The Formation of Pyridine Haemochromogen

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1. Titration of haem with pyridine in alkaline media of low ionic strength yields a true pyridine haemochromogen, compound III, at very low concentrations of pyridine. 2. Graphical analysis of this titration gives the first spectrophotometric evidence for a dimeric haem. 3. Compound III is unstable and tends to aggregate to a second compound, compound II, whose formation is enhanced under those conditions favourable to hydrophobic bonding. 4. At higher concentrations of pyridine, compound II is dispersed to yield the classical pyridine haemochromogen, compound I, whose spectral properties are essentially those of pyridine haemochromogen in a non-aqueous medium.

Since the elucidation of the nature of haemochromogen by Anson & Mirsky (1925) and the discovery by Keilin (1925) of the central role of certain haemochromogens in energy metabolism, the interrelationships of haematin, haem and haemochromogen have been the subject of intensive study by a number of workers. Their results have been summarized by Lemberg & Legge (1949) and more recently by Falk (1964). Smith (1958) reported the formation of at least three distinct pyridine–haem compounds during the titration of alkaline haem with pyridine. In view of the importance of pyridine haemochromogen formation for the quantitative determination of haematin compounds it was considered that a better understanding of this titration was desirable. We have therefore determined the spectral properties of the compounds, the conditions for the optimum development of each and the relationships that exist between them during the titration.

MATERIALS AND METHODS

Chemicals. Baker Analyzed reagent-grade chemicals or their equivalent were used throughout.

Haemins. Four haemin samples were used. Two commercial samples of Hemin Recrystallized, one from Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.) and one (lot 1043) from Mann Research Laboratories (New York, N.Y., U.S.A.), were used without further purification. Another highly purified sample was supplied by Dr M. E. Chilcote and the fourth sample was prepared and recrystallized in this Laboratory as described by Fischer (1941). There was no detectable difference in the behaviour of the different samples.

Spectrophotometry. Absorption spectra and changes in extinction were recorded with the Cary model 11 recording spectrophotometer by using silica cells with light-paths ranging from 5 to 0·1cm.

Preparation of solutions. Fresh haematin solutions were made up daily and used within 6hr. Crystalline haemin was dissolved in 20mN-NaOH to yield stock solutions that were diluted to give the required concentrations. All haem concentrations are given on the basis of a dimeric haem (i.e. 2 atoms of iron/mol. of haem). Compound III was formed by mixing the haematin and pyridine and diluting to volume before reduction with the minimum amount of $Na_2S_2O_4$, with less than 1mg./3ml. of solution in the spectrophotometer cuvette. Compound II was formed by mixing the haematin and freshly prepared $Na_2S_2O_4$ solution (2·5%, w/v) before dilution with 20mN-NaOH and stock pyridine solution (0·1M-pyridine in 20mN-NaOH). The final $Na_2S_2O_4$ concentration was 0·25% (w/v). Compound I was prepared in two ways: (*a*) by pre-mixing the reagents and reducing with the minimum amount of solid $Na_2S_2O_4$ (final pyridine concentration: 20%, v/v); (*b*) by reduction of haematin with freshly prepared $Na_2S_2O_4$ solution (2·5%, w/v) and then dilution with 20mN-NaOH and addition of pyridine to 20% (v/v) final concentration; the final $Na_2S_2O_4$ concentration was 0·25% (w/v). Pyridine haemochromogen in ethylene glycol was prepared by making compound II and then diluting with 2vol. of ethylene glycol. After mixing, the solutions were transferred to cuvettes and the spectra recorded.

Each point on a titration curve represents a separate experiment in which the only variable was the pyridine concentration. The absorption spectrum of each solution was read within 1–2min. of the formation of the haemochromogen.

RESULTS

The three compounds noted by Smith (1958, 1959) in the titration of alkaline haem with pyridine are referred to below as compounds III, II and I in order of their appearance in the titration. Compound I is the final product of the titration, the classical pyridine haemochromogen. Table 1 lists the spectral properties of these compounds. The extinction coefficients given are the highest

Table 1. *Spectral properties of compounds I, II and III*

Compound	α-Band		β-Band		475–480 mμ Band		Soret band	
	(mμ)	(ϵ_{mM})	(mμ)	(ϵ_{mM})	(mμ)	(ϵ_{mM})	(mμ)	(ϵ_{mM})
Compound III at 25°	555	25·5	523	14·1	475	9·45	416	132
Compound II at 25°	562	28·6	528	15·6	480	11·0	432·5	83
Compound II at 30°	559	29·9	528	16·1	475	12·5	431·5	77·5
Compound I at 25°	558	30·6	526·5	17·0	478	12·3	418·8	157
Compound I at 30°	556·5	22·5	523	12·9	475	10·5	417·5	117
Pyridine haemochromogen in glycol at 25°	556	30·0	525	15·5	477	11·4	417	160
Pyridine haemochromogen in benzene + pyridine at 25°	558	—	527	—	481	—	419·7	—

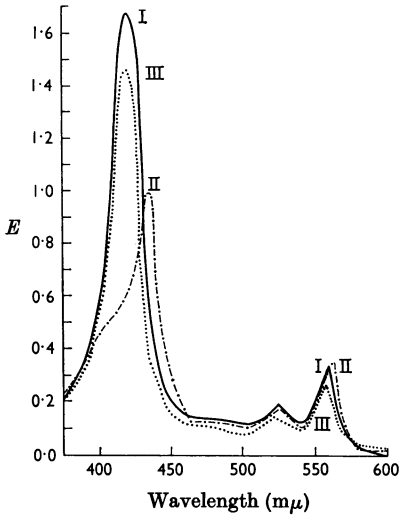


Fig. 1. Absorption spectra of compounds I (——), II (–·–·–) and III (·····). The spectra of compounds I and III were obtained with 10·3 μM-haemochromogen solutions. The spectrum of compound II was obtained with 100 μM-pyridine haemochromogen by using a 0·1cm. light-path instead of the 1cm. path used for the other two spectra.

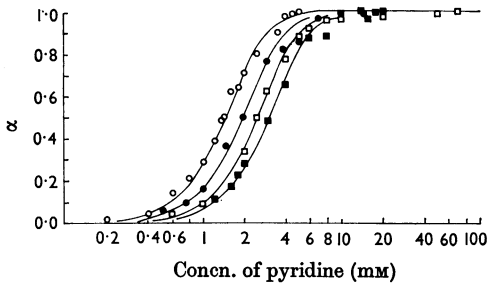


Fig. 2. Data from titrations of four different concentrations of haem with pyridine plotted as α, the fraction of haem converted into haemochromogen, against the millimolar pyridine concentration (on a logarithmic scale). The concentrations of dimeric haem were: ○, 0·43 μM; ●, 2·15 μM; □, 5·3 μM; ■, 10·0 μM.

readings that were obtained. Because of the chemical equilibria that exist between the three compounds, these coefficients must not be considered as the true coefficients. The separately determined spectra of the three compounds are shown in Fig. 1.

Compound III. The formation of compound III is favoured by mixing the haematin and pyridine and diluting to volume before reduction with solid dithionite. (Titration of haematin with pyridine in 20mN-sodium hydroxide showed that less than 1% of the haematin formed pyridine parahaematin when the pyridine concentration was 10mM (Gallagher, 1965). In Fig. 2 data from several titrations of haem with pyridine over a range of haem concentrations from 0·43 to 10 μM are plotted as α, the fraction of haem converted into haemochromogen, against millimolar pyridine concentration (on a logarithmic scale). The experimental points are in good agreement with the solid (theoretical) curves, which represent the reaction of a dimeric haem with 4mol. of pyridine to form 2mol. of pyridine haemochromogen, as in eqn. (1):

$$Hm_2 + 4\ Pyr \rightleftarrows 2\ HmPyr_2 \qquad (1)$$

At 25° $K_D = 3{\cdot}68 \times 10^{-6}$ moles^{3}l.$^{-3}$.

Fig. 3 represents the ultimate criterion for the splitting of a dimer as recommended by Cowgill & Clark (1952). Data from Fig. 2, at a constant pyridine concentration of 2mM, are plotted as $M\alpha$ (on a logarithmic scale) against $M(1-\alpha)$ (also on a logarithmic scale), where M is the molar concentration of dimeric haem. The fit of the experimental data to a theoretical curve with slope of 0·5 shows that the dimeric haem is being split by 4mol. of pyridine to form 2mol. of pyridine haemochromogen. The broken line in Fig. 3 repre-

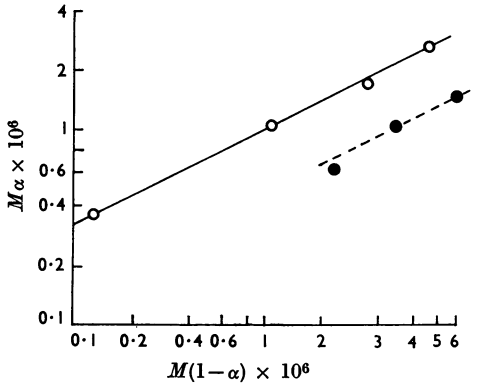


Fig. 3. 'Dilution test plot': a plot of $M\alpha$ (on a logarithmic scale) against $M(1-\alpha)$ (also on a logarithmic scale), where M is the molar concentration of dimeric haem, at constant pyridine concentration (2 mM). The solid line is the theoretical curve, with slope 0·5, for the pyridine haemochromogen system, and the broken line is that for the imidazole haemochromogen system.

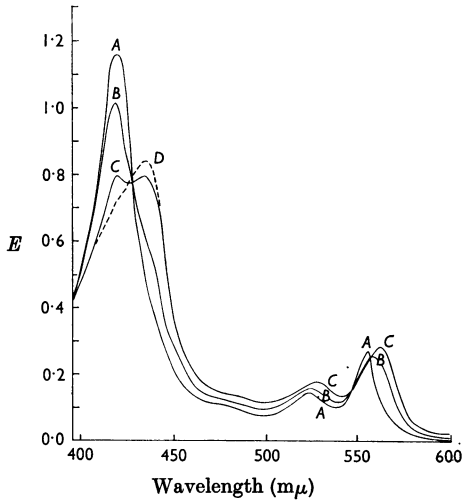


Fig. 4. Conversion of compound III into compound II. Curve A represents freshly formed compound III, and curve B was obtained with the same sample 10 min. later. Curve C shows the spectrum of the same sample immediately after the addition of 3 mg. of Na_2SO_4, and curve D shows the Soret spectrum of the same sample 5 min. later.

sents the analogous imidazole haemochromogen compound where at 28° K_D $1{\cdot}09 \times 10^{-5}$ moles^{3}l.$^{-3}$. The method used for the formation of imidazole haemochromogen was identical with that for pyridine haemochromogen.

The titration of haem with pyridine in 67% alkaline ethylene glycol solution, as described in the Materials and Methods section, indicates that haem titrates as a monomer in this medium with K_D $1{\cdot}76 \times 10^{-5}$ moles^{2}l.$^{-2}$ at 25°.

Compound II. The formation of compound II is favoured by conditions of high ionic strength, high haemochromogen concentration, higher temperatures and by reduction of the haematin before the addition of pyridine. The spontaneous conversion of compound III into compound II is illustrated in Fig. 4. Curve A was recorded immediately on reduction of the haematin–pyridine mixture in the cuvette to form compound III, and curve B was recorded 5 min. later. During this time there was a decrease in the Soret peak and the development of a shoulder at 433 mμ together with a shift of the bands in the visible spectrum to longer wavelengths. This change to compound II does not go to completion and usually reaches equilibrium in 40 min. at 25° and in 10 min. at 30°. After curve B had been recorded about 3 mg. of solid sodium sulphate was added to the cuvette and curve C was immediately recorded. Soret absorption bands of both compounds III and II were apparent and a further shift to longer wavelengths in the visible spectrum occurred. The same effect was produced by sodium chloride or by excess of dithionite, showing it to be a simple salt effect and excluding the possibility that the change was due to autoxidation. Curve D, recorded in the Soret region only, was recorded 5 min. after curve C and showed further conversion of compound III into compound II.

Compound I. When minimum amounts of dithionite were used to reduce the haematin–pyridine mixtures only compound III was detected in the titration. However, if several minutes elapsed between the time of reduction and the recording of the spectrum, mixtures of compounds III and II were present. With increasing pyridine concentrations, i.e. above 0·2 M, there was a gradual shift and sharpening of the absorption spectrum of the haemochromogen corresponding to a change from compound III to compound I. The shift appeared to be complete when the pyridine concentration reached 2·5 M (20%, v/v) and it is probably a solvent effect. In any case, by premixing the haematin and pyridine before reduction with the minimum of solid dithionite, it is possible to pass from compound III to compound I without any detectable sign of compound II and with no isosbestic points in the titration.

Formation of compound I by pre-reduction of haematin with dithionite, as in method (*b*), gave stable reproducible Soret peaks. Preparation of compound I by method (*a*) gave initial Soret peaks which were 10–20% higher than those by method (*b*) but which decreased with time (Fig. 5).

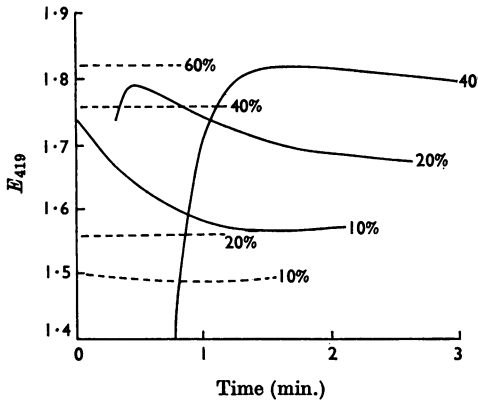


Fig. 5. Effect of order of mixing reagents on the formation of compound I. Broken lines show stability of pyridine haemochromogen with time when haematin is reduced with $Na_2S_2O_4$ before the addition of pyridine. Solid curves show the decay in Soret bands with time that occurs in samples which are pre-mixed and then reduced with the minimum of solid $Na_2S_2O_4$. The % values represent the pyridine concentration (v/v).

Spectra of haemochromogen at the temperature of liquid nitrogen. By using techniques developed in this Laboratory (Elliott & Tanski, 1962; Doebbler & Elliott, 1965), we detected the expected shift of the absorption bands of compounds I and II to shorter wavelengths at the lower temperature (Hartridge, 1921). The absorption bands of compound III shifted to longer wavelengths, which suggests an aggregation phenomena, due to freezing, similar to that reported by Hartree (1955) in his studies on turacin compounds at low temperatures.

DISCUSSION

Hogness, Zscheile, Sidwell & Barron (1937) have given excellent spectrophotometric evidence for the existence of haematin as a dimer in alkaline aqueous media. The dimeric haematin reacts with CN^- ion to form 2mol. of dicyanohaematin according to the equation:

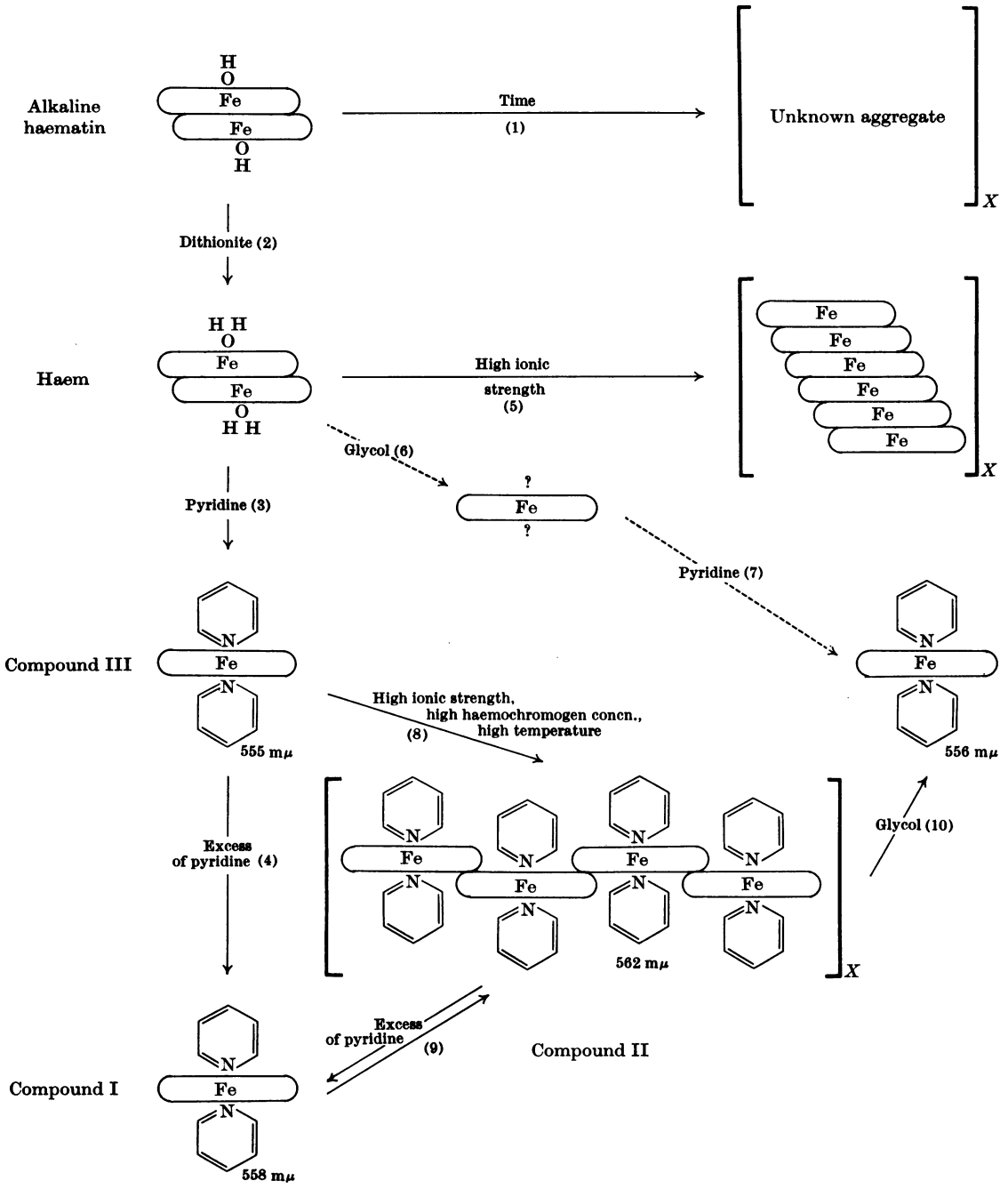
$$Hm_2(OH)_2 + 4CN^- \rightleftarrows 2Hm(CN^-)_2 + 2OH^- \quad (2)$$

This work has been confirmed by Shack & Clark (1947) and extended to show the existence of a dimeric haematin at pH values as low as 6·72.

The evidence for the existence of a dimeric haem was not convincing in spite of intensive work on the problem (Davies, 1940; Shack & Clark, 1947; Walter, 1952). However, our investigations on the formation of compound III in alkaline aqueous media provide good evidence for dimeric haem. Under conditions of low ionic strength, reduction of the pre-mixed haematin and pyridine with the minimum of solid dithionite gives titration curves agreeing closely with the theoretical curve for a dimeric haem reacting with 4mol. of pyridine to yield 2mol. of pyridine haemochromogen (Fig. 2). Further, the 'dilution test', first used by Hogness *et al.* (1937) and later by Shack & Clark (1947), confirms the splitting of the dimeric haem (Fig. 3). Compound III is unstable and spontaneously aggregates to compound II, as shown by the shift of absorption bands to longer wavelengths. This aggregation is favoured by high haemochromogen concentration, high ionic strength and higher temperatures. All of these factors militate against the use of compound III, the pyridine haemochromogen formed at low pyridine concentrations, for quantitative determination of haematin compounds.

Anson & Mirsky (1925) noted that several different haemochromogens exist in what they called an α- and β-form, each with its characteristic absorption bands. Their α- and β-forms correspond to our compounds II and I respectively. The α-form was the less soluble and was formed at lower ligand concentrations. The β-form was the more soluble and could be formed from the α-form by increasing the ligand concentration. The absorption bands of the β-haemochromogen appeared at shorter wavelengths than those of the α-compound. Keilin (1926) related this difference in absorption-band positions to the physicochemical state of the pigment. He recognized five different positions for the α-band of pyridine haemochromogen, each of which was dependent on the particular state of aggregation of the pigment or on the solvent system or both. These early observations with the Zeiss micro-spectroscopic ocular and the Hartridge reversion spectroscope are in complete harmony with our results. Analysis of our titration data supports this earlier interpretation of the variations in absorption-band positions.

Compound II is an aggregate, but its nature is not clear at present. Davies' (1940) potentiometric experiments with the nicotine–haemochromogen system in aqueous media suggested that both the parahaematin and the haemochromogen exist as dimers or at least that the reacting moieties appeared to be dimeric. These dimeric units might be further loosely aggregated, as was found by Shack & Clark (1947) for dimeric haematin. The fact that aggregation of compound III into compound II is favoured by higher ionic strengths and higher temperatures but that compound II is dispersed as a monomeric pyridine haemochromogen by such solvents as pyridine and ethylene glycol suggests that the formation of aggregates involves hydrophobic bonding. In view of the marked spectral differences between compound II and compounds I



Scheme 1. Summary of equilibria involved in the formation of pyridine haemochromogen in alkaline media.

and III the hydrophobic bonding probably involves an overlap of the haem rings.

Parallel experiments with the imidazole haemochromogen system yield a similar shift in absorption spectra, although the system is not as sensitive to the different parameters as is the pyridine haemochromogen system. This suggests that the formation of imidazole haemochromogen might be a

better method for quantitative determination of haematin pigments. It also suggests that the haem of cytochrome *b* (α-band 563 mμ) may not be bound as a simple haemochromogen but may be involved in further interaction between the haem and the protein so as to produce a shift of the α-band of the haemochromogen to longer wavelength.

Compound I. The formation of compound I, the classical pyridine haemochromogen, has been carefully studied by Paul, Theorell & Åkeson (1953) and Hartree (1955). Reproducible formation of pyridine haemochromogen requires at least 10% (v/v) pyridine not only 'to keep the haemochromogen in solution' (Hartree, 1955) but also to disperse compound II into the monomeric form. The effect of reducing the haematin before the addition of the pyridine, as opposed to pre-mixing and then reducing, has been discussed above. Apparently the pre-reduction recommended by Smith (1959) and others favours an aggregated system that quickly reacts with the added pyridine and is rapidly dispersed to an equilibrium mixture of monomeric and aggregated forms. If the pyridine is pre-mixed with the haematin the high concentration of pyridine (20%, v/v) probably disperses the haematin to a monomeric parahaematin compound. When this system is reduced with the minimum of solid dithionite the immediate reaction product is a monomeric pyridine haemochromogen that slowly equilibrates to an aggregated form.

The environment of compound I in 20% (v/v) pyridine appears to be essentially that of pyridine haemochromogen in a non-polar medium. Extraction of pyridine haemochromogen into benzene (Caughey, Alben & Beaudreau, 1964) gives rise to absorption bands of pyridine haemochromogen in the pyridine–benzene mixture that are very similar to those of pyridine haemochromogen in pyridine–20 mN-sodium hydroxide (1:4, v/v) (Table 1).

A scheme to explain our results is given in Scheme 1. Both haem and haematin are pictured as dimers, and though the degree of overlap of the rings is uncertain it must be insufficient to permit any iron–iron interactions except at high pH (Barron, 1937). Reaction (1) is the well-documented deterioration of aqueous haematin solutions on standing in light and air (Maehly & Åkeson, 1958). We noted a marked deviation of the experimental data from the theoretical curves for reactions involving a dimeric haem if the haematin solution stood at room temperature for more than 6 hr. Reaction (2) represents the reduction of dimeric haematin to dimeric haem. The tendency for haem in concentrations as low as 10 μM to aggregate (reaction 5) is greatly enhanced in solutions of high ionic strength. The formation of compound III (α-band 555 mμ) is represented by reaction (3). At very low ionic strength the order of mixing reagents had little effect on its formation. However, the most effective and reproducible experimental methods required mixing the haematin and pyridine before reduction with solid dithionite. The reaction of haematin and pyridine at these concentrations was almost non-existent (Gallagher, 1965). The presence of pyridine at the time of reduction allows reaction (3) to compete successfully with reaction (5) and to give full formation of compound III. Reaction (4) involves a change in solvent system from 10 mM-pyridine in 20 mN-sodium hydroxide to 2·5 M-pyridine in 16 mN-sodium hydroxide. The shift in band positions of the pyridine haemochromogen are attributed solely to the change in solvent system. The higher pyridine concentration prevents the aggregation of compound III into compound II. The formation of compound II from compound III (reaction 8) has all the properties of a hydrophobic interaction. This aggregate is pictured as one involving a haem–haem overlap, principally on the basis of marked changes in spectrum. Reactions (9) and (10) both demonstrate the dispersing effect of the non-polar medium on compound II and thus give further evidence for an aggregation involving hydrophobic bonding. On this basis and from the experimental observations by Smith (1959) it is probable that reactions (6) and (7) also represent a pathway for the formation of compound II. It has not been shown whether the effect of ethylene glycol is essentially a solvent effect, the fifth and sixth co-ordination positions of the monomeric haem being occupied by water molecules, or whether the ethylene glycol itself co-ordinates with the haem iron.

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REFERENCES

Anson, M. L. & Mirsky, A. E. (1925). *J. Physiol.* **60**, 50.
Barron, E. S. G. (1937). *J. biol. Chem.* **121**, 285.
Caughey, W. S., Alben, J. O. & Beaudreau, C. A. (1964). *Proc. int. Symp. Oxidases and Related Oxidation Reduction Systems, Amherst, Mass.*, Ed by King, T. E., Mason, H. S. & Morrison, M. New York: John Wiley and Sons Inc.
Cowgill, R. W. & Clark, W. M. (1952). *J. biol. Chem.* **198**, 33.
Davies, T. H. (1940). *J. biol. Chem.* **135**, 597.
Doebbler, G. F. & Elliott, W. B. (1965). *Biochim. biophys. Acta*, **94**, 317.
Elliott, W. B. & Tanski, W. (1962). *Analyt. Chem.* **34**, 1672.
Falk, J. E. (1964). *Porphyrins and Metalloporphyrins*, p. 181. Amsterdam: Elsevier Publishing Co.

Fischer, H. (1941). *Org. Synth.* **21**, 53.
Gallagher, W. A. (1965). Ph.D. Thesis: State University of New York at Buffalo.
Hartree, E. F. (1955). In *Modern Methods of Plant Analysis*, vol. 4, p. 197. Ed. by Paech, K. & Tracey, M. Berlin: Springer-Verlag.
Hartridge, H. (1921). *J. Physiol.* **54**, cxxviii.
Hogness, T. R., Zscheile, F. P., Sidwell, A. E. & Barron, E. S. G. (1937). *J. biol. Chem.* **118**, 1.
Keilin, D. (1925). *Proc. Roy. Soc.* B, **98**, 312.
Keilin, D. (1926). *Proc. Roy. Soc.* B, **100**, 129.
Lemberg, R. & Legge, J. W. (1949). *Hematin Compounds and Bile Pigments*, pp. 159–205. New York: Interscience Publishers Inc.
Maehly, A. C. & Åkeson, Å. (1958). *Acta chem. scand.* **12**, 1259.
Paul, K. G., Theorell, H. & Åkeson, Å. (1953). *Acta chem. scand.* **7**, 1284.
Shack, J. & Clark, W. M. (1947). *J. biol. Chem.* **171**, 143.
Smith, M. H. (1958). *Biochem. J.* **68**, 27P.
Smith, M. H. (1959). *Biochem. J.* **73**, 90.
Walter, R. I. (1952). *J. biol. Chem.* **196**, 151.