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Solution Structures of Ferrihaem in some Dipolar Aprotic Solvents and their Binary Aqueous Mixtures

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1. Conductivity and u.v. and visible spectroscopic techniques were used to investigate the solution structure of the prosthetic group of the ferric haemoproteins (ferrihaem) in dimethyl sulphoxide, *NN*-dimethylacetamide, *NN*-dimethylformamide and sulpholane, and certain of their aqueous mixtures. 2. In neutral or acid dimethyl sulphoxide, chlorohaemin is monomeric and completely dissociated into Cl^- ion and a ferrihaem species with dimethyl sulphoxide molecules in the fifth and sixth co-ordination positions on iron. 3. In neutral *NN*-dimethylacetamide and *NN*-dimethylformamide chlorohaemin is monomeric but is largely undissociated, giving different spectra from that of chlorohaemin in dimethyl sulphoxide. On acidification, dissociation occurs and the dimethyl sulphoxide type of spectrum results. 4. Studies in a fourth solvent, sulpholane, indicate that solvent co-ordinating power (ligand strength) rather than bulk dielectric constant is responsible for dissociation of chlorohaemin. 5. In neutral dimethyl sulphoxide–water mixtures chlorohaemin remains monomeric and completely dissociated, and spectra are independent of mixture composition, except at high water concentrations, when precipitation occurs. In alkaline dimethyl sulphoxide–water mixtures, where the complete solvent mixture range is accessible, ferrihaem is polymeric (probably dimeric) and spectra are dependent on solvent composition. A quantitative analysis indicates that the spectral changes are due to replacement by water of one molecule of co-ordinated dimethyl sulphoxide per ferrihaem aggregate, and do not involve a two-molecule replacement as has been suggested for the alkaline pyridine–water system.

Although homogeneous catalysis by the ferrihaemoproteins and their simple inorganic analogues has been comprehensively studied and reviewed (Saunders, Holmes-Siedle & Stark, 1964; Nicholls & Schonbaum, 1963; Brown, Jones & Suggett, 1969), neither the biological systems nor the model systems are well understood in terms of the precise solution structure of the catalytic species. That this is a general problem in both biochemistry and inorganic chemistry is well illustrated by the continued attention given to mechanistic aspects of homogeneous catalysis. A major feature of recent work on ferric iron systems has been the significance of discrepancies between formal molecular structures and solution structures of the catalytic species (Sund, Weber & Molbert, 1967; Brown, Jones & Suggett, 1968; Jones & Suggett, 1968). This feature is typified by the complex haem, iron protoporphyrin IX, in which irreversible modification of the tetradentate ligand can occur (Brown & Jones, 1968*a*) in addition to polymerization reactions (Falk, 1964) and ligand substitution axial to the planar porphyrin ligand. In the ferric form this complex exhibits, albeit relatively crudely, catalase and peroxidase activity, and has been the subject of several studies as a model for these haemoproteins (Kremer, 1965, 1967; Brown & Jones, 1968*b*).

We have recently found that ferrihaem has a high solubility in some dipolar aprotic solvents and certain of their aqueous binary mixtures. We believe that our studies of ferrihaem in these solvent systems are important (*a*) in the correlation of solution structures of haems and haemoproteins (particularly near the active site) with electronic spectra, (*b*) in extrapolating data from non-aqueous to aqueous media and (*c*) in providing a basis for the n.m.r. study of haem compounds where low solubility has previously been an inhibiting factor. In this context we suggest that ferrihaem in a purely aqueous environment is not necessarily a better haemoprotein model than ferrihaem in a dipolar aprotic environment. Indeed, the ferrihaem–water system is associated with several complicating

features (e.g. haem–haem dimerization, ligand oxidation) that are absent from both ferrihaem–dipolar aprotic solvent systems and haemoprotein–water systems.

When isolated from chloride medium, ferrihaem contains a chloro ligand in the fifth co-ordination position and is usually termed chlorohaemin. In this work, chloroprotohaemin (ferric iron chloroprotoporphyrin IX) has been used throughout as starting material. The X-ray analysis by Koenig (1965) has shown that chlorohaemin is a penta-co-ordinate complex of iron with the iron atom 0·475 Å out of the plane of the porphyrin in the direction of chlorine.

The electronic spectrum of ferrihaem in any solvent is dominated by the highly intense band near 400 nm. (the Soret band) with several less intense bands occurring between 450 and 1000 nm. A notable feature of ferrihaem spectra is that in certain solvents, e.g. DMSO,* the Soret band is a narrow single band of high molar extinction coefficient ($>10^5$), whereas in other solvents, e.g. water, there are two distinct bands in this region. There have been several suggestions (e.g. see Urry, 1967) that a sharp single Soret band corresponds to monomeric ferrihaem, whereas a doublet shows the presence of dimers. We have recently isolated and characterized a ferrihaem dimer (Brown, Jones & Lantzke, 1969) and confirmed that this view is substantially correct. In the present work, using this spectroscopic criterion, we clearly distinguish between solutions in which only monomeric species exist and those where dimers or higher polymers are possible. The whole question of the existence and nature of ferrihaem dimers is complex, and of considerable importance, but is not discussed in detail here. In this paper we report spectra of ferrihaem under various conditions and discuss the spectra in terms of equilibria involving the various ferrihaem species.

Solutions of compounds of biological importance in dipolar aprotic solvents are of additional interest since DMSO, with its excellent solvent properties and low short-term toxicity, has found considerable clinical applications. These have included storage of biological fluids, e.g. haemoglobin and semen, use as a vehicle for the direct skin transfer of drugs and use in the treatment of fibrositis.

EXPERIMENTAL

Chlorohaemin was prepared from fresh defibrinated ox blood by the method of Fischer (1941) and recrystallized once. Determination as pyridine haemochrome indicated a purity of 99·9%. DMSO, DMF and DMA were BDH Laboratory Reagent grade chemicals [BDH (Chemicals) Ltd., Poole, Dorset], and were stored for 24 hr. over BDH type 4A molecular sieves and fractionally distilled at 5 mm. Hg pressure, the middle 60% fraction being collected. These purification techniques were carried out immediately before use of the solvent. Purified sulpholane (supplied by Fisons Ltd., Loughborough) was dried over type 4A sieves and stored in the frozen state. Other materials used were of A.R. grade. Oxygen-free nitrogen (less than 10 p.p.m. oxygen) was supplied by British Oxygen Co., Birtley, Co. Durham.

Spectrophotometric measurements were made on an Optica CF 4 recording instrument and a Cary 16 manual instrument. The recording instrument was used for wavelength scans in solvent mixtures and to monitor any change that occurred after preparation of the various solutions. The Cary 16 instrument was used for precise measurement of the spectra reproduced in Figs. 1, 2 and 3. The instruments agreed to within 1% in extinction and 1 nm. in wavelength. The Cary 16 instrument was equipped for measurement in a nitrogen atmosphere. All spectrophotometric measurements were made at $25\pm0·1°$. Conductivity measurements were made on a Wayne Kerr Universal Bridge type B221, fitted with a glass cell with shiny platinum electrodes (cell constant, 1·42 $cm.^{-1}$), thermostatically controlled at $25·04\pm0·01°$. Volumetric apparatus was calibrated grade B glassware.

In general, ferrihaem solutions of suitable concentrations for spectrophotometric measurements were prepared by dilution of stock solutions in pure solvents. This technique permitted various reagents to be added during the dilution stage so that a constant final ferrihaem concentration was maintained and spectra were directly comparable. Oxygen-free solutions were prepared in a nitrogen-purged glove-box, by using freshly distilled solvent that was first purged for 30 min. with dry nitrogen. The cuvettes were filled in the dry box, tightly stoppered and rapidly transferred to the nitrogen-purged cell compartment of the Cary 16 spectrophotometer.

RESULTS AND DISCUSSION

DMSO, DMA and DMF are relatively stable liquids with dielectric constants in the range 37–46 and have found extensive use because of their excellent solvent properties (Parker, 1962). They are known to form complexes with ferric iron (Langford & Chung, 1968; Drago, Carlson & Purcell, 1965), the co-ordinating power of DMSO being particularly strong. Nevertheless slow solvent decomposition can occur (Ferrari & Heider, 1963; Zaugg & Schaefer, 1964) and may be particularly important for DMA and DMF in ferric iron systems, since the dimethylamine produced is a stronger ligand than the solvent itself (Lantzke & Watts, 1967). Redox reactions involving DMSO are also possible; Berney & Weber (1968) reported the slow oxidation of ferrous iron by DMSO, but we have no evidence that such reactions are important under our experimental conditions.

Chlorohaemin was found to have a relatively high solubility in DMSO, DMF and DMA, but a negligible solubility in sulpholane or acetonitrile.

* Abbreviations: DMSO, dimethyl sulphoxide; DMF, *NN*-dimethylformamide; DMA, *NN*-dimethylacetamide.

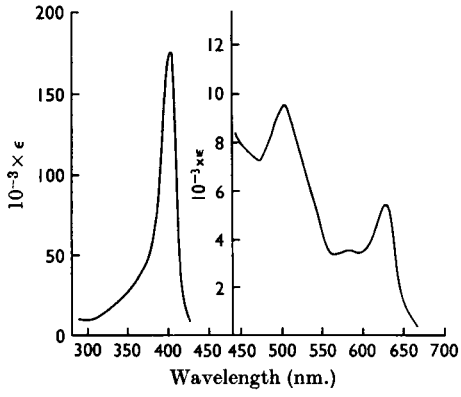

Fig. 1. Spectrum of chlorohaemin in DMSO.

At 25° a saturated DMF solution is approx. 0·5M; the solubility in DMSO is somewhat higher. We present our results and discussion first for pure solvent systems and then for certain solvent mixtures.

DMSO solutions. The spectrum of chlorohaemin in pure DMSO is shown in Fig. 1. The solution showed no significant spectral changes on standing for several days, and identical results were obtained whether air-saturated or nitrogen-purged solutions were used. Dilution experiments showed that Beer's Law was obeyed for chlorohaemin concentrations between 0·4 and 0·008mM. The sharpness of the bands in Fig. 1 is striking. Clearly there are four distinct bands in the visible region in addition to the intense Soret band. This sharp single band is indicative of monomeric ferrihaem. The spectrum is analogous to those of the ferrihaemoproteins in water, but is different from that of ferrihaem in water. Since haem–haem-bound polymeric units are very unlikely in the proteins, this is consistent with the view that ferrihaem is dimeric in aqueous systems but monomeric in DMSO. This is not unexpected, since ferrihaem is considered to be monomeric in strongly co-ordinating systems (Davies, 1940) and DMSO is known to be a moderately strong ligand (Lantzke & Watts, 1967). Certainly the sharpness of the Soret band suggests that absorption is due to a single chemical species. Assuming that ferric iron is hexa-co-ordinate in this solvent, there may be one or two DMSO solvent molecules bound to iron, depending on whether the chloro ligand has been replaced or not. We have resolved this problem by carrying out conductivity measurements on chlorohaemin solutions of different concentrations. Allowance for solvent conductivity and extrapolation to infinite dilution yielded a value of $29{\cdot}5\,\Omega^{-1}\text{cm.}^2\,\text{mole}^{-1}$ at $25{\cdot}04 \pm 0{\cdot}01°$ for the molar conductance of chlorohaemin in DMSO. Since Kolthoff, Chantooni & Bhownik (1968) have shown that the pK of acetic acid in DMSO is 12·6, it seems reasonable to assume that the ferrihaem propionic acid groups are largely undissociated in neutral DMSO and make little contribution to the conductivity. In addition, the proportion of the conductance attributable to the large ferrihaem species would be small. The mobility of Cl^- ion in DMSO has been estimated by Millen & Watts (1967) as $25{\cdot}2 \pm 0{\cdot}2\,\Omega^{-1}\text{cm.}^2\,\text{mole}^{-1}$ at 25°. From these data it is clear that in DMSO solutions chlorohaemin is almost fully dissociated, with little ion-pairing. The small discrepancy between 29·5 and $25{\cdot}2\,\Omega^{-1}\text{cm.}^2\,\text{mole}^{-1}$ probably represents the contribution of the ferrihaem cation. The structure of ferrihaem in neutral DMSO may therefore be represented by a species having two DMSO molecules bound to iron at the fifth and sixth positions and an overall charge of +1. We have confirmed this structure by showing that formiatohaemin in DMSO is spectroscopically identical with chlorohaemin in DMSO, which is to be expected if the fifth position ligand is replaced by solvent. Comparison with the work of Erdman & Corwin (1947) shows that the DMSO type of spectrum is obtained for dioxan solutions of ferrihaem perchlorate (assumed to be fully dissociated). The values of peak extinction coefficients in the two solvents are in excellent agreement [e.g. for the Soret band, ϵ_{dioxan} (ferrihaem perchlorate) $= 176 \times 10^3$, ϵ_{DMSO} (chlorohaemin) $= 174 \times 10^3$]. These results are consistent with our observations that chlorohaemin is fully dissociated in DMSO and suggest that the DMSO type of spectrum refers to the ferrihaem monocation with solvent molecules co-ordinated to iron in the fifth and sixth positions.

The presence of water in DMSO-ferrihaem solutions had, significantly, no effect on the spectrum up to a mole fraction of water $x_{H_2O} \leqslant 0{\cdot}75$. At this point the solution became colloidal and, on standing, ferrihaem was precipitated. It seems likely (and consistent with our work in alkaline solutions discussed below under 'DMSO–water mixtures') that, at $x_{H_2O} \geqslant 0{\cdot}75$, water molecules are significant competitors with DMSO molecules for co-ordination and that the aquo complex is insoluble in this particular solvent mixture. Perchloric acid up to a concentration of 110mM also had no effect on the spectrum. We may conclude that there is no spectroscopically observable change in the protolytic state of ferrihaem. The effect of alkali on solutions of ferrihaem in DMSO is complicated and is discussed in detail below under 'DMSO–water mixtures'.

DMA solutions. Spectra of chlorohaemin solutions in nitrogen-purged DMA were unchanged for at least 1hr. after preparation. Thereafter slow changes were observed, consistent with the slow

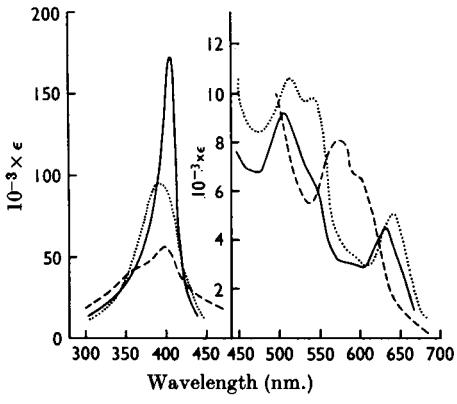

Fig. 2. Spectra of chlorohaemin in DMA. ....., Chlorohaemin in neutral DMA; ——, chlorohaemin in acid DMA (concn. of $HClO_4$ 23mM, x_{H_2O} 0·056); – – –, chlorohaemin in alkaline DMA (concn. of OH^- ion 10mM, x_{H_2O} 0·056).

decomposition of DMA, and consequent build-up of dimethylamine (cf. below under 'DMF solutions'). Spectra in non-nitrogen-purged DMA changed more rapidly. Spectra of chlorohaemin in DMA are shown in Fig. 2. The pure solvent spectrum is noticeably different from that of chlorohaemin in pure DMSO. The Soret-band extinction coefficient is almost halved and the visible band near 540 nm. is now well resolved. These differences are greater than any bulk solvent effects due to differential solvation of the ground and excited states and suggest some difference in the co-ordination state of ferrihaem. We suggest that the DMA type of spectrum results from a ferrihaem species that has retained the chloro ligand, has a solvent molecule in the sixth position and is uncharged. This idea is supported by the fact that the spectra of the halohaemins in dioxan (Erdman & Corwin, 1947) are different from those of ferrihaem perchlorate in dioxan, but very similar to those of chlorohaemin in DMA. This structure is further supported by substantial additional experimental evidence. Addition of aqueous perchloric acid to a solution of chlorohaemin in DMA (acid concentration 23mM, $x_{H_2O} = 0{\cdot}056$) gives a spectrum identical with that of chlorohaemin in neutral DMSO (Fig. 2). Water alone at this mole fraction had very little effect. The same spectrum was obtained by the addition of water to DMA for $x_{H_2O} \simeq 0{\cdot}7$. Intermediate spectra were obtained for addition of less water. It is likely that the effect of the addition of acid or excess of water is to solvate and thereby stabilize Cl^- ion, permitting replacement of the chloro ligand by a DMA molecule, or perhaps at high x_{H_2O} a water molecule. Assuming co-ordination of DMA via oxygen (nitrogen co-ordination is seriously sterically hindered), the observation of a spectrum very similar to that of chlorohaemin in DMSO is readily explained. Addition of hydrochloric acid rather than perchloric acid to a solution of chlorohaemin in DMA did not change the spectrum, which is consistent with the equilibrium being predominantly in favour of the chloro complex in the presence of excess of Cl^- ion. Final confirmation of these ideas was obtained when chlorohaemin solutions in DMA were shown to have only very small conductivities above that of the pure solvent. With the chloride molar conductance value in DMA of $44{\cdot}4\,\Omega^{-1}\,cm.^2\,mole^{-1}$ (Millen & Watts, 1967) our results indicate that chlorohaemin is less than 2% dissociated in DMA. The spectrum obtained on addition of aqueous alkali to chlorohaemin in DMA is shown in Fig. 2. This is noteworthy because of the decrease in extinction coefficient of the Soret band and its resolution into two distinct components. Under these conditions, spectra are very similar to those obtained in aqueous alkali and it is likely that ferrihaem dimerization occurs (see below under 'DMSO–water mixtures').

DMF solutions. Whereas DMSO is comparatively stable and DMA adequately so, DMF decomposes comparatively rapidly and it was necessary to make spectroscopic measurements immediately after preparation of solutions. The initial spectrum is very similar to that of chlorohaemin in DMA and it is likely that chlorohaemin is also largely undissociated in DMF. Addition of acid and water also produced the same effects as in DMA. The change with time of the spectrum of chlorohaemin in DMF was followed on the Optica CF4 instrument. The spectra thus obtained, together with that of chlorohaemin in DMF with a small amount of added diethylamine, showed clear isosbestic points, confirming that spectral aging is due to decomposition of DMF and co-ordination of the dimethylamine produced. Since dimethylamine is a considerably stronger ligand for ferric iron than is DMF, a very small amount of solvent decomposition thus has a large spectral effect.

DMSO–sulpholane mixtures. It is important to determine whether dielectric constant or solvent co-ordinating power is the more significant factor in determining the degree of dissociation of chlorohaemin in a particular solvent. It is hardly surprising that no dissociation occurs in dioxan (Erdman & Corwin, 1947), since this solvent has a low dielectric constant and is also a weak ligand. The case for the dipolar aprotic solvents concerned in this work is more complicated. The dielectric constants of DMSO, DMA and DMF are approx. 46, 37 and 38 respectively at 25° (Parker, 1962). DMSO therefore has somewhat more dissociating power on this basis than has DMA or DMF, but

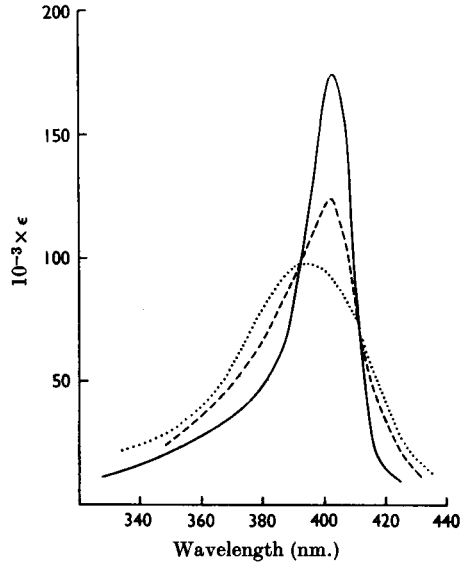

Fig. 3. Spectra of chlorohaemin in DMSO–sulpholane mixtures. ——, Pure DMSO; – – – –, $x_{\text{sulpholane}}$ 0·85; . . . . . , $x_{\text{sulpholane}}$ 0·97.

since it is also a much stronger ligand it is not possible to say which is the determining factor in the dissociation of chlorohaemin. We have resolved this problem by using an additional solvent, sulpholane, which has a dielectric constant close to that of DMSO (43) but is known to be a relatively poor ligand (Fitzgerald & Watts, 1967). Chlorohaemin does not dissolve in pure sulpholane at 30° but is soluble in sulpholane–DMSO mixtures for $x_{\text{sulpholane}} \leqslant 0{\cdot}97$. This fact alone suggest that co-ordinating power rather than dielectric constant governs the solubility of chlorohaemin in these solvents. Fig. 3 shows spectra of chlorohaemin in some sulpholane–DMSO mixtures in the region of the Soret band. Analogy with spectra in DMA and DMSO indicates that dissociation of chlorohaemin is small in mixtures at high $x_{\text{sulpholane}}$ and increases with increasing x_{DMSO}. The isosbestic points show that spectra intermediate between the DMA and DMSO types correspond to equilibrium mixtures of chlorohaemin and its dissociated form. Assuming that the dielectric constant of any sulpholane–DMSO mixture lies between the values for the pure solvents, little error is incurred in regarding the dielectric constant as invariant over the complete solvent mixture range. The changes in spectra may then be ascribed to the change in ligand properties of the solvent, indicating that solvent co-ordinating power is more important than dielectric constant in determining the degree of dissociation of chlorohaemin.

DMSO–water mixtures. The nature of chlorohaemin in DMSO–water mixtures was studied with three points in view: (*a*) that the catalytic importance of ferrihaem refers chiefly to aqueous solution, (*b*) that it is interesting to compare water and DMSO as ligands and (*c*) that previously reported ferrihaem spectra in aqueous solutions have sometimes been difficult to reproduce. Having characterized chlorohaem in pure DMSO we hoped that a study in DMSO–water mixtures would permit extrapolation to aqueous solution. Chlorohaemin has a negligible solubility in pure water or in aqueous acid, but becomes significantly soluble in aqueous alkali. The complete mole-fraction range of DMSO–water mixtures becomes accessible if a small concentration of sodium hydroxide is added to each mixture. Therefore the discussion below refers to the nature of ferrihaem in DMSO–water mixtures containing a constant [OH^-] of 5mM. Solutions could be prepared in any of several ways; for example, chlorohaemin could be dissolved in aqueous alkali and DMSO added, in DMSO and aqueous alkali added or directly in a suitable alkaline DMSO–water mixture. Spectra were obtained, usually within 5min. of solution preparation. After attainment of equilibrium spectra of solutions were independent of the method of preparation, but in DMSO-rich mixtures results were not reproducible. Such solutions underwent a rapid initial change with time (over several minutes) followed by a slower change (over several hours). In all cases a decrease in Soret-band extinction coefficient was observed as compared with pure DMSO as solvent, but sometimes this band appeared as a well resolved doublet with peaks at 360 and 403nm., whereas on other occasions only a small inflexion occurred on the high-energy side of the usual band. Similar irreproducible changes occurred for the visible bands. It seems likely that several factors contribute to the difficulties in observing meaningful spectra in DMSO-rich mixtures in the presence of alkali. The OH^- ions would be significant ligand competitors with DMSO for co-ordination to iron, but the most complicating feature is probably the dimerization of ferrihaem. DMSO–water mixtures at high x_{DMSO} are extremely viscous and complete mixing takes a significant time. Also, a very large heat of mixing is involved. These factors probably affect the relative extent to which OH^- ion substitution and dimerization take place initially and result in difficulty in reproducing initial spectra. Further changes with time in DMSO-rich mixtures may be due to redox reactions involving solvent or iron. Since we are primarily interested in the water-rich mixtures, we have not investigated these effects further.

For $x_{\text{H}_2\text{O}} \geqslant 0{\cdot}77$ spectra were reproducible and did not change significantly within a week after

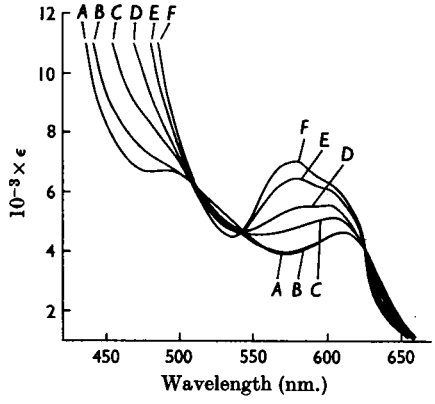

Fig. 4. Spectra of chlorohaemin in alkaline DMSO–water mixtures. Concn. of OH^- ion 5mM; x_{H_2O}: *A*, 0·990; *B*, 0·978; *C*, 0·968; *D*, 0·953; *E*, 0·904; *F*, 0·771.

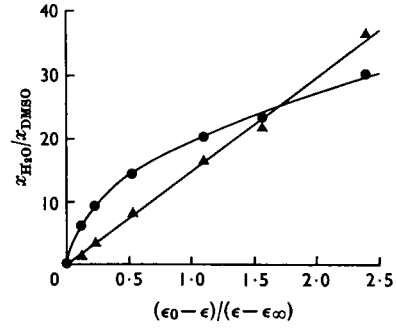

Fig. 5. Ligand replacement of DMSO by water on ferrihaem. Relationship between $(x_{H_2O}/x_{DMSO})^n$ and $(\epsilon_0-\epsilon)/(\epsilon-\epsilon_\infty)$. ●, $n=1$; ▲, $n=2$.

preparation of solutions. These spectra in the range 420–660nm. are shown in Fig. 4 for various mixtures between $x_{H_2O}=0{\cdot}77$ and $x_{H_2O}=1$. The sharp isosbestic points suggest that only two absorbing species exist in this mole-fraction range. These spectral changes are produced at constant $[OH^-]$ by increasing x_{H_2O} and, although it is not impossible that they are due to ligand replacements involving OH^- ion (since the nucleophilicity of OH^- ion varies with x_{H_2O}; McGregor, 1967), it seems more likely that the spectral changes are caused by ligand replacement of DMSO by water. Gallagher & Elliott (1968) have observed similar behaviour for ferrihaem spectra in alkaline pyridine–water solutions and have concluded that the insertion of two molecules of pyridine into a ferrihaem dimer is responsible for the spectroscopic changes. Our data can be tested quantitatively in the following way.

The spectral changes may be represented by the replacement reaction:

$$(\text{Ferrihaem})(\text{DMSO})_n + n\text{H}_2\text{O} \underset{}{\overset{K}{\rightleftharpoons}} (\text{Ferrihaem})(\text{H}_2\text{O})_n + n\text{DMSO}$$

where the polymeric nature of the two haemin species is unspecified. If ϵ_0 is the extinction coefficient of $(\text{Ferrihaem})(\text{DMSO})_n$, ϵ_∞ is the extinction coefficient of $(\text{Ferrihaem})(\text{H}_2\text{O})_n$ and ϵ is the extinction coefficient of any mixture, then by using the equilibrium above, and assuming that DMSO–water mixtures are ideal, it is readily shown that:

$$K(x_{H_2O}/x_{DMSO})^n = (\epsilon_0-\epsilon)/(\epsilon-\epsilon_\infty) \qquad (1)$$

where K is the equilibrium constant. Plots of $(x_{H_2O}/x_{DMSO})^n$ for $n=1$ and $n=2$ are shown in Fig. 5. The data show a reasonably linear relationship for $n=2$ and are apparently in good agreement with the results of Gallagher & Elliott (1968). However, an examination of activities for water and DMSO in mixtures at high x_{H_2O} (Table 1) reveals that these solutions are far from ideal, especially for very low values of x_{DMSO}. We have computed these activity values from freezing-point data (Crown Zellerback Corp., 1966) and they refer to slightly varying temperatures below the freezing point of water. It is probable that we shall introduce a small error in using these activity values, since sodium hydroxide is present in our system. However, since the $[OH^-]$ and the total ionic strength are small and constant, the relative values of our calculated activities will be essentially correct. To account for non-ideality, it is necessary to modify eqn. (1) to:

$$K'(a_{H_2O}/a_{DMSO})^n = (\epsilon_0-\epsilon)/(\epsilon-\epsilon_\infty) \qquad (2)$$

where a_{H_2O} and a_{DMSO} are activities on the mole-fraction scale at 25° and K' is the thermodynamic equilibrium constant. Assuming that a_{H_2O} and a_{DMSO} do not vary greatly between the freezing point and 25°, this function has been plotted in

Table 1. *Activities and spectrophotometric data at 580nm. for ferrihaem in alkaline DMSO–water mixtures*

$\epsilon_\infty = 4{\cdot}01\times10^3$, $\epsilon_0 = 7{\cdot}02\times10^3$, $[OH^-]=5$mM.

x_{DMSO}	x_{H_2O}	a_{DMSO}	a_{H_2O}	$10^{-3}\epsilon$
0	1·000	—	—	4·01
0·010	0·990	—	—	4·03
0·032	0·968	0·0030	0·981	4·89
0·041	0·959	0·0046	0·971	5·18
0·047	0·953	0·0058	0·961	5·47
0·065	0·935	0·0108	0·927	5·98
0·096	0·904	0·0281	0·869	6·46
0·139	0·861	0·110	0·710	6·71
0·229	0·771	—	—	7·02

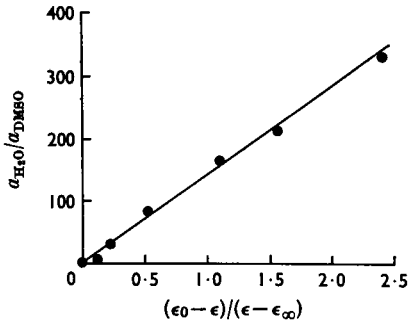

Fig. 6. Ligand replacement of DMSO by water on ferrihaem. Relationship between $(a_{H_2O}/a_{DMSO})^n$ and $(\epsilon_0-\epsilon)/(\epsilon-\epsilon_\infty)$ for $n=1$.

Fig. 6 for $n=1$ and shows good linearity. The value of K' from this graph is $7{\cdot}1\times10^{-3}$. Further, if $n=2$, we might expect to observe the formation of the intermediate complex ($n=1$) spectroscopically. This would give at least three absorbing species and, in the absence of fortuitous coincidence of extinction coefficients at several wavelengths, isosbestic points would not be observed. On this basis we conclude that only one molecule of co-ordinated DMSO is being replaced by water for each ferrihaem aggregate. It is possible that, if Gallagher & Elliott (1968) had worked with activities rather than concentrations, their data would have led to the same conclusion. These results give no information about the polymeric nature of ferrihaem, other than that it does not change under the observed conditions. Assuming that at $[OH^-]=5$ mM at least one ligand is OH^- ion, these changes may be represented by:

$$\text{DMSO–(ferrihaem)–OH} + H_2O = H_2O\text{–(ferrihaem)–OH} + \text{DMSO}$$

where the polymeric nature of ferrihaem is unspecified, except that at least two co-ordination positions on iron atoms must be available. This representation is consistent with previously suggested structures for ferrihaem in aqueous alkali (haematin) (Falk, 1964). The above analysis suggests that water becomes a significant competitor with DMSO for ferrihaem co-ordination at $x_{H_2O}\geqslant0{\cdot}77$, in good agreement with our work in neutral DMSO–water mixtures, when precipitation occurred for $x_{H_2O}\geqslant0{\cdot}75$. It is difficult to give quantitative significance to the relative ligand strengths of water and DMSO for ferrihaem since in DMSO–water mixtures the medium is not constant, but it is clear qualitatively that DMSO is much the better ligand in this environment.

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