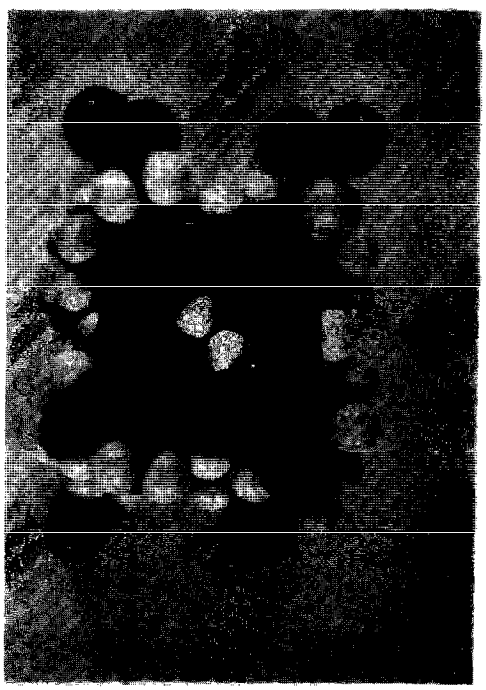
The Model

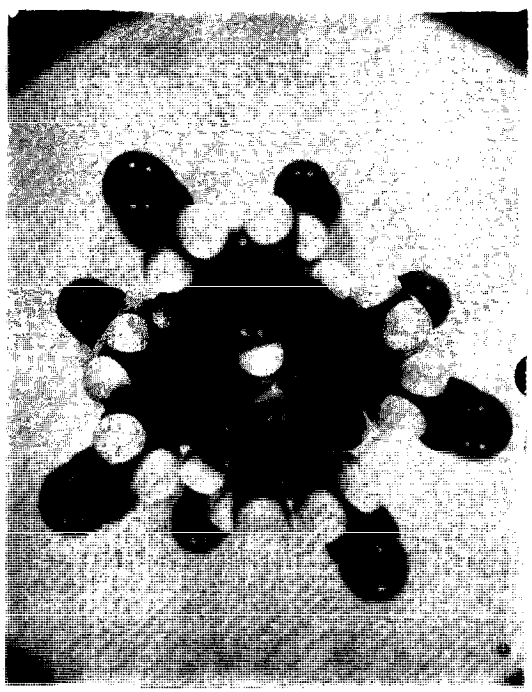
In an effort to more easily relate the dimerization constant to the molecular structure (see Table 1) the porphyrins used in this study were chosen because they have identical

FIG. 8. Space-filling models (C–P–K) of protoporphyrin and coproporphyrin dimers. (a) Top view of the protoporphyrin dimer assuming full overlap of the hydrophobic surfaces of the two protoporphyrin monomers. Note the extension of the propanoate side chains as far from each other as possible. (b) Side view of the same protoporphyrin dimer. (c) Top view of the coproporphyrin dimer. Notice how the relative orientation of the two monomers yields a more or less regular interval between the propanoate side chains. (d) Side view of the same coproporphyrin dimer showing the "zig–zag" relationship existing between most of the adjacent propanoate side chains.









structures (Type III) except for the substituents in positions 2 and 4. The differences in the dimerization constants seem to be directly related to the hydrophobic/hydrophilic character of the substituents and follow a predictable pattern even in the case of coproporphyrin, where the additional aspect of electrostatic repulsion yields a dimerization constant which is an order of magnitude lower than those of the uncharged substituents. This electrostatic repulsion was previously noted by Mauzerall (*11*) as the principle reason why it was virtually impossible for uroporphyrin with its eight carboxylate groups to dimerize in aqueous alkali.

From the experimental evidence available, it appears that the major factors in the dimerization of these porphyrins are the resistance of the aqueous environment to disruption by the hydrophobic component of the porphyrin molecule and the consequent juxtapositioning of these "flat" hydrophobic surfaces to minimize the total hydrophobic area exposed to the aqueous medium. When the concentration of most porphyrins is increased, the aggregation does not stop at the dimerization stage but results in "micellization" (*3*) and ultimately precipitation.

Our experimental evidence and that of others (*12–14*) suggests a dimeric structure consisting of two overlapping rings with the 2- and 4- substituents being part of the overlapped surfaces. This would allow the vinyl groups of protoporphyrin and the ethyl groups of mesoporphyrin to enter into the π–π interaction between the two aromatic rings and thus partially account for the extraordinary stability of these dimeric porphyrins. The negatively charged propanoate groups would be extended as far from each other as possible. This model is probably reflective, at least in part, of the structure of freshly prepared dimeric heme and dimeric hematin. In the dimerization of coproporphyrin, the additional electrostatic repulsion causes a greater separation of the porphyrin rings, which is reflected by a lower value for K_D. The orientation of the overlapping coproporphyrin III monomers in the formation of the coproporphyrin dimer (see Fig. 8) was found by Abraham *et al.* (*15*) to be accompanied by a slight lateral displacement which staggers the propanoate side chains in such a way as to keep them as far from each other as possible. The construction of molecular models of these porphyrin structures is very instructive on this point.

The exceptionally high molar absorptivity of the porphyrins, coupled with the sensitivity of both their absorption spectra and their fluorescence spectra to changes in ionic strength, temperature, dielectric constant, and pH, to their binding of ligands and their binding as ligands, suggests that porphyrin systems are excellent models for the study of the hydrophobic/hydrophilic interactions so prevalent in biochemical systems.

REFERENCES

1. R. F. PASTERNACK, *Ann. N.Y. Acad. Sci.* **206**, 614 (1973).
2. W. I. WHITE AND R. A. PLANE, *Bioinorg. Chem.* **4**, 21 (1974).
3. S. B. BROWN, M. SHILLCOCK, AND P. JONES, *Biochem. J.* **153**, 279 (1976).
4. W. A. GALLAGHER AND W. B. ELLIOTT, *Ann. N.Y. Acad. Sci.* **206**, 463 (1973).
5. "Specifications and Criteria for Biochemical Compounds," 3rd ed. National Academy of Science, Washington, D.C., 1972.
6. E. RABINOWITCH AND L. F. EPSTEIN, *J. Amer. Chem. Soc.* **63**, 69 (1941).
7. S. P. MOULIK, S. GHOSH, AND A. R. DAS, *Indian J. Chem.* **14A**, 306 (1976).
8. W. G. HANSTEIN, K. A. DAVIS, AND Y. HATEFI, *Arch. Biochem. Biophys.* **147**, 534 (1971).

9. B. C. NAIR AND W. B. ELLIOTT, *Bioorg. Chem.* **4**, 326 (1975).
10. P. MUKERJEE AND A. GHOSH, *J. Amer. Chem. Soc.* **92**, 6419 (1970).
11. D. MAUZERALL, *Biochemistry* **4**, 1801 (1965).
12. H. SCHEER AND J. J. KATZ, "Porphyrins and Metalloporphyrins" (K. M. Smith, Ed.). Elsevier, Amsterdam/New York, 1975.
13. T. R. JANSON AND J. J. KATZ, *J. Magn. Reson.* **6**, 209 (1972).
14. R. J. ABRAHAM, F. EIVAZI, H. PEARSON, AND K. SMITH, *J. Chem. Soc. Chem. Commun.*, 699 (1976).
15. R. J. ABRAHAM, P. A. BURBIDGE, A. H. JACKSON, AND D. B. MACDONALD, *J. Chem. Soc. B*, 620 (1966).