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Equilibrium and Kinetic Studies of the Aggregation of Porphyrins in Aqueous Solution

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An investigation of the behaviour of protoporphyrin IX, deuteroporphyrin IX, haematoporphyrin IX and coproporphyrin III in aqueous solution revealed extensive and complex aggregation processes. Protoporphyrin appears to be highly aggregated under all conditions studied. At concentrations below 4 μM, aggregation of deutero-, haemato- and coproporphyrin is probably restricted to dimerization. At approx. 4 μM each of these three porphyrins exhibits sharp changes in spectra consistent with a 'micellization' process to form large aggregates of unknown size. This critical concentration increases with increasing temperature and pH, but is not very sensitive to variation in ionic strength. Temperature-jump kinetic studies on deuteroporphyrin also imply an initial dimerization process, the rate constants for which are comparable with those for various synthetic porphyrins, followed by a further extensive aggregation. The ability of a particular porphyrin to dimerize appears to parallel that of the corresponding iron(III) complexes (ferrihaems), although it is thought that ferrihaems do not exhibit further aggregation under these conditions.

The aggregation of ferrihaems in aqueous solution has recently been reinvestigated in detail. Dimerization has been found to occur in all the systems studied (Brown *et al.*, 1970*a*; Fleischer *et al.*, 1971; Jones *et al.*, 1974; Katz *et al.*, 1963) although the earlier evidence for more extensive aggregation, reviewed by Lemberg & Legge (1949), has not been substantiated. For the naturally occurring protoferrihaem and the related deuteroferrihaem, dimerization occurs according to the equation:

$$2M \underset{}{\overset{K}{\rightleftharpoons}} D + H^+ \quad \text{(Brown } et\ al.\text{, 1970}a\text{)}$$

where M and D represent monomeric and dimeric ferrihaem respectively. The values of K for protoferrihaem and deuteroferrihaem differ (surprisingly) by more than two orders of magnitude. The proportions of monomeric and dimeric species in equimolar solutions of protoferrihaem and deuteroferrihaem may therefore be quite different. For example, at a total ferrihaem concentration of 100 μM (pH 7), only 1% of the protoferrihaem molecules exist as monomers, whereas the corresponding value for deuteroferrihaem is 15%. Moreover, the reactivities of the monomeric and dimeric ferrihaem molecules in any given system may differ markedly. This differential reactivity has in fact been observed in all the aqueous ferrihaem reactions investigated in detail, including the degradation of ferrihaem to bile pigment (Jones *et al.*, 1973*a*; Brown *et al.*, 1974) and the use of ferrihaem in catalase model systems (Brown *et al.*, 1970*b*; Jones *et al.*, 1973*b*). In the many biochemical systems in which investigators have used ferrihaems, it is important therefore that account should be taken of monomer–dimer equilibria in the interpretation of results.

The structure of ferrihaem dimers is only partially understood. A wealth of evidence, including i.r. spectroscopy (Brown *et al.*, 1969) and X-ray crystallography (Fleischer & Srivastava, 1969) has shown that the two ferrihaem units are linked via their iron atoms in an oxo-bridged structure. In this structure, the iron atoms are displaced from the plane of their respective nitrogen atoms (towards one another) by 0.05 nm. Although protoferrihaem and deuteroferrihaem both contain Fe–O–Fe bonds, the reason for the extra stability of the protoferrihaem dimers is not clear. It is unlikely that such a large difference could be due solely to an indirect influence of the substitution of –H for –CH=CH$_2$ on the Fe–O–Fe bond strength. It therefore appears probable that the extra stability of protoferrihaem dimers is due to some extra bonding (additional to Fe–O–Fe) between the porphyrin rings, which is not possible in deuteroferrihaem. Such bonding might also be expected to occur in the metal-free protoporphyrin and thereby cause aggregation.

Aggregation processes in synthetic porphyrins have been observed and investigated by Das *et al.*

(1970) and by Pasternack *et al.* (1972), who found that those porphyrins with peripheral groups which are negatively charged at pH7 exhibit dimerization, whereas those porphyrins with positively charged peripheral groups show no indication of aggregation.

The naturally occurring porphyrins have not yet been studied in this respect in aqueous solution. However, Abraham *et al.* (1966) have observed dimerization in solutions of coproporphyrin in chloroform. They concluded that aggregation occurred by electrostatic interaction between parallel, overlapping porphyrin rings and suggested the possibility of stacking to form larger aggregates. The aims of the present work were therefore to investigate the behaviour of these porphyrins in aqueous solution and to study any aggregation processes that might be revealed.

Experimental

Materials

Protohaemin was obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. Deuterohaemin was prepared by heating protohaemin in a resorcinol melt (Falk, 1964). Protoporphyrin IX and deuteroporphyrin IX were prepared from the corresponding haemins by removal of iron as described by Falk (1964). Haematoporphyrin IX was obtained as the 'purum' grade from Fluka, supplied by Fluorochem Ltd., Dinting Vale Trading Estate, Glossop, Derbyshire, U.K. Coproporphyrin III was obtained biosynthetically by the method of Lascelles (1956), which involved the exposure to light of cultures of *Rhodopseudomonas spheroides* suspended in a medium appropriate for porphyrin synthesis. All porphyrin samples were checked for possible ferrihaem impurity by dissolving in pyridine/water (1:1, v/v) and adding dithionite. No trace of pyridine haemochrome peaks was found in the absorption spectrum in the region of 526–580nm. In addition the porphyrin samples were dissolved in pyridine or in HCl and the wavelengths (λ) of the various peaks compared with literature values (Falk, 1964). In all cases, observed λ values were within $\pm$1nm of those quoted by Falk (1964).

Buffer components (Na_2HPO_4, KH_2PO_4, NaCl) were A.R.-grade materials and were dissolved in doubly distilled water.

Preparation of solutions

Buffer solutions were prepared from Na_2HPO_4/KH_2PO_4 mixtures (pH range 6.64–8.04) and a Na_2HPO_4/NaOH mixture (pH11.0). NaCl was added to maintain constant ionic strength, usually 0.1M. pH measurements were made on a Pye 290 pH-meter.

Stock solutions (approx. 0.4mM) were prepared by dissolution of a weighed quantity of the appropriate haemin or porphyrin in a minimum volume of 0.1M-NaOH (usually about 1ml) followed by dilution by buffer to a known volume (usually 50 or 100ml). The pH of the stock solution prepared in this way was always the same as that of the buffer. Test solutions could then be obtained by suitable dilution of the stock solution.

Spectrophotometric measurements

All extinction measurements were made on a Unicam SP.1800 recording spectrophotometer. The temperature of solutions under test was maintained within limits of $\pm$0.1°C, by using a thermostatically controlled cuvette holder, except for 100mm cuvettes, when the room temperature was held constant.

Scans of extinction against wavelength were made in the region of the Soret peak (330–460nm), over a wide range of porphyrin concentration. This was made possible by the use of cuvettes varying in path length between 1 and 100mm, and measurement of extinction values between 2 and 0.01. Because of favourable variation of extinction coefficients it was thereby possible to make measurements over a concentration range covering five orders of magnitude.

Kinetic measurements

Rate measurements were made on a Messenlagen type 5BA7 Temperature-Jump Spectrophotometer and photographic recordings of oscilloscope traces were made with a Polaroid camera. Test solutions in the temperature-jump cuvette were partially degassed in a vacuum desiccator before insertion into the apparatus. Kinetic measurements were made at 25°C after allowing for a temperature jump of 2.6°C on solutions initially thermostatically maintained at 22.4°C.

Results and Discussion

Concentration-dependence of spectra

Fig. 1(*a*) shows spectra of equimolar proto-, deutero-, copro- and haemato-porphyrin solutions in phosphate buffer, pH6.98. These may be compared with the spectra of the same porphyrins in diethyl ether (Fig. 1*b*). The latter are similar for all four porphyrins, except for the small shifts in $\lambda_{max.}$ expected for the substitution of different groups at the periphery of the porphyrin molecule. The differences in spectra for the four porphyrins in Fig. 1(*a*), however, are much more extensive than would be expected for such substitution, and suggest that aggregation processes occur in aqueous solution. Moreover, since aggregation is associated with a lowering of extinction

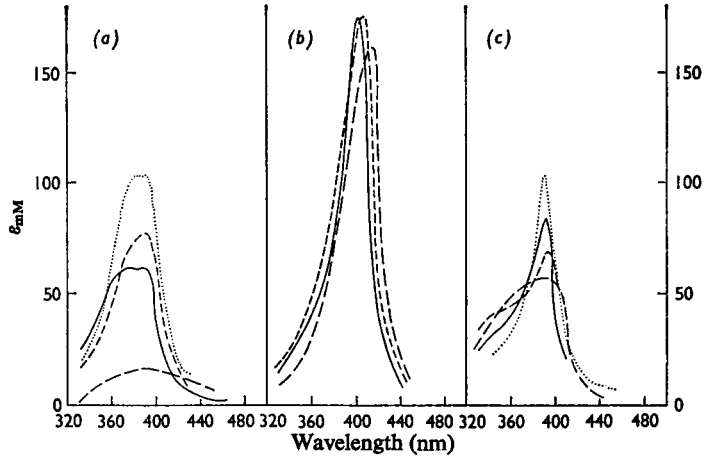

Fig. 1. *Soret-band spectra of porphyrins and corresponding ferrihaems*

(*a*) 4μM solutions of porphyrins in phosphate buffer, pH6.98 at 25°C; (*b*) 4μM solutions of porphyrins in ether at 25°C; (*c*) 4μM solutions of ferrihaems in phosphate buffer, pH6.98, at 25°C. ····, Copro-; ----, haemato-; ————, proto-; ——, deutero-porphyrin. ε_{mM}, millimolar extinction coefficient.

and broadening of the Soret band, the extent of aggregation differs between the various porphyrins at a concentration of 4μM and is much greater for protoporphyrin than for deutero-, copro- or haemato-porphyrins. These spectra may be compared with those of the corresponding ferrihaems at similar concentrations (Fig. 1*c*). By using the magnitude of extinction coefficients and sharpness of the Soret peak as crude indicators of aggregation state, there is a clear correlation between the identity of the side chains and degree of aggregation, i.e. aggregation in the ferrihaem series appears to be approximately parallel to that in the free porphyrin series at approx. 1μM concentration. (There is a possible reversal between the deutero and haemato compounds, but this is small and does not detract from the main trend.) This result would not necessarily be predicted, since a covalent Fe–O–Fe bond is formed in the ferrihaem dimers, whereas no such bond is possible in the free porphyrins. Fig. 2 shows the spectrum (millimolar extinction coefficient, ε_{mM}, versus wavelength) of deuteroporphyrin at pH6.98 for concentrations between 0.4mM and 80nM. Over this range, deuteroporphyrin shows a large variation in spectrum. Since pH, ionic strength and temperature are all constant this variation is proof of the existence of aggregation. Further, since there is no single isosbestic point through which all spectra pass, it is evident that the aggregation is not confined to a single process. Similar spectral changes with various concentrations, although to differing extents, were observed with the other three porphyrins studied.

Careful observation of the Soret-band spectra for deuteroporphyrin revealed an extremely sensitive

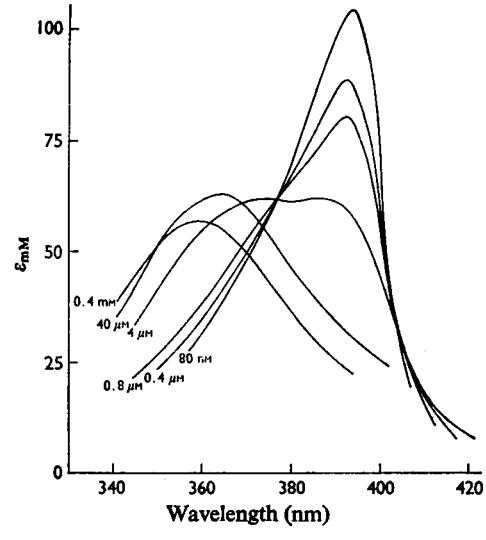

Fig. 2. *Deuteroporphyrin spectra in phosphate buffer, pH6.98, at 25°C*

Deuteroporphyrin concentrations are shown on the Figure.

dependence of the peak wavelength ($\lambda_{max.}$) on concentration ([E]^0) in the region of 4μM (pH6.98, 25°C). This is illustrated in Fig. 3(*a*) (concentration plotted logarithmically). Indeed the change in $\lambda_{max.}$ is so sharp as to represent almost a discontinuity in the plot of $\lambda_{max.}$ versus log[E]^0. The dramatic change in

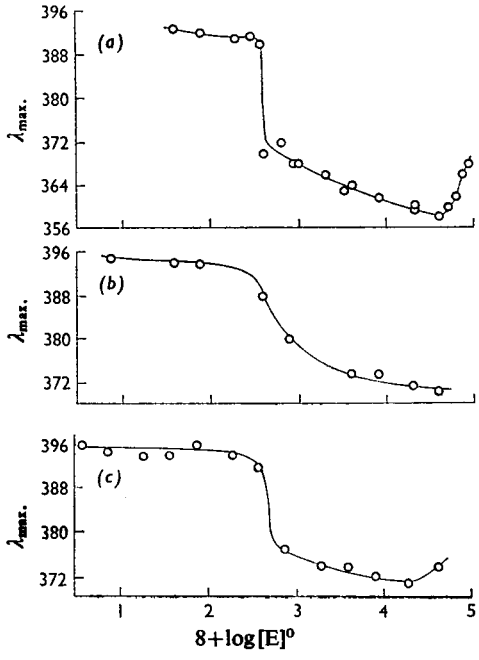

Fig. 3. *Dependence of the wavelength of the Soret peak ($\lambda_{max.}$) on total porphyrin concentration ([E]^0)*

Data were obtained at 25°C in phosphate buffer, pH 6.98. (*a*) Deuteroporphyrin, (*b*) haematoporphyrin, (*c*) coproporphyrin.

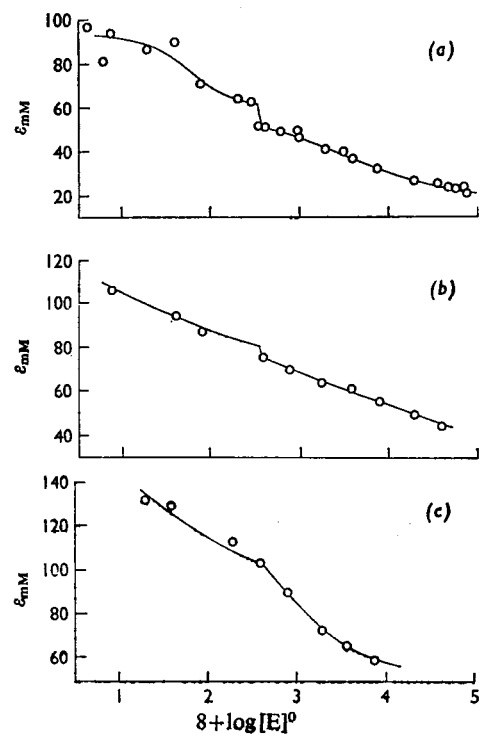

Fig. 4. *Dependence of ε_{mM} on total porphyrin concentration ([E]^0)*

Data were obtained at 25°C in phosphate buffer, pH 6.98. (*a*) Deuteroporphyrin (measurements made at 390nm), (*b*) haematoporphyrin (measurements made at 390nm), (*c*) coproporphyrin (measurements made at 394nm). The discontinuity in curve (*b*) has been drawn largely by comparison with the other porphyrins.

$\lambda_{max.}$ from 390 to 370nm could be produced experimentally by adding a single drop of buffer to a 10mm-light-path cuvette containing deuteroporphyrin at the critical concentration (approx. 0.05ml of buffer to 3ml of deuteroporphyrin solution). The change in $\lambda_{max.}$ could then be reversed by adding a drop of more concentrated deuteroporphyrin (e.g. 0.1mM) solution. Similar spectral changes were observed for haematoporphyrin and coproporphyrin (Figs. 3*b* and 3*c*). Protoporphyrin appeared to be too highly aggregated to permit study of such an effect.

These changes in porphyrin spectra were further investigated by accurate measurement of ε_{mM} at fixed wavelength (390nm for deuteroporphyrin and haematoporphyrin, 394nm for coproporphyrin) for concentrations between 0.8mM and 2nM (Fig. 4). The sharp changes around 4μM are also seen in these plots. In regions where no aggregation process is occurring, plots of ε_{mM} versus log[E]^0 should be straight lines parallel to the log[E]^0 axis. These regions might be expected at very low concentration (when almost all porphyrin molecules would exist as monomers) and perhaps at very high concentration, when no further aggregation takes place. Clearly, none of the curves shown in Fig. 4 levels out at high [E]^0. There is some evidence that at very low [E]^0, the curve for deuteroporphyrin may become horizontal, but the experimental scatter on points in this region renders this conclusion doubtful.

The changes in spectra in the region of 4μM are clearly due to changes in state of aggregation of the porphyrin molecules. Since the changes are so sharp, the aggregation may reasonably be classified as 'micellization', with a well-defined critical micelle concentration. However, this is clearly not a simple equilibrium of the type:

$$n(\text{monomer}) \rightleftharpoons (\text{monomer})_n$$

since we observe spectral changes both above and below the critical concentration. Below this concentration the simplest process consistent with the data would be dimerization:

$$2M \overset{K}{\rightleftharpoons} D \qquad (1)$$

where M and D represent monomeric and dimeric species respectively. Such a process would require good isosbestic points for all spectra at $[E]^0 \leqslant 4\mu M$ for any given porphyrin. Experimentally this is difficult to determine, since at these high dilutions large experimental scatter is inevitable. However, examination of Fig. 2 shows that, allowing for experimental error, the results for deuteroporphyrin are consistent with a process involving only dimerization occurring below the critical point. Kinetic results (see below) also support this possibility.

At concentrations above the critical point, some spectral variation may be expected owing to the change in the ratio of the various species involved and hence the observed extinction coefficient. Effects in this region would normally depend on the number of small units (monomers or dimers) condensing into the aggregate, and in this case we have the added complication of the pre-critical-point equilibrium. The molecular situation after 'micellization' might therefore be expected to be fairly complex.

Estimate of K

Assuming that the slopes of the curves in Fig. 4 before the critical point are due to a simple monomer–dimer process, it is possible to estimate a very approximate value of the dimerization constant. For the reaction of eqn. (1), it is readily shown that:

$$[M](2K[M]+1) = [E]^0 \qquad (2)$$

where $[E]^0$ is the total or stoicheiometric porphyrin concentration and K is the dimerization constant as defined in eqn. (1). A non-zero slope on the plot of ε_{mM} versus $8+\log[E]^0$ indicates a region where neither monomer nor dimer predominates greatly over the other. Hence we may write $[M] \simeq \frac{1}{2}[E]^0$, and substituting in eqn. (2) we get $K \simeq 1/[E]^0$. This, of course, is a gross approximation, but would yield a K value at least of the correct order of magnitude. For all three porphyrins studied, therefore, the K values would be of the same order of magnitude, i.e. $1 \times 10^6 M^{-1}$, at pH6.98.

Temperature, ionic strength and pH variation

The dependence of these aggregation phenomena on temperature, ionic strength and pH are shown for selected cases in Fig. 5. So far as could be ascertained, all the porphyrins studied behaved similarly except for protoporphyrin.

The effect of temperature is particularly noteworthy. On increasing the temperature from 25° to 50°C, a critical point was again observed, but at a concentration that was an order of magnitude greater. On decreasing the temperature to 8.5°C, a much smaller shift in the opposite direction was observed. Apparently, increase in temperature favours dissociation of the micelle. This contrasts with the behaviour of deuteroferrihaem, when dimerization increased with increasing temperature (Jones *et al.*, 1974).

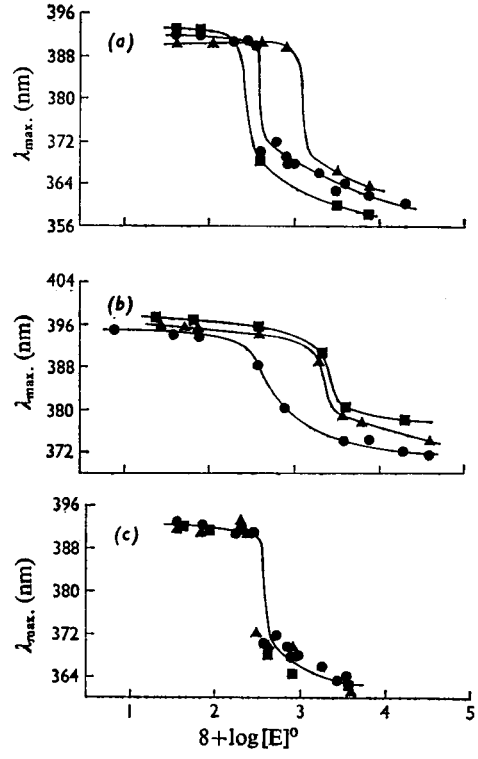

Fig. 5. *Dependence of porphyrin aggregation on temperature, pH and ionic strength*

$[E]^0$ = total porphyrin concentration. (*a*) Dependence of aggregation on temperature for deuteroporphyrin, pH6.98, $I = 0.1$ M: ■, 8.5°C; ●, 25°C; ▲, 50°C. (*b*) Dependence of aggregation on pH for haematoporphyrin at 25°C: $I = 0.1$ M; ■, pH11; ▲, pH7.38; ●, pH6.98. (*c*) Dependence of aggregation on ionic strength for deuteroporphyrin at 25°C, pH6.98: ●, $I = 0.1$; ■, $I = 0.2$; ▲, $I = 0.4$ M.

The pH-dependence (shown for haematoporphyrin) was also significant. It is likely that it arises from the effect of differing states of protonation on the stability of the aggregates, since it is well established that porphyrins have an acid–base dissociation with $pK_a \simeq 5$–6.

The variation of ionic strength between 0.1 and 0.4 M had no significant effect on aggregation (Fig. 5).

Kinetic results

For deuteroporphyrin, a kinetic study was carried out by using temperature-jump spectrophotometry.

Reproducible single relaxation curves of comparatively large amplitude were observed in the concentration range 1–30 μM. For the reaction of eqn. (1), it may be shown that the relaxation time (τ) is given by:

$\tau^{-2} = 8k_{+1}k_{-1}[E]^0 + k_{-1}^2$ (Pasternack *et al.*, 1972) (3)

where k_{+1}, k_{-1} are the forward and reverse rate constants respectively and $K = k_{+1}/k_{-1}$. For a dimerization process, a plot of τ^{-2} versus $[E]^0$ should therefore be linear. In theory k_{-1} may be obtained from the intercept and k_{+1} from the slope of such a plot, but in practice the intercept is often too small to permit accurate measurement. In this case, the values of k_{+1} and k_{-1} may be obtained separately by use of the equilibrium constant K, measured by a non-kinetic method. Fig. 6 shows a plot of the temperature-jump data for deuteroporphyrin according to eqn. (3). Allowing for the inevitable scatter at low $[E]^0$, these data are clearly consistent with the dimerization model at low $[E]^0$. However, at concentrations between 4 and 6 μM, there is a dramatic change in dependence of τ^{-2} on $[E]^0$, and at higher $[E]^0$, τ is almost invariant. This sharp change occurs exactly at the 'critical point' observed in the spectrophotometric experiments. These data therefore lend further support to the postulate of an initial dimerization process, followed by 'micellization' at $[E]^0 \simeq 4\,\mu$M. Presumably the constancy of τ values at higher $[E]^0$ reflects the probability that increasing $[E]^0$ above 4 μM does not increase the monomer/dimer concentration but only the concentration of the higher aggregate, a well-described phenomenon in 'micellization' theory.

It is possible to analyse the data in Fig. 6 according to the parameters of eqn. (3), by using the previously determined value of K, since the intercept is too small to permit accurate measurement. Such an analysis yields $k_{+1} = 5\times10^8\,M^{-1}\cdot s^{-1}$, $k_{-1} = 5\times10^2\,s^{-1}$. Again, it should be emphasized that these values reflect an order of magnitude rather than precise data. However, it is noteworthy that the k_{+1} values are very similar to the accurate values obtained by Pasternack *et al.* (1972) for synthetic porphyrins and by Jones *et al.* (1974) for deuteroferrihaem. In all of these dimerizations, the values of k_{+1} are virtually the same and approach the diffusion-controlled value. The k_{-1} values vary somewhat and hence effectively determine K, the dimerization constant. If it is assumed that protoporphyrin and protoferrihaem fit into this general pattern (for practical reasons study by temperature-jump has not proved possible), the extra stability of the dimer (at least in the case of protoferrihaem) must be due to a lower rate of its dissociation by a factor of approx. 1×10^2.

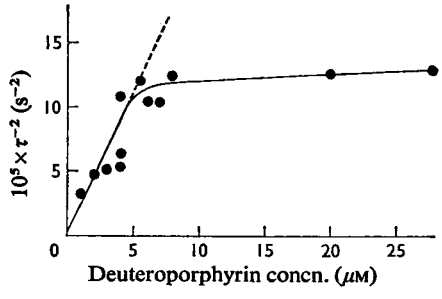

Fig. 6. *Dependence of τ^{-2} on deuteroporphyrin concentration at 25°C, pH6.98*

τ = the relaxation time, defined by $E = E_0e^{-t/\tau}$, where E_0, E are the extinctions at time zero and time t respectively.

General Discussion

In the ferrihaem dimer, the strength of the covalent Fe–O–Fe bonds is probably virtually independent of the nature of the porphyrin, provided that their formation is not restricted on steric grounds. Indeed, there are many reports of oxo-bridged iron dimers in non-porphyrin systems (Vogt *et al.*, 1967; Hewkin & Griffith, 1966). In considering the large additional stability of dimeric protoferrihaem over dimeric deuteroferrihaem, it is reasonable to search for additional bonding owing to the vinyl groups. If such bonding were specific in the sense of a covalent bond, and occurred also in the iron-free porphyrins, it might be expected that protoporphyrin would dimerize, whereas deuteroporphyrin would remain monomeric. The present work has shown that this is clearly not the case. Protoporphyrin is indeed more highly aggregated than the other porphyrins studied, but nevertheless all of these porphyrins are relatively strongly dimerized, with the superimposition of 'micellization' at higher concentration. The molecular explanation for this tendency for porphyrin molecules to stick together even in very dilute solution (<1 μM) is not yet clear. An explanation might be sought in terms of hydrophobic interaction between the large 'aromatic-like' delocalized porphyrin systems, and this may also explain the additional stability of protoporphyrin and protoferrihaem dimers over those of the deutero, haemato and copro compounds. If this were the case the meso compounds might also be expected to show such extra stability, since they also possess additional 'hydrophobic' structure.

It is also noteworthy that despite the finding by Abraham *et al.* (1966) that dimerization is not affected by the polarity of the solvent, their K value for coproporphyrin tetramethyl ester in chloroform is some six orders of magnitude higher than that found in the present study for coproporphyrin-free acid in aqueous medium. This comparison suggests

the possibility that in aqueous solution, aggregation involves the charged carboxyl groups in the porphyrin side chains. Pasternack *et al.* (1972) has shown that synthetic porphyrins with anionic side chains dimerize readily, whereas those with cationic side chains remain monomeric. This may be due to the presence of cations being 'sandwiched' between two porphyrin molecules and holding them together by ionic attraction. Unfortunately, the obvious experimental test of using the mono- or di-methyl esters is difficult or impossible because of consequent solubility problems.

Apparently the only way in which the naturally occurring ferrihaems can be maintained in monomeric form in aqueous solution is by use of a strong ligand, e.g. pyridine, to bind at the fifth position on the iron atom and hence effectively compete with another ferrihaem molecule to prevent dimerization. However, such strong ligands usually bind at both the fifth and the sixth positions (forming haemochromes) and hence would prevent the ferrihaem functioning, e.g. as a catalase (binding H_2O_2) or as an oxygen binder. Nature has surmounted this problem by direct binding of protein to ferrihaem in the fifth position. The design of the apoproteins is such that double binding does not occur in the oxygen-binding proteins nor the hydroperoxidase enzymes. On the other hand, where the binding of substrate ligands is not part of the protein function, as in cytochrome *c*, the apoprotein is bound to both the fifth and the sixth positions on the ferrihaem.

The possible role of these aggregation processes *in vivo* has not yet been evaluated. Suggestions of the occurrence of a haem pool in mammalian systems have been made occasionally, but without firm evidence. Nevertheless, if such a reservoir exists in which there is an accumulation of protein-free haem, it would be expected that dimerization would be extensive. For the porphyrins, and especially protoporphyrin, aggregation *in vivo* is a distinct possibility, particularly so since aggregation cannot be easily relieved, for haems, by binding of a protein to the iron atom. The aggregation of porphyrins and its consequences should certainly be taken into account in studies of haem and porphyrin biosynthesis.

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