Biochem. J. (1970) **117**, 733–739
Printed in Great Britain

Aggregation of Ferrihaems

DIMERIZATION AND PROTOLYTIC EQUILIBRIA OF PROTOFERRIHAEM AND DEUTEROFERRIHAEM IN AQUEOUS SOLUTION

BY S. B. BROWN, T. C. DEAN AND PETER JONES
Department of Physical Chemistry, University of Newcastle upon Tyne, Newcastle upon Tyne NE1 7RU, U.K.

(*Received* 8 *December* 1969)

1. The absorption spectra of deutero- and proto-ferrihaem in aqueous solution at 25°C show marked changes with concentration and pH in the Soret band region. Quantitative studies of these phenomena imply that they are associated with ferrihaem dimerization and with protolytic equilibria involving monomeric (M) and dimeric (D) ferrihaem species according to the scheme:

$$2M \underset{}{\overset{K}{\rightleftharpoons}} D + H^+$$

$$M \overset{K_{a(M)}}{\rightleftharpoons} M' + H^+$$

$$D \overset{K_{a(D)}}{\rightleftharpoons} D' + H^+$$

2. For deuteroferrihaem we obtain $K = 1.9 \times 10^{-2}$, $pK_{a(M)} = 7.1$, $pK_{a(D)} = 7.4$. Protoferrihaem has a much higher dimerization constant, $K = 4.5$ and $pK_{a(D)} = 7.5$ ($pK_{a(M)}$ is not accessible). 3. Possible structural relationships between monomeric and dimeric ferrihaem species in solution are discussed in relation to recent work on the oxo-bridged nature of crystalline ferrihaem dimers.

Several physical and chemical properties of aqueous ferrihaem solutions change with experimental conditions in a manner consistent with the occurrence of aggregation processes, but the interpretation of the nature and extent of polymerization has varied with the property studied. In early work, reviewed by Lemberg & Legge (1949), dialysis, diffusion and ultracentrifuge experiments suggested that ferrihaem solutions are polydisperse, a potentiometric study indicated both monomeric and dimeric species, and a second potentiometric study implied that only monomers exist. These differences were explained to some extent by Shack & Clark (1947), who suggested the possibility of strongly bound dimeric units existing within weakly bound micellae of variable particle size.

The more recent literature, reviewed by Falk (1963, 1964) and by Phillips (1963), supports the view that protoferrihaem is dimeric in aqueous solution. This conclusion depends chiefly on spectrophotometric (Clark & Perkins, 1940; Cowgill & Clark, 1952) and polarographic (Jordan & Bednarski, 1964) measurements. The only recent work to emphasize the importance of aggregation beyond dimers is the kinetic spectrophotometric work of Inada & Shibata (1962). Problems in the interpretation of these experiments are discussed below.

A number of crystalline compounds has recently been characterized as ferrihaem dimers by either i.r. or X-ray studies. The species studied include a protoferrihaem dimer (Brown, Jones & Lantzke, 1969) and a number of related compounds (Vogt, Zalkin & Templeton, 1967; Earnshaw & Lewis, 1961; Cohen, 1969; Fleischer & Srivastava, 1969; Gerloch, McKenzie & Towl, 1968; Bancroft, Maddock & Pandl, 1968). It now seems certain that dimers are formed only in the presence of alkali, the monomeric units being linked by an oxo-bridge between the iron centres (Fe–O–Fe). On addition of acid, in solvents where precipitation does not occur, monomers are re-formed, demonstrating the reversibility of dimer formation (Brown & Lantzke, 1969). Assuming that each iron atom is 0.5 Å out of the plane of the corresponding porphyrin nitrogen atoms in the direction of the bridging oxygen atom (compare the crystal structure of chloroprotoferrihaem; Koenig 1965), that the Fe–O bond length is 1.8 Å, and that the van der Waals distance for the influence of the ligand is 1.8 Å, Brown *et al.* (1969) have constructed a molecular model for protoferrihaem dimer. Dimerization was only

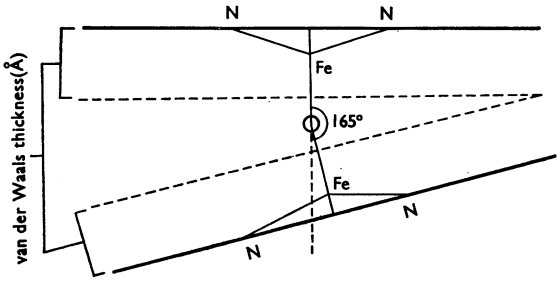


Fig. 1. Section through model of an oxo-bridged ferrihaem dimer.

possible, without distortion of the porphyrin plane, provided that the Fe–O–Fe bond angle was greater than 165° (Fig. 1). The assumptions of this model are supported by the X-ray study of crystalline dimeric iron(III) tetraphenylporphin (Fleischer & Srivastava, 1969), in which the Fe–O–Fe bond angle is reported as 168°, the Fe–O bond length as 1.76 Å and the iron atoms 0.48 Å out of the plane of the corresponding nitrogen atoms. It is clear that the formation of higher polymers by processes of this type is hardly feasible.

The dimerization process in solution may be formally represented by:

$$2M \underset{}{\overset{K}{\rightleftharpoons}} D + nH^+ \qquad (1)$$

where M and D are the monomeric and dimeric species respectively, K is the dimerization constant and n may be a positive or negative integer or zero. The relative proportions of monomer and dimer in a particular solution will depend on the total ferrihaem concentration. Since these species have very different spectra in the region of the Soret band (Brown & Lantzke, 1969) the process may be studied and K and n evaluated, by measuring the absorption spectra of solutions over a range of ferrihaem concentrations and pH. We initially investigated deuteroferrihaem, since it is not susceptible to autoxidation, and it was found that K is in a range that permits precise evaluation. On the basis of this work we extended our study to protoferrihaem.

EXPERIMENTAL

Materials. Chloroprotohaemin was prepared from defibrinated fresh ox blood (Fischer, 1941), and recrystallized once. Determination as pyridine haemochrome indicated a purity of 99.9%, compared with a sample of chromatographically pure material (Fluka A. G., Buchs, Switzerland). Chlorodeuterohaemin was prepared by heating chloroprotohaemin in a resorcinol melt (Falk, 1964) and by recrystallizing once. Pure CO_2-free NaOH solution was supplied by BDH (Chemicals) Ltd., Poole, Dorset, U.K., and buffer components (Na_2HPO_4 and KH_2PO_4) were A.R.-grade materials. All solutions were prepared in distilled water that had been redistilled once from dilute alkaline $KMnO_4$ solution and subsequently from dilute H_3PO_4. The O_2-free N_2 was supplied by British Oxygen Co. Ltd., Birtley, Co. Durham, U.K. Traces of CO_2 and water vapour were removed by passing the gas through columns containing Sofnolite (Sofnol Ltd., London S.E.7, U.K.) and $CaCl_2$.

Preparation of solutions. To avoid problems associated with the effects of atmospheric O_2 on ferrihaem solutions (Brown, Jones & Suggett, 1968), all solutions were prepared under an atmosphere of N_2 that, together with degassing techniques, assured a concentration of molecular O_2 in solution negligible compared with that of ferrihaem. Test solutions were prepared by dilution of a suitable quantity of freshly prepared stock ferrihaem solution (approx. 4.5–5mM) with phosphate buffer of the required pH. For very dilute test solutions (0.1–1.0 μM), the small quantities of stock solution required were accurately measured out with an Agla micrometer syringe. For more concentrated solutions, a weighing technique was employed. In this way, the range of ferrihaem concentrations investigated was 0.1 μM–0.2mM. Buffer solutions (67mM) were prepared from Na_2HPO_4 and KH_2PO_4 mixtures (pH range 6.64–8.04) and an Na_2HPO_4–NaOH mixture (pH 11.0). NaCl was added to maintain an ionic strength of 0.1. pH measurements were made on a Pye Dynacap pH-meter, reading to ±0.01 pH unit.

Spectrophotometric measurements. Extinction measurements were made on a Cary 16 recording spectrophotometer. The cuvette compartment was purged with N_2 during measurements and was thermostatically controlled at 25.0±0.1°C. Scans of extinction against wavelength were made in the region of the Soret peak (370–420nm). The use of cuvettes with a range of path lengths (0.1–10cm) ensured that accurate extinction measurements were possible on the full range of ferrihaem concentrations studied.

RESULTS AND DISCUSSION

Spectra were always obtained within 10min of solution preparation and did not change significantly during the subsequent 24h. Fig. 2 shows spectra obtained at a number of ferrihaem concentrations for both deuteroferrihaem and protoferrihaem at pH 6.98. Several features are immediately apparent from Fig. 2. There are marked spectroscopic changes with varying ferrihaem concentration for both deuteroferrihaem and protoferrihaem, i.e. Beer's Law is not obeyed. For deuteroferrihaem, a sharp single Soret band is observed at very low concentrations that, with increasing concentration, has decreased extinction and broadens considerably. These changes are consistent with those associated with dimerization observed by Urry (1967), Inada & Shibata (1962) and Brown & Lantzke (1969). For protoferrihaem, spectrum (*e*) of Fig. 2, corresponding in concentration to spectrum (*a*) for deuteroferrihaem, the Soret band is certainly not a single peak and is broad and of low extinction. Neverthe-

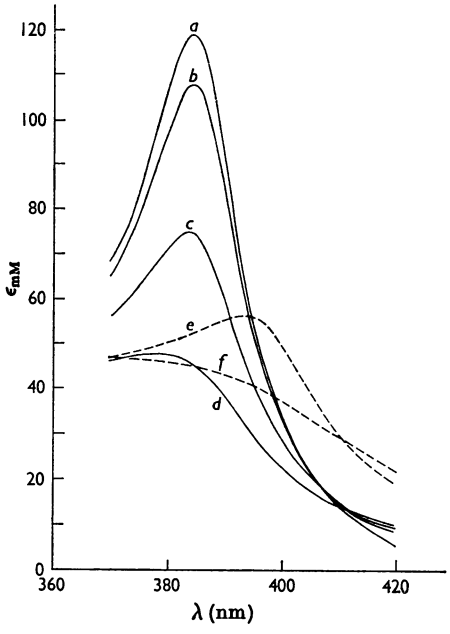


Fig. 2. Illustration of the dependence on ferrihaem concentration of the absorption spectra of ferrihaem solutions (pH 6.98, 25°C). Curves (*a*)–(*d*) deuteroferrihaem solutions, concentrations: (*a*), 0.198 μM; (*b*), 0.743 μM; (*c*), 7.43 μM; (*d*), 198 μM. Curves (*e*)–(*f*) protoferrihaem solutions, concentrations: (*e*), 0.201 μM; (*f*), 191 μM.

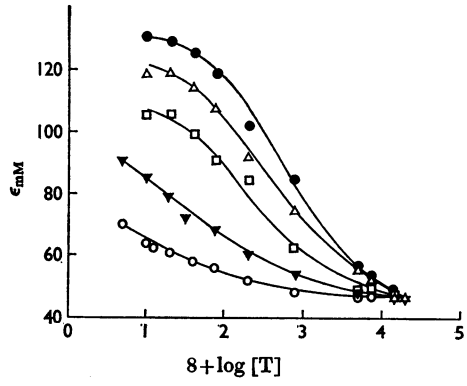


Fig. 3. Concentration and pH-dependence of ϵ_{384} for deuteroferrihaem solutions at 25°C. ●, pH 6.64; △, pH 6.98; □, pH 7.38; ▼, pH 8.04; ○, pH 11.0. [T] is the total deuteroferrihaem concentration (mol l^{-1}).

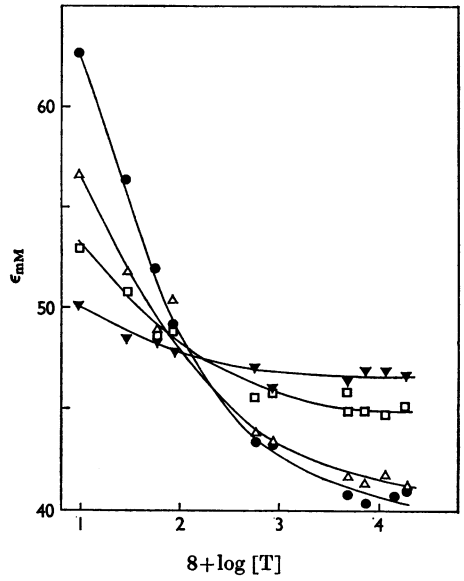


Fig. 4. Concentration and pH-dependence of ϵ_{394} for protoferrihaem solutions at 25°C. ●, pH 6.98; △, pH 7.38; □, pH 8.04; ▼, pH 11.0. [T] is the total protoferrihaem concentration (mol l^{-1}).

less, on increasing the concentration (spectrum *f*), the extinction again decreases and further broadening occurs. The immediate explanation of these observations is that at the lowest concentration studied (approx. 0.1 μM) deuteroferrihaem is largely monomeric, whereas at the same concentration protoferrihaem contains a much higher proportion of dimers.

For purposes of analysis, we have measured the values of the millimolar extinction coefficients, $\epsilon_{obs.}$, at wavelengths corresponding to the Soret maximum for the most dilute solution in each case (i.e. solutions containing the maximum proportion of monomer). For deuteroferrihaem this wavelength was 384nm and for protoferrihaem it was 394nm. The results for the complete range of ferrihaem concentrations at various pH values are shown graphically in Figs. 3 and 4 for deuteroferrihaem and protoferrihaem respectively. For a single process, such as that given by eqn. (1), at constant pH we would expect a sigmoid curve in which $\epsilon_{obs.}$ becomes constant at both high- and low-concentration extremes. Inspection of Fig. 3 shows that this is the case for deuteroferrihaem. At pH $\leqslant$ 7.38, the curve is flat on the low-concentration side, although the high concentration limit has not been reached. The reverse becomes true as the pH increases. The absence of any intermediate 'steps' in the curves suggests that only a single process is operative, and that aggregation beyond dimers does not occur, at least in this concentration range. Further, the pH-dependence of the dimerization

curves suggests that deuteroferrihaem monomer is more stable at low pH than at higher pH.

The corresponding curves for protoferrihaem (Fig. 4) are, perhaps, not so obviously interpreted when taken alone, but by comparison with Fig. 3 it is a reasonable inference that they are portions of similar sigmoid curves at the high-concentration extreme. This is consistent with our previous observation (Fig. 2) and suggests that even at the lowest protoferrihaem concentration studied dimers are the predominant species.

To determine K and n (eqn. 1) the following analytical treatment has been adopted.

From eqn. (1);

$$K = \frac{[D][H^+]^n}{[M]^2} \quad (2)$$

where [D] and [M] are concentrations of dimer and monomer respectively.

Also we define:

$$K_{obs.} = \frac{[D]}{[M]^2} = \frac{K}{[H^+]^n} \quad (3)$$

where $K_{obs.}$ is then the observed dimerization constant at fixed pH.

The observed extinction coefficient at any fixed wavelength and pH may then be expressed in the form:

$$\epsilon_{obs.}[T] = \epsilon_M[M] + \epsilon_D[D] \quad (4)$$

where ϵ_M and ϵ_D are the extinction coefficients of pure monomer and pure dimer respectively, and [T] is the total ferrihaem concentration.

If α is the fraction of ferrihaem in the form of monomer then:

$$[M] = \alpha[T] \quad (5)$$

and

$$[D] = \tfrac{1}{2}(1-\alpha)[T] \quad (6)$$

From eqns. (4), (5) and (6) we obtain:

$$\alpha = \frac{\epsilon_{obs.} - \frac{1}{2}\epsilon_D}{\epsilon_M - \frac{1}{2}\epsilon_D} \quad (7)$$

and from eqns. (5), (6) and (3):

$$K_{obs.} = \frac{(1-\alpha)}{2\alpha^2[T]}$$

or rearranging:

$$\alpha^2[T] = \frac{1}{2K_{obs.}} - \frac{1}{2K_{obs.}}\alpha \quad (8)$$

In principle therefore, provided that ϵ_M and ϵ_D may be obtained, we may calculate α. A plot of $\alpha^2[T]$ against α should be linear and $K_{obs.}$ may be calculated from either slope or intercept.

From eqn. (3):

$$\log K_{obs.} = \log K + n\mathrm{pH} \quad (9)$$

A plot of $\log K_{obs.}$ against pH should therefore be a straight line, from which K and n may be calculated.

Deuteroferrihaem. As shown in Fig. 3, at low pH, although the value of ϵ_M is fairly obvious, the value of ϵ_D is much less easy to determine. At high pH the reverse is true. To determine accurate values of ϵ_M and ϵ_D, we have therefore adopted the following extrapolation procedures.

At sufficiently high [T], i.e. when [D] $\gg$ [M], it is readily shown that:

$$\epsilon_{obs.} = \frac{\epsilon_M}{\sqrt{2K_{obs.}}} \cdot \frac{1}{\sqrt{[T]}} + \frac{\epsilon_D}{2} \quad (10)$$

A plot of $\epsilon_{obs.}$ against $1/[T]$ should therefore give a linear extrapolation to determine the intercept $\frac{1}{2}\epsilon_D$.

Similarly when [M] $\gg$ [D] we obtain:

$$\epsilon_{obs.} = \epsilon_M + \epsilon_D K[T] \quad (11)$$

In this case, a plot of $\epsilon_{obs.}$ against [T] gives a linear extrapolation from which ϵ_M may be calculated. Preliminary values of $K_{obs.}$ may also be calculated from eqns. (10) and (11).

For deuteroferrihaem, extrapolations using eqns. (10) and (11) yielded values for ϵ_M and ϵ_D shown in Table 1 (it should be noted that ϵ_D values refer to 1 mmol of dimer/l). The data do not permit the meaningful use of this procedure at pH 11. By using these values for ϵ_M and ϵ_D, values of α were computed and plots of $\alpha^2[T]$ against α are shown in Fig. 5 for each pH value studied. These plots represent good straight lines within the limits of error imposed by the use of derived data and have been used to calculate the $K_{obs.}$ values given for deuteroferrihaem in Table 1. Fig. 7 shows a plot of $\log K_{obs.}$ against pH for deuteroferrihaem. The plot is the best straight line of slope +1 drawn through the

Table 1. *Dependence on* pH *of* ϵ_M, ϵ_D *and* $K_{obs.}$ *for deutero- and proto-ferrihaem at* 25°C

	Deuteroferrihaem			Protoferrihaem	
pH	ϵ_M	ϵ_D	$10^{-5}\,K_{obs.}$ (M^{-1})	ϵ_D	$10^{-7}\,K_{obs.}$ (M^{-1})
6.64	132.5	70.0	1.47	—	—
6.98	121.5	74.0	1.87	78.6	4.70
7.38	110.0	80.0	3.50	81.6	10.50
8.04	95.0	88.0	16.60	89.2	38.90
11.0	89.0	92.8	—	93.2	—

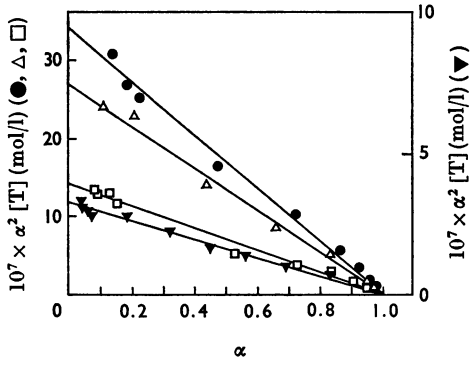


Fig. 5. Graphs of data for deuteroferrihaem according to eqn. (8). ●, pH 6.64; △, pH 6.98; □, pH 7.38; ▼, pH 8.04.

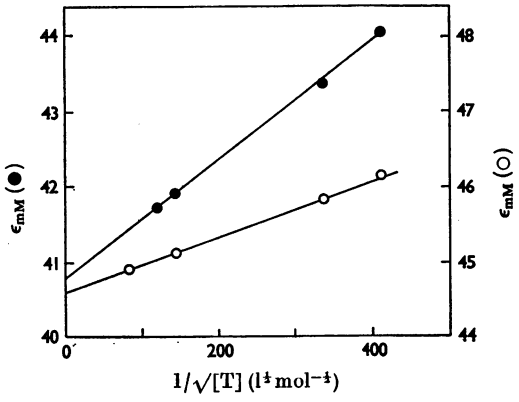


Fig. 6. Graphs of data for protoferrihaem according to eqn. (10). (●), pH 7.38, $K = 10.5 \times 10^7$. (○), pH 8.04, $K = 38.9 \times 10^7$.

experimental points. We conclude that $n = 1$, and that eqn. (1) may be written more explicitly as:

$$2M \rightleftharpoons D + H^+$$

The value of K calculated from the intercept is 1.9×10^{-2}.

Protoferrihaem. The results for protoferrihaem are more difficult to analyse since they refer only to solutions containing large proportions of dimer. Thus, although ϵ_D is easily calculated, determination of ϵ_M is particularly difficult. For the same reason, plots corresponding to those of Fig. 5 for a reasonably wide range of α values are not possible for protoferrihaem. To determine $K_{obs.}$ values we have therefore used eqn. (10), which is operative when $[D] \gg [M]$. The limiting slope of a plot of $1/\sqrt{[T]}$ against $\epsilon_{obs.}$ as $1/\sqrt{[T]} \rightarrow 0$ is equal to $\epsilon_M/\sqrt{2K_{obs.}}$, i.e. $K_{obs.}$ may be calculated provided that ϵ_M is known. Since it is well known that changing porphyrin side-chain substituents, while altering the position of the Soret peak, does not significantly affect its extinction (Falk, 1964), we have assumed that ϵ_M for protoferrihaem is identical with that of deuteroferrihaem at the same pH. This assumption embodies the further assumption that the pH variation of ϵ_M is the same in both cases (see below). An alternative procedure is to assume that the ϵ_M/ϵ_D ratios are identical for protoferrihaem and deuteroferrihaem at the same pH. In practice this yields almost the same ϵ_M values as the previous assumption. Plots of $1/\sqrt{[T]}$ against $\epsilon_{obs.}$ at pH 7.38 and pH 8.04 are shown in Fig. 6. By using the values of ϵ_M in Table 1, values of $K_{obs.}$ were readily calculated from the gradients of such plots. Fig. 7 also shows the $\log K_{obs.}$ against pH plot for protoferrihaem, the line drawn being the best straight line of integral slope. Although only three points are available, as with deuteroferrihaem, the data

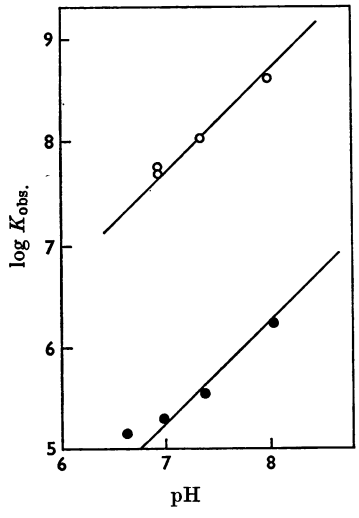


Fig. 7. Dependence on pH of $K_{obs.}$ for deuteroferrihaem (●) and protoferrihaem (○).

clearly conform to the case $n = 1$. The value of K calculated from the intercept is 4.5.

pH *variation of* ϵ_M *and* ϵ_D. The pH-dependence of both ϵ_M and ϵ_D may be taken to represent acid dissociation equilibria involving monomer and dimer separately. These processes may therefore be represented by:

$$M \overset{K_{a(M)}}{\rightleftharpoons} M' + H^+$$

$$D \overset{K_{a(D)}}{\rightleftharpoons} D' + H^+$$

where M′ and D′ are the conjugate bases of M and D respectively.

For dissociation of the monomer, by using:

$$K_{a(M)} = \frac{[M'][H^+]}{[M]}$$

it is readily shown that:

$$\frac{K_{a(M)}}{[H^+]} \cdot (\epsilon'_{M'} - \epsilon_M) = \epsilon_M - \epsilon^0_M$$

where $\epsilon'_{M'}$ and ϵ^0_M are the extinction coefficients of M′ and M respectively; i.e.:

$$\frac{K_{a(M)}\Delta\epsilon}{[H^+]} = \epsilon_M - \epsilon^0_M \qquad (12)$$

where $\Delta\epsilon = \epsilon'_{M'} - \epsilon_M$.

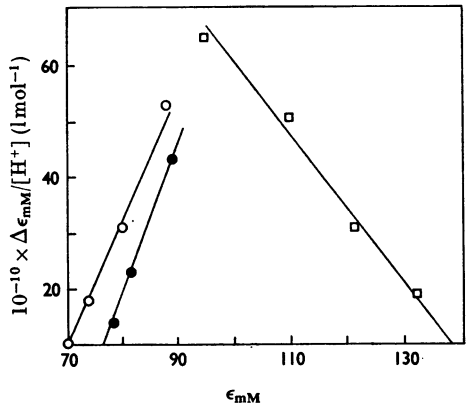
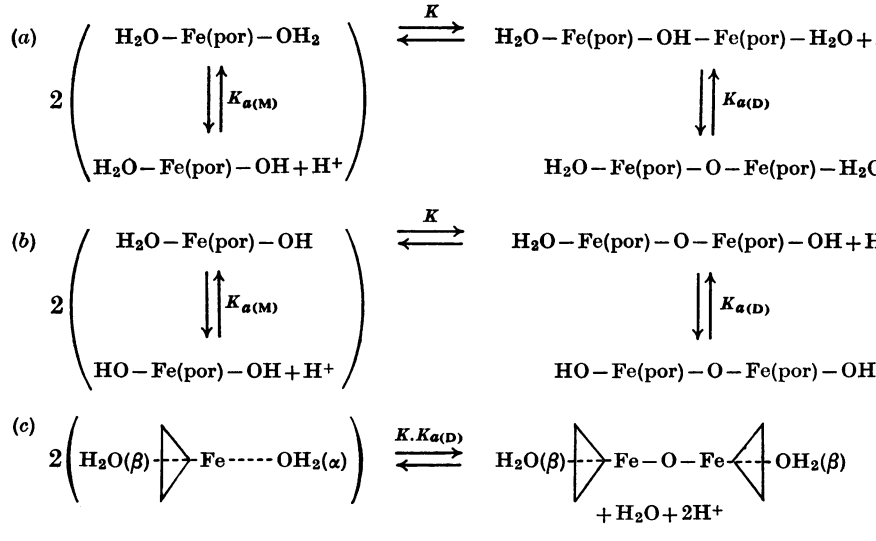


Fig. 8. Graphs for the determination of $K_{a(D)}$ for deuteroferrihaem dimer (○), $K_{a(D)}$ for protoferrihaem dimer (●) and $K_{a(M)}$ for deuteroferrihaem monomer (□), according to eqn. (12).

An analogous relationship is readily derived for dissociation of the dimer. To express this relationship graphically, it is necessary to evaluate $\epsilon'_{M'}$, and the corresponding dimer parameter $\epsilon'_{D'}$. Since inspection shows large changes in ϵ_M and ϵ_D between pH 6.7 and pH 8.04, it is reasonable to assume that $\epsilon'_{M'}$ and $\epsilon'_{D'}$ are essentially identical with the values of ϵ_M and ϵ_D at pH 11. For both deuteroferrihaem and protoferrihaem the values of ϵ_D at pH 11 are entered in Table 1. To determine $\epsilon'_{M'}$ for deuteroferrihaem, however, we have plotted ϵ_M against $[H^+]$ and extrapolated to $[H^+] = 10$ pM. For protoferrihaem ϵ_D at pH 11.0 is also entered in Table 1. Since ϵ_M values were assumed to be identical for protoferrihaem and deuteroferrihaem, their pH variation must necessarily be the same, and we are unable to calculate a $pK_{a(M)}$ value for protoferrihaem monomer. Plots of $\Delta\epsilon/[H^+]$ against ϵ_M for deuteroferrihaem monomer and dimer and for protoferrihaem dimer are shown in Fig. 8. In spite of the small number of points in each case, the good linearity confirms the single acid dissociation process. pK_a values calculated from the slopes are as follows: deuteroferrihaem monomer, $pK_{a(M)}$ 7.1; deuteroferrihaem dimer, $pK_{a(D)}$ 7.4; protoferrihaem dimer, $pK_{a(D)}$ 7.5.

Structural interpretations. There are two possible structural interpretations that are consistent with our results and with the results of investigations of



Scheme 1. Possible structural relationships between monomeric and dimeric ferrihaem species.

crystalline ferrihaem dimers. If M is a bisaqua-ferrihaem species then Scheme 1(*a*) is appropriate; if M is a hydroxyaqua-ferrihaem Scheme 1(*b*) applies. The direct experimental evidence does not permit a firm decision as to which scheme is valid. Only a single deprotonation process for the monomer is observed (M → M′) and this might be regarded as support for Scheme 1(*b*). However, more detailed consideration of the stereochemical aspects of the process suggests that the system may be rather more subtle.

Recalling that in chlorohaemin the iron atom is about 0.5 Å out of the plane of pyrrole nitrogen atoms of the porphyrin ring (Koenig, 1965), we must consider the possibility that, in a bisaqua-ferrihaem monomer, one water molecule may be bound in a different manner from the other. This situation is shown in Scheme 1(*c*), where the triangle with an iron atom at one apex represents a cross-section of the square–pyramidal core of the ferrihaem unit. The two water molecules in a bisaqua monomer may then be distinguished as apical [$H_2O(\alpha)$] and basal [$H_2O(\beta)$], with the apical water molecule presumably the more tightly bound. The olation reaction by which dimer is formed would then most probably involve the α-H_2O molecules. [Olation involving two $H_2O(\beta)$ molecules is highly improbable, although an $H_2O(\alpha) + H_2O(\beta)$ process is possible.] This then yields the structure shown in Scheme 1(*c*) for the oxo-bridged dimer.

We suggest that, according to this model, $K_{a(M)}$ refers to the acid dissociation of $H_2O(\alpha)$, the pK values observed being appreciably higher than that for the aqua-ferric ion (p$K \simeq 2.2$), in part because of the decreased effective field (net charge +1) at the co-ordination centre (Basolo & Pearson, 1958). The $H_2O(\beta)$ molecules would be much less tightly bound and expected to have a pK value near that of bulk water (p$K = 16$) and therefore, perhaps, not separately measurable. We believe that this model may also have important implications for the catalytic behaviour of ferrihaems, since effective substrate binding in the dimer may not readily be achieved.

Concluding remarks. Further studies of the energetics, kinetic and structural aspects of ferrihaem dimerization are required before attempting a theoretical explanation both for the high stability constants of ferrihaem dimers and for the large differences in dimer stability that we find as a consequence of changes in peripheral groups on the porphyrin ligand. The results of Inada & Shibata (1962) suggest that the kinetics of the dimerization process are complex, since it appears that, in the absence of buffer, metastable monomer solutions may be obtained. These workers also reported slow changes in the absorption spectrum of protoferrihaem solutions with time that they considered to be associated with a dimer → tetramer reaction. No precautions to exclude oxygen from the solutions are noted and the time-scale of the process is very similar to that of the 'aging' process, which was first reported by Shack & Clark (1947) and later shown to be a partial autoxidation of protoferrihaem (Rothschild, 1960; Brown *et al.* 1968).

We thank the Shell International Petroleum Co. for a research fellowship (to S. B. B.) and the Science Research Council for a research studentship (to T. C. D.).

REFERENCES

Bancroft, G. M., Maddock, A. G. & Pandl, R. P. (1968). *J. chem. Soc.* A, p. 2939.

Basolo, F. & Pearson, R. G. (1958). *Mechanisms of Inorganic Reactions*, chapter 9. New York: John Wiley and Sons Inc.

Brown, S. B., Jones, P. & Lantzke, I. R. (1969). *Nature, Lond.*, **223**, 960.

Brown, S. B., Jones, P. & Suggett, A. (1968). *Trans. Faraday Soc.* **64**, 986.

Brown, S. B. & Lantzke, I. R. (1969). *Biochem. J.* **115**, 279.

Clark, W. & Perkins, M. E. (1940). *J. biol. Chem.* **135**, 643.

Cohen, I. A. (1969). *J. Am. chem. Soc.* **91**, 1980.

Cowgill, R. W. & Clark, W. M. (1952). *J. biol. Chem.* **198**, 33.

Earnshaw, A. & Lewis, J. (1961). *J. chem. Soc.* p. 396.

Falk, J. E. (1963). In *Comprehensive Biochemistry*, vol. 9, chapter 1. Ed. by Florkin, M. & Stotz, E. H. Amsterdam: Elsevier Publishing Co.

Falk, J. E. (1964). *Porphyrins and Metalloporphyrins*, pp. 46, 117. Amsterdam: Elsevier Publishing Co.

Fischer, H. (1941). In *Organic Syntheses*, vol. 21, p. 53. Ed. by Drake, N. L. New York: John Wiley and Sons Inc.

Fleischer, E. B. & Srivastava, T. S. (1969). *J. Am. chem. Soc.* **91**, 2403.

Gerloch, M., McKenzie, E. D. & Towl, A. D. C. (1968). *Nature, Lond.*, **220**, 907.

Inada, Y. & Shibata, K. (1962). *Biochem. biophys. Res. Commun.* **9**, 323.

Jordan, J. & Bednarski, T. M. (1964). *J. Am. chem. Soc.* **86**, 5690.

Koenig, D. F. (1965). *Acta cryst.* **18**, 663.

Lemberg, R. & Legge, J. W. (1949). *Hematin Compounds and Bile Pigments*, p. 172. New York: Interscience Publishers Inc.

Phillips, J. N. (1963). In *Comprehensive Biochemistry*, vol. 9, chapter 2. Ed. by Florkin, M. & Stotz, E. H. Amsterdam: Elsevier Publishing Co.

Rothschild, M. (1960). *Archs Biochem. Biophys.* **90**, 229.

Shack, J. & Clark, W. M. (1947). *J. biol. Chem.* **171**, 143.

Urry, D. W. (1967). *J. Am. chem. Soc.* **89**, 4190.

Vogt, L. H., jun., Zalkin, A. & Templeton, D. H. (1967). *Inorg. Chem.* **6**, 1725.