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Studies on Haemin in Dimethyl Sulphoxide/Water Mixtures

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The nature of the complexes and equilibria shown by solutions of protohaemin in dimethyl sulphoxide/water mixtures and in the presence of acid and base were studied by u.v.–visible spectrophotometry. In neutral solutions containing from 40 to 100% dimethyl sulphoxide, haemin is present as a monomeric complex in which the Cl^- ion is not co-ordinated. Only a single pH-dependent equilibrium pK_{12} is observed over the range 40–80% dimethyl sulphoxide, corresponding to formation of the μ-oxo dimer. As the dimethyl sulphoxide content is lowered below 35%, so the single equilibrium (pK_{12}) is replaced by two equilibria (pK_1 and pK_2); with solutions of 5μM-haemin, pK_1 decreases (from pK_{12} 7.55 in 65% dimethyl sulphoxide to pK_1 approx. 1.5 in 0.01% dimethyl sulphoxide), whereas pK_2 hardly changes (from pK_{12} 7.55 in 65% to pK_2 approx. 7.5 in 0.01%).

We have studied the properties of horseradish peroxidase in mixtures of Me_2SO and water and have shown that the enzyme retains its activity up to 74% (v/v) (0.4mole fraction) Me_2SO, whereas inactive solutions of the enzyme in 100% Me_2SO can be at least partly re-activated after dilution with water (Adams *et al.*, 1979). We now report parallel studies on the properties of the prosthetic group (protohaemin) without the protein in Me_2SO/water mixtures. Aqueous solutions of haemin have, of course, been extensively studied; they exhibit a complex set of equilibria and readily form dimers and perhaps higher aggregates (see the Discussion section).

Our aim has been to establish (1) the range of composition over which haemin remains monomeric and hence whether there exists any region of solvent composition over which one can study in parallel both the active enzyme and the protein-free monomeric haemin, and (2) whether the reversible inactivation of peroxidase in Me_2SO/water mixtures involves the formation of free haemin or not. The former would, for the first time, allow a direct comparison between the properties of monomeric Fe(III) protoporphyrin IX complexes with and without the protein, which is essential for understanding how the protein modifies the properties of the cofactor or prosthetic group (Pratt, 1975). We also wished to examine (3) how the nature of the complexes and equilibria exhibited by haemin vary as the environment is gradually changed from 100% Me_2SO to 100% water. This might indicate some of the possible effects on the prosthetic group of changes in the environment offered by the protein and also provide information relevant to the unravelling of the complex equilibria observed in aqueous solution.

We report here a qualitative study of the species and equilibria exhibited by protohaemin in water/Me_2SO mixtures ranging from 0.01 to 100% Me_2SO and from approx. 1 M-$HClO_4$ to 1 M-NaOH, together with a quantitative study of the pH-dependent equilibrium observed in 65% Me_2SO.

Surprisingly little work has been reported on haemin in Me_2SO/water mixtures. The co-ordination of imidazole and/or chloride by haemin in pure Me_2SO has been studied by Ellfolk & Mattsson (1969), by Brown & Lantzke (1969) and by ourselves (Adams *et al.*, 1978). Mohr & Scheler (1964) merely state that the spectrum of haemin in Me_2SO is similar to that of methaemoglobin in Me_2SO. The only previous work on haemin in Me_2SO/water mixtures is that of Brown & Lantzke (1969), who worked mainly with solutions containing NaOH, and of Beaven *et al.* (1974), who merely recorded the spectrum of haemin in aq. 80% (v/v) Me_2SO for comparison with that of methaemalbumin. Scheler (1960) studied solutions of haemin in a wide range of solvents (but excluding Me_2SO) and found dimethylformamide to be by far the best solvent. Brown & Lantzke (1969) reported that a saturated dimethylformamide solution at 25°C contains approx. 0.5M-haemin and that the solubility in Me_2SO is even higher.

Abbreviation used: Me_2SO, dimethyl sulphoxide.

Experimental

$HClO_4$, NaOH, KOH, $NaClO_4$ and $AgClO_4$ were all of AnalaR grade. Me_2SO (Merck, Darmstadt, W. Germany) was purified, when so required, by twice distilling 250ml portions under vacuum (approx. 10mmHg) and discarding the first and last 30ml portions; the solvent was stored over Linde 5A molecular sieve. Haemin (BDH, Poole, Dorset, U.K.) was used without further purification. Concentrated stock solutions of haemin in pure Me_2SO were made up fresh each day and diluted with water as required.

U.v.–visible spectra were recorded on a Pye-Unicam SP.1800 or a Jasco Uvidec-1 digital spectrophotometer with 1cm-path-length cells thermostatically maintained at 25°C. pH measurements were made with a Beckman glass electrode, which was equilibrated in the solvent (65% Me_2SO) for several hours before use; after each addition of reagent ($HClO_4$ or KOH), the solution was stirred and left for up to 10min before a steady reading was attained.

Results

(1) *Haemin in neutral Me_2SO/water mixtures*

Preliminary studies on the effect of varying the concentration of (unpurified) Me_2SO in water/Me_2SO mixtures on the wavelength and intensity of the Soret band (approx. 400nm) of haemin showed the occurrence of several different reactions, e.g. in solutions containing 80–100% Me_2SO the intensity of the Soret band usually fell for several hours before reaching a steady value, whereas solutions in 50% Me_2SO slowly deposited an almost black precipitate and eventually became colourless. These changes were eliminated by the use of a newly opened or freshly distilled sample of Me_2SO (see the Experimental section) but were usually observed again after about 2 days or 2 weeks respectively. All further experiments were therefore carried out with fresh samples of Me_2SO. We have not attempted to identify the impurities responsible for these side reactions. We also showed that, at least in 100% Me_2SO, there were no significant differences between the spectra observed in air and under N_2 over several hours, and all further experiments (except for some in aqueous solution; see below) were therefore carried out in the presence of air.

Table 1 shows the effect of varying the Me_2SO content in Me_2SO/water mixtures (with no other reagents added) on the wavelength and intensity of the Soret band and also, in order to provide a means for comparing the changes on a common basis, on the A_{400} of 5μM solutions of haemin. The changes in A_{400} are shown graphically in Fig. 1. The solutions were prepared by adding 9μl of a concentrated stock solution of haemin in 100% Me_2SO to 2ml of the given solvent composition in a 1cm spectrophotometer cell. Possible changes in the spectrum were monitored over the range 350–450nm for 10–20min. At or above 35% Me_2SO, where only monomeric complexes are involved (see below), the changes in spectrum on dilution were 'instantaneous'. With

Table 1. *Spectra of haemin in the Soret-band region in neutral Me_2SO/water mixtures*

The haemin concentration was 5μM; 1cm cells were used.

Concn. of Me_2SO(%)	$\lambda_{max.}$ (nm)	$A_{max.}$	A_{400}
100	404	0.94	0.88
95	404	0.94	0.88
90	403	0.93	0.89
85	402	0.92	0.90
80	402	0.94	0.92
75	402	0.92	0.90
70	402	0.93	0.91
65	402	0.92	0.90
60	402	0.93	0.91
50	401	0.91	0.91
40	400	0.90	0.90
37.5	400	0.87	0.87
35	400	0.85	0.85
32.5	400	0.82	0.82
30	400	0.76	0.76
25	400	0.62	0.62
20	399	0.49	0.49
10	~398	0.36	0.35
5	~397	0.31	0.30
3			0.27
1			0.24
0.5			0.24

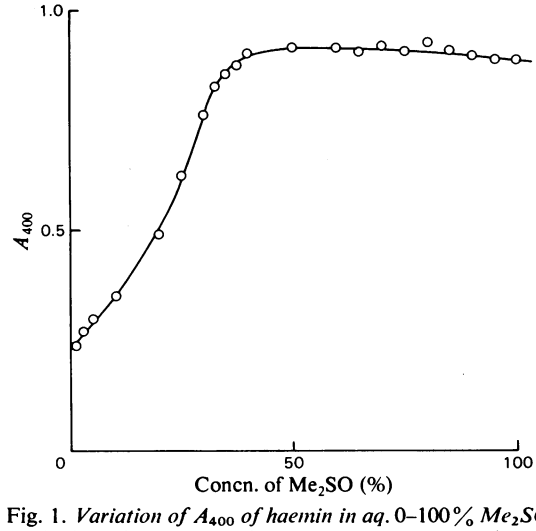

Fig. 1. *Variation of A_{400} of haemin in aq. 0–100% Me_2SO*
The haemin concentration was 5μM. Data are taken from Table 1.

lower Me_2SO content, the rate of equilibrium was finite, but complete within 10min.

Solutions of haemin in 35% Me_2SO (or over) show only a single sharp band in the Soret region, but, as the Me_2SO content is decreased, so the Soret band falls in intensity, the absorption band becomes much broader and a prominent shoulder appears at about 375nm. Superposition of the successive spectra from 35 down to 1% Me_2SO (see Fig. 2) produces a semblance of isosbestic points at 368 and 415nm. This strongly suggests that we are observing the gradual displacement of a single type of equilibrium (see also below) with effects of changing solvent composition on the spectra of the two complexes involved.

Further experiments showed that one could routinely obtain clear homogeneous solutions with as little as 0.01% Me_2SO, which still give a reasonable absorption (>0.5) in the Soret region, by dilution of the appropriate solution in 100% Me_2SO with water. Even here relatively little change was observed over 1h or so (see below). However, we were unable to prepare solutions with a similar absorption containing 0.005 and 0.001% of Me_2SO by diluting either a more concentrated solution of haemin in 100% Me_2SO (the haemin precipitated from the final solution) or a concentrated solution with a lower Me_2SO content (which only dissolved a limited amount of haemin).

Qualitative tests showed that at room temperature haemin dissolves very readily in 80% Me_2SO or over, but not at any appreciable rate in solvents containing less than 40% Me_2SO. On the other hand, haemin at the concentrations used in Table 1 was precipitated only from solutions containing 0.01% Me_2SO or less. Precipitation from solutions containing 0.01% Me_2SO could not be induced by 'seeding' with freshly ground crystals of haemin. It is clear that equilibrium between haemin in solution and in the crystalline state is not readily established except near 100% Me_2SO, and it is possible that solutions of haemin in solvents containing low concentrations of Me_2SO are, in fact, metastable. In view of the hysteresis shown by solutions of peroxidase in Me_2SO/water mixtures (Adams *et al.*, 1979), the possible occurrence of hysteresis in the reactions and equilibria of haemin described above was tested as follows. One portion of a sample of haemin in 100% Me_2SO was diluted first to 20% Me_2SO and then returned to 96% Me_2SO to give $A_{403} = 0.592$, 0.620, 0.590 (average 0.599), and a second portion was diluted directly to 96% Me_2SO to give the same final concentration and $A_{403} = 0.595$, 0.610, 0.611 (average 0.605). Equilibrium between the species observed in 100 and 20% Me_2SO is therefore completely reversible in both directions.

It has already been shown that the chloride present in solid haemin is displaced from co-ordination in

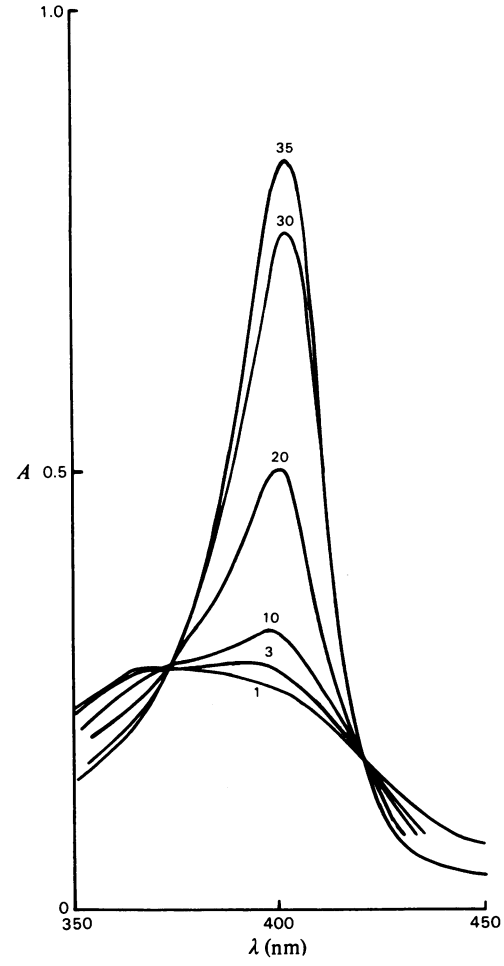

Fig. 2. *Variation of absorption spectrum of haemin in aq. 0–35% Me_2SO*

The haemin concentration was 5μM. Numbers indicate % of Me_2SO.

100% Me_2SO (Brown & Lantzke, 1969; Adams *et al.*, 1978). In the same way, the addition of small amounts of solid $AgClO_4$ to solutions of haemin in 50% Me_2SO produced a very slight turbidity (due to AgCl), and hence a slight increase in the background absorption, but had no effect on the wavelength or shape of the Soret band. Maehly (1958) has already shown that haemin in aqueous solutions of pH1 and 3 binds one chloride ion, with 50% formation requiring 0.2M-Cl^-; the degree of formation of the chloro complex in 5μM aqueous solutions of haemin would therefore be negligible. We conclude that chloride is not co-ordinated to the Fe(III) ion to any significant extent over the whole range from 0 to 100% Me_2SO.

Solutions of haemin in both 50 and 100% Me_2SO obeyed the Beer–Lambert Law, both at the lower concentrations (up to 4.6 μM) used when studying the Soret-band region and at the high concentrations (up to 0.1 mM) needed for studying the $\alpha\beta$ region. The spectra in 50 and 100% Me_2SO are virtually superimposable in the $\alpha\beta$ region, only minor differences being detected in the small band at approx. 570nm. Three separate determinations of the molar absorption coefficient of the Soret band of haemin in 100% Me_2SO gave values of $\varepsilon_{404} = 1.86$, 1.82 and 1.80 (average $1.83) \times 10^5 M^{-1} \cdot cm^{-1}$.

(2) *Qualitative study of the acid–base equilibria shown by haemin in Me_2SO/water mixtures*

The pattern of pH-dependent equilibria exhibited by haemin has been examined qualitatively in 0.01, 10, 25, 50, 65 (i.e. 0.3 mole fraction, as used in studies on horseradish peroxidase by Adams *et al.*, 1979), 80, 90 and approx. 100% Me_2SO over the range from 1M-$HClO_4$ to 1M-NaOH. In most cases solutions for study were prepared by adding a small volume of concentrated haemin in 100% Me_2SO to neutral solvent of the desired composition and the pH was then varied by adding acid or base. Only with 0.01% Me_2SO were solutions also prepared by adding the concentrated haemin solution directly to an acid or alkaline solution. It appeared that the same spectrum was eventually obtained, regardless of the method of preparation, below pH 6, but that above pH 7 the spectra were somewhat variable (see below).

The simplest situation obtains in 50–80% Me_2SO. Only a single pH-dependent equilibrium was observed, which was reversible, showed isosbestic points at 378, 420 and approx. 615nm and was established 'instantaneously'; no further changes were observed over at least 1 h. This equilibrium was studied quantitatively in 65% Me_2SO (see below), where the alkaline form (see Fig. 3) has a relatively low Soret band at 398nm ($\varepsilon = 1.1 \times 10^5$ per g-atom of Fe per cm) and bands in the visible region at approx. 560 and 590nm (both with ε approx. 0.1×10^5 per g-atom of Fe).

Additional effects were, however, observed in the presence of added electrolytes such as $NaClO_4$; these were further investigated in 65% Me_2SO. The addition of $NaClO_4$ or $KClO_4$ to a neutral solution of haemin caused a slight decrease in the Soret band (10–15% decrease when the solutions were saturated with the added salts), but relatively little change in the visible region. Their addition to a solution of the alkaline form, however, caused a much bigger fall in the Soret band and significant changes in the visible region. In both neutral and alkaline solutions the rates of reaction with $NaClO_4$ or $KClO_4$ were relatively slow. The addition of $NaClO_4$ or $KClC_4$, followed immediately by that of NaOH, caused the instantaneous formation of the usual alkaline form, followed by further slow reaction with $NaClO_4$ or $KClO_4$. The spectrum of the alkaline form of haemin in the presence of excess of $NaClO_4$ showed a Soret band at 397nm with a molar absorption coefficient (per g-atom of Fe) of approx. 0.55×10^5 and a pronounced shoulder at approx. 350nm, while the first band in the visible spectrum (approx. 600nm) was less intense than the second (approx. 580nm).

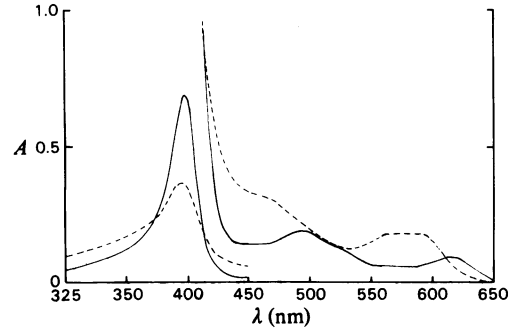

Fig. 3. *Absorption spectra of haemin in aq. 65% Me_2SO*
Neutral (——) and alkaline (– – – –) forms of haemin at concentrations of 4 and 20 μM (left- and right-hand spectra respectively).

The situation in 90 and 100% Me_2SO is complicated by the existence of more than one pH-dependent equilibrium and by the occurrence of slow as well as fast reactions, and has not been investigated in detail.

Solutions of approx. 5 μM-haemin in 0.01% Me_2SO exhibit two pH-dependent equilibria. The pK of the first lies in the region pH 1–1.5, appears to be simple and reversible with isosbestic points at 366 and 412nm, but is established relatively slowly. The complex formed below pH 1 is characterized by a prominent Soret band at 396nm with a molar absorption coefficient of $0.8 \times 10^5 M^{-1} \cdot cm^{-1}$ in 0.5M-H_2SO_4; this spectrum has been given by Maehly (1958) in his Fig. 5. The spectrum observed in more neutral solutions is characterized by much broader and lower absorption in the Soret-band region with a maximum at approx. 370nm and a shoulder at approx. 400nm, together with a band at approx. 640nm in the visible; this spectrum was not recorded by Maehly (1958). The spectra of these two forms in the Soret region are shown in Fig. 4. The second pH-dependent equilibrium occurs at pH 7–7.5 and is also not established instantaneously. The spectrum observed at pH above 8 again shows broad and low absorption in the Soret-band region, usually with a maximum at approx. 385nm, a shoulder at approx. 360nm and a band in the visible at 610nm; cf. Fig. 5 of Maehly (1958) and Fig. 2 of Maehly & Åkeson (1958). However, the relative heights of the two bands in the Soret region showed considerable variation

(depending on conditions such as ionic strength) and sometimes changed with time, which suggested the presence of (at least) two species in a pH-independent equilibrium. There appeared to be no further pH-dependent equilibria detectable up to pH 14. To test whether O_2 is involved in any of these equilibria 2μl portions of a concentrated solution of haemin in 100% Me_2SO (in equilibrium with air) were added to 2ml portions of aqueous solutions of different pH values (0.5M-H_2SO_4, water pH5.4, and 0.1mM-NaOH) in the presence and absence of O_2 (solutions deoxygenated with N_2, in equilibrium with air, and saturated with O_2) and changes in the 400nm region followed for 30–60min. The three different spectra described above for the three different pH values were again observed under N_2. There were no significant changes (<2%) in the spectra in N_2, air or O_2 in the neutral and alkaline solutions. At pH1 there was no significant change (<2%) over 30min under N_2, a slight fall in the Soret band (about 3%) in air, and a greater fall (about 10%) in O_2; this may be the same reaction that was reported by Brown *et al.* (1964).

The situation in 10% Me_2SO is even more complex than that in 0.01%, apparently owing to the overlap of the two pH-dependent equilibria, relatively slow equilibration and the unexpected effects of added electrolytes. The successive addition of small amounts of solid $NaClO_4$ to a neutral solution of haemin, for example, caused an immediate lowering of the total absorption in the Soret-band region and also changed the shape of the spectrum in a way which suggested the preferential removal of the species with the intense Soret band and the formation of a species absorbing at 360–370nm; yet on standing the spectrum slowly, and with good isosbestic points, partially reverted to the shape seen before the previous addition of $NaClO_4$. In 25% Me_2SO there appears to be only a single equilibrium, but here again the equilibrium is strongly affected by $NaClO_4$ as well as by $HClO_4$ and NaOH.

(3) *Quantitative study of the pK in 65% Me_2SO*

The single pK observed over the range 40–80%

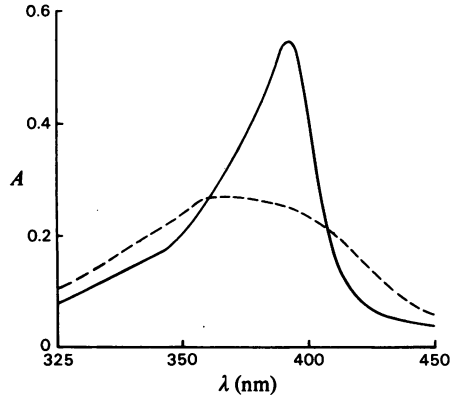

Fig. 4. *Absorption spectra of haemin in aq. 0.01% Me_2SO*
Approx. 7μM-haemin in 0.5M-H_2SO_4 (——) and water, pH approx. 5 (----), both containing 0.01% Me_2SO.

Table 2. *pK of haemin in 65% Me_2SO*

[M] and [D] represent the concentrations (M) of the monomer and dimer respectively in eqn. (1). For further details see the text.

Expt. A			Expt. B		
pH	A_{404}	log([D]/[M]²)	pH	A_{404}	log([D]/[M]²)
6.84	0.892		6.72	0.909	
6.93	0.892		6.91	0.910	
7.01	0.892				
7.09	0.891		7.05	0.909	
7.17	0.883				
7.27	0.864	3.79	7.24	0.887	3.67
7.35	0.825	4.24			
7.44	0.762	4.66	7.41	0.799	4.54
7.52	0.681	5.08			
7.61	0.585	5.58	7.58	0.629	5.42
7.71	0.471	6.45			
7.82	0.403		7.77	0.434	7.19
7.91	0.384				
8.01	0.384		7.98	0.394	
8.12	0.384		8.19	0.394	
8.31	0.384		8.38	0.394	
			9.29	0.394	

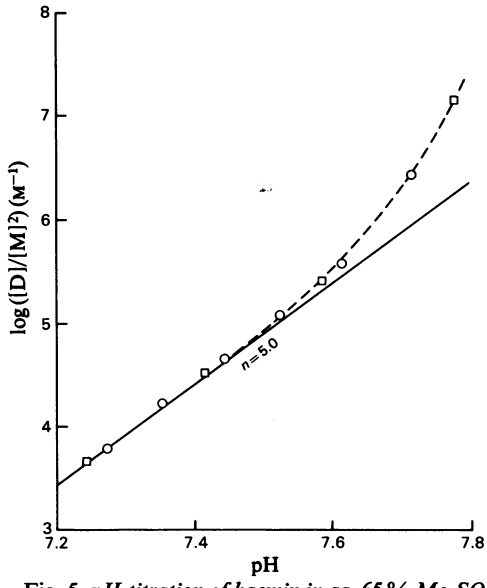

Fig. 5. *pH-titration of haemin in aq. 65% Me_2SO*
Plot for determining the number of protons (n) involved in the pH-dependent equilibrium of haemin. Data are taken from Table 2 (Expt. A, ○; Expt. B, □).

Me_2SO was studied quantitatively in two separate experiments (A, B) by using 5 μM solutions of haemin in 65% Me_2SO with no added $NaClO_4$ etc. The solutions were first acidified with $HClO_4$ (to pH 4.84 and 5.73 in Expts. A and B respectively). The pH was then gradually raised by the addition of small volumes of a solution of KOH in pure Me_2SO. After each addition the pH was measured with a glass electrode and the A_{404} recorded. The values of pH (from pH 6.5) and A_{404} (corrected for dilution) are given in Table 2.

The data were evaluated in terms of equilibria (1) and (2):

$$2M = D + nH^+ \quad (1)$$

$$M \text{ (or } D) = M' \text{ (or } D') + nH^+ \quad (2)$$

where M and M′ are monomeric, and D and D′ dimeric, complexes. The calculations using eqn. (1) are given in Table 2 and the plots of $\log([D]/[M]^2)$ against pH are shown in Fig. 5. In both experiments $n = 5$–5.5 over at least half of the titration, but then apparently increases to $n \geq 6$ towards the end of the titration. In both experiments the mid-point of the titration corresponded to pH 7.55. Plots using eqn. (2) also gave $n = 5$–6, but were more curved than those shown in Fig. 5.

Discussion

In the present paper we have used u.v.–visible spectrophotometry to study the nature of the complexes formed by the prosthetic group of haemoproteins (haemin) in mixtures of water and Me_2SO (from 0.01% to 100% Me_2SO) and in the presence of acid ($HClO_4$) and base (NaOH). Haemin, when prepared by the usual method from chloride-containing media, is a chloro complex in the solid state; X-ray analysis (Koenig, 1965) has shown that the Fe(III) ion is five-co-ordinate and displaced 0.0475 nm out of the plane of the four porphyrin nitrogen atoms towards the chlorine atom. In solution in donor solvents and in the absence of added chloride, however, the chloride may be dissociated from the Fe(III) ion, e.g. in water (Maehly, 1958), aq. 50% Me_2SO (the present paper) or 100% Me_2SO (Brown & Lantzke, 1969; Adams *et al.*, 1978). Under all the conditions used in the present study, therefore, haemin can be regarded as the true cofactor of the haemoproteins without any complications owing to the presence of co-ordinated chloride.

Brown & Lantzke (1969) reported that solutions of haemin in aqueous Me_2SO containing less than 0.25 mole fraction (i.e. 60%) Me_2SO were colloidal and deposited a precipitate on standing. We find that no precipitate is formed if the Me_2SO is rigorously purified and conclude that the precipitate is due to a reaction of the haemin with some (so far unidentified) impurity normally present in the Me_2SO. In fact, by diluting a concentrated solution of haemin in 100% Me_2SO we have been able to obtain aqueous solutions of haemin containing as little as 0.01% Me_2SO, which are reasonably stable in acid, neutral or alkaline solution. Aqueous solutions of haemin are usually prepared by dissolving haemin in strongly alkaline solution and then adjusting the pH to the desired value; such solutions, which contain a relatively high concentration of inorganic salts (but no Me_2SO), readily deposit solid haemin on neutralization or acidification (Maehly, 1958). The present method of preparing aqueous solutions of haemin (containing 0.01% Me_2SO or over, but little or no added inorganic salts) therefore provides a useful alternative method, which opens up a wider range of pH for study.

Solutions of haemin have been shown to obey Beer's Law in 100% (Brown & Lantzke, 1969; Adams *et al.*, 1978; the present paper) and 50% Me_2SO (the present paper). This suggests, but does not prove, that haemin exists as a monomer in these solutions; solutions of a dimer with a sufficiently high value of K_d (=[dimer]/[monomer]²) would also obey Beer's Law. However, the high value of the molar absorption coefficient of the Soret band also strongly suggests (Brown & Lantzke, 1969) that

haemin is a monomer under these conditions and, in the absence of any evidence to the contrary, we conclude that these are monomeric complexes. Our values for the wavelength and molar absorption coefficient of the Soret band in 100% Me_2SO (405nm; 1.83×10^5 litre·mol^{-1}·cm^{-1}) agree well with the values (approx. 403nm, read from their Fig. 3; 1.74×10^5) found by Brown & Lantzke (1969) and those (404nm; approx. 1.87×10^5, read from their Fig. 1) recorded by Ellfolk & Mattsson (1969). The data of Table 1 and Fig. 1 show that $\lambda_{max.}$ moves gradually from 405nm in 100% Me_2SO to 401nm in 35% Me_2SO, and the molar absorption coefficient falls to about 90% of its value in 100% Me_2SO, i.e. to about 1.66×10^5 litre·mol^{-1}·cm^{-1} in 35% Me_2SO. We conclude that haemin remains monomeric with no significant changes over the range of solvent composition from 35 to 100% Me_2SO.

These data answer the two questions posed in the introduction. Firstly, haemin in neutral solution is monomeric if the Me_2SO content is greater than 35%, whereas horseradish peroxidase is reversibly inactivated when the Me_2SO content rises above 75% (Adams *et al.*, 1979). One can therefore study both the monomeric protein-free haemin and the enzymically active haemoprotein horseradish peroxidase in neutral solution over the range of solvent composition from 35 to 75% (v/v) Me_2SO; the range of overlap could probably be considerably extended by the use of acidic solutions (see below). Secondly, the variation of the absorption of the 'inactive' forms of peroxidase with variation in the Me_2SO content (see Table 1 and Fig. 1 of Adams *et al.*, 1979) is very different from that of haemin (Table 1 and Fig. 1). Over the range 0.25–1.00 mole fraction (i.e. 60–100%) Me_2SO the observed molar absorption coefficient of peroxidase at 403.5nm shows hysteresis (depending on whether one dilutes a concentrated stock solution of peroxidase in water or Me_2SO) and goes through a sharp maximum, e.g. the values rise from 1.20×10^5 at approx. 75% Me_2SO to 1.50×10^5 at approx. 80% Me_2SO and back to 1.25×10^5 or less at higher percentages of Me_2SO when diluting from water, and from approx. 1.25×10^5 at approx. 60% Me_2SO to 1.63×10^5 at 75% Me_2SO and back to 1.0×10^5 or less at higher percentages of Me_2SO when diluting from Me_2SO. By contrast, the molar absorption coefficient of the Soret band of free haemin at 402–404nm (and the values at 403.5nm will be very similar) remains almost constant at 1.8×10^5 M^{-1}·cm^{-1} and shows no hysteresis. We conclude that haemin derived from the 'inactive' forms of horseradish peroxidase in aqueous Me_2SO is not present as free haemin, but must remain bound in some way to the protein.

These results are also relevant to some of the conclusions drawn by Beaven *et al.* (1974) about the binding of haemin to human serum albumin. They compared the spectrum of methaemalbumin in the Soret-band region (values not stated in the text, but Fig. 1 of their paper gives $\lambda_{max.}=403$nm, ε approx. 0.87×10^5) with that of haemin in aq. 80% (v/v) Me_2SO (for which they apparently found $\lambda_{max.}=403$nm and ε approx. 0.86×10^5, as indicated in their Fig. 1). They stated that the two spectra were indistinguishable and concluded that the haemin in methaemalbumin is therefore not co-ordinated to any nitrogenous ligand. There appears to be a large discrepancy between their value of the molar absorption coefficient for haemin (0.86×10^5) and ours (approx. 1.8×10^5). However, they do not state if (and how) they purified their Me_2SO, and it seems likely that the use of unpurified Me_2SO could, as we have found, have caused the deposition of some solid and hence led to a lower apparent molar absorption coefficient. On the other hand, their values for methaemalbumin (403nm, 0.87×10^5) are close to those reported (Maehly, 1955) for horseradish peroxidase (403nm, 0.89×10^5–0.91×10^5), as pointed out by Maehly (1958). Since there is now good evidence that the iron in peroxidases is co-ordinated to a nitrogenous ligand (Yonetani & Yamamoto, 1973), the most reasonable conclusion is, in fact, that the haemin in methaemalbumin is co-ordinated to a nitrogenous base, just as it is in horseradish peroxidase.

The results in part (2) of the Results section and Fig. 1 show that over the range of 40–80% Me_2SO there exists only a single pH-dependent equilibrium with good isosbestic points, but that as the Me_2SO content decreases (and the water content increases), so the situation becomes more complex. The situation in 90–100% Me_2SO is also more complex than that in the 40–80% Me_2SO region, but has not been further studied. The effect of increasing the water content from 60 to 100% is apparently to split the single equilibrium (with a pK designated here as pK_{12}) into two equilibria (with pK_1 and pK_2). At the haemin concentrations used in this study pK_1 steadily decreases (from pK_{12} 7.5 in 65% Me_2SO to pK_1 approx. 1.5 in 0.01% Me_2SO), whereas pK_2 hardly changes (from pK_{12} 7.5 to pK_2 approx. 7.5). The changes observed in Fig. 1 below 35% Me_2SO reflect the change in pH, at which the first equilibrium is observed, from the alkaline to the acid side of the neutral solutions.

Maehly (1958) reported that haemin in 0.05M-H_2SO_4 formed a complex with $\lambda_{max.}=397$nm and $\varepsilon=0.81\times10^5$. Our results ($\lambda_{max.}=396$nm, $\varepsilon=0.8\times10^5$) are in good agreement. It appears that no-one else has reported this form of haemin. Maehly & Åkeson (1958) concluded that this was the di-aquo complex in which the carboxylic acid side chains were undissociated. Furthermore, Maehly (1958) found that the molar absorption coefficient of protohaemin in acid was lower (0.81×10^5) than

those of either mesohaemin (1.63×10^5) or deuterohaemin (1.44×10^5), but was increased (to 1.30×10^5) by binding to haemalbumin, whereas that of deuterohaemin was not: he therefore suggested that the lower absorption coefficient of protohaemin might be due to the partial formation of some aggregate with a lower absorption coefficient. Since solutions of protohaemin in Me_2SO/water mixtures of any composition at a pH lower than pK_1 (or pK_{12}) are characterized by a sharper Soret band than those observed at higher pH, it seems reasonable to conclude that there is some common denominator and that the main species present in these solutions are the monomeric complexes FeS_x, where S is a neutral solvent molecule (water or Me_2SO). Further work is needed to establish whether x is 1 or 2 (or whether a mixture is present) in any given solvent composition and whether and where any aggregation of these species occurs.

The equilibrium observed in 40–80% Me_2SO was studied quantitatively in 65% Me_2SO. Two separate spectrophotometric titrations of 5μM solutions of haemin with NaOH both gave $pK_{12} = 7.55$ and an apparent value of $n = 5$–6 (or even higher) in eqn. (2). Such a high value of n can only readily be explained on the basis of equations such as (3) or (4):

$$2(HO_2C)_2FeS_x + 2H_2O = [(^-O_2C)_2Fe\cdot OH^-]_2 + 6H^+ + 2xS \quad (3)$$

$$2(HO_2C)_2FeS_x + H_2O = (^-O_2C)_2Fe\cdot O^{2-}\cdot Fe(CO_2^-)_2 + 6H^+ + 2xS \quad (4)$$

which involve the formation of a dimer together with the (nearly) simultaneous ionization of most or all of the four carboxylic acid side chains. The spectrum of the alkaline product [see Fig. 3 and section (2) of the Results section] is similar in general shape and in the position of the bands to those reported by O'Keefe *et al.* (1975) for well-characterized μ-oxo derivatives, even though the Soret band is considerably higher (see below); the spectrum is, however, quite different from the spectra shown by haemin in aqueous alkali [see the data and references in section (2) of the Results section], which can probably be ascribed to a dimeric hydroxo complex formed by hydrophobic interaction between the two conjugated rings (Goff & Morgan, 1976). We conclude that the alkaline product in 65% Me_2SO is a μ-oxo complex. No evidence was seen for the intermediate formation of the monomeric hydroxo complex, i.e. equilibrium (5) must lie far to the right:

$$2Fe\cdot OH^- = Fe\cdot O^{2-}\cdot Fe + H_2O \quad (5)$$

Since the μ-oxo complex with four ionized carboxylate side chains would carry four negative charges, it is reasonable to expect some ion-pairing to occur with the added Na^+ ions. We therefore suggest that the apparent increase in the value of n in eqn. (2) from 5 to 6 or even higher during the course of the titration could be explained by the increasing stabilization of the μ-oxo product through ion-pair formation as the concentration of Na^+ ions increases.

We have found that the addition of 'inert' electrolytes such as $NaClO_4$ or $KClO_4$ has a marked effect on the μ-oxo complex. Our provisional conclusion is that the μ-oxo complex forms further ion-pairs with Na^+ and K^+ cations (in addition to those mentioned in the previous paragraph) and that the finite rate of interaction and the change in the spectrum are both due to some change in the structure of the μ-oxo complex, probably involving a change in the disposition of the carboxylate side chains. It is worth noting that the presence of excess $NaClO_4$ decreases the intensity of the Soret band from approx. 2.2×10^5 per mol of dimer per cm (at 398nm) to about 1.1×10^5 (at 397nm), and that the latter agrees with the values of 1.1×10^5–1.2×10^5 (at 397nm) reported for the sodium salt of the μ-oxo derivative of protohaemin dimethyl ester in benzene and pyridine (O'Keefe *et al.*, 1975), in which solvents ion-pair formation must obviously be greatly enhanced. The quantitive pH titration was carried out under conditions where the formation of these particular ion-pairs was negligible, as shown by the constancy of A_{404} with further additions of KOH (see Table 2).

The situation in aqueous solution is much more complex. The first pH-dependent equilibrium has previously been reported only by Maehly (1958), who found $pK_1 = 1.5$; our value of $pK_1 = 1$–1.5 is in good agreement. The second equilibrium was reported by Shack & Clark (1947), who found $pK_2 = 7.4$–7.6, and studied more quantitatively by Brown *et al.* (1970), who found $pK_2 = 7.5$; cf. our value 7–7.5. The variability in spectra that we have observed above pH7 presumably reflects the existence of the same pH-independent equilibrium between high-spin and low-spin complexes revealed by measurements of magnetic susceptibility (see Cohen, 1969; Goff & Morgan, 1976). We conclude that the presence of 0.01% Me_2SO has no significant effect on the species and equilibria shown by haemin in aqueous solution.

The present results provide numerous examples of the occurrence of two factors which often complicate and frustrate the study of equilibria involving haemin, namely (1) the marked effect of the presence of electrolytes which would normally be considered 'inert', and (2) the occurrence of both 'instantaneous' and slow reactions/equilibria under the same conditions. The clearest example is seen in 65% Me_2SO, where there exists only a single pH-dependent equilibrium. The added ions ($NaClO_4$ or $KClO_4$) appear to interact mainly with the μ-oxo complex and to cause significant changes in its spectrum, but to have little or no effect on the acid form; and the

simple pH-dependent equilibrium is established 'instantaneously', whereas the reaction with added electrolytes is relatively slow. Electrolytes have been reported to have marked effects in promoting the aggregation of haemin (Inada & Shibata, 1962) and pyridine haemochromogen (Nair & Elliott, 1975). It appears that ions can play a very critical and specific role in promoting the formation of dimers and perhaps even higher aggregates of these complexes.

A very instructive parallel is provided by Co(II) tetrasulphophthalocyanine, where cations can promote dimerization and change the spectrum of the dimer in both aqueous and non-aqueous solvents and where the rate of monomer–dimer equilibration can vary markedly depending on conditions (Abel *et al.*, 1976). It was also shown, in studying the effect of the solvent, that dimerization was promoted only by water and not by organic solvents with either higher (formamide) or lower (e.g. Me_2SO) dielectric constant. Preliminary experiments indicate that haemin also forms the same type of monomeric complex (in which Cl^- is no longer co-ordinated) in neutral formamide as in neutral Me_2SO.

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