

Stabilization of Aqueous Hemin Solutions*

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The deterioration of aqueous hemin solutions upon standing in light and air is studied and ways of stabilizing such solutions are described. Alcohols are found to be efficient stabilizing agents, apparently due to the formation of hemin-alcohol compounds. The paper describes spectra, titration data and magnetic properties of these hemin compounds. Their possible structures are included in the discussion which also covers data of the preceding publication.

The properties of aqueous solutions of protohemin change quite rapidly on standing. This is apparently due to several processes closely related to each other. Polymerization is one of these processes and has been studied by Zeile, Haurowitz, Gralén, and especially by Shack and Clark¹ in 1947 who also cite earlier literature. The particle size depends on the pH, the kind of buffer and time, and the apparent molecular weight varies between about 3 000 and 100 000¹.

The spectral changes of hemin solutions as a function of time have also been measured by Shack and Clark¹ from 400 $m\mu$ to 700 $m\mu$. They find that, under their experimental conditions, the spectrum of a freshly prepared solution undergoes a change for several days when a new spectrum is formed which then remains stable for more than 60 days. Similar results were obtained more recently by Blyumenfeldt² who studied the spectrum in the Soret region.

At least one method for preventing or slowing down the reactions responsible for the instability of protohemin in water will be described here.

EXPERIMENTAL

The hemins and hemoproteins used in these studies are described in the preceding paper³. The horse myoglobin (HMb) mentioned here was prepared as described by Theorell and Åkeson⁴. The spectra and the pH were measured with the same apparatus mentioned in *Ref.*³. The magnetic measurements were performed by A. Ehrenberg (Nobel Medical Institute, Stockholm) using a highly sensitive apparatus designed by Theorell and Ehrenberg⁵.

* A preliminary report of some of the data presented here was made by one of us (A. C. M.) at the 128th Meeting of the Am. Chem. Soc. in Minneapolis (1955), p. 68 C.

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RESULTS

Spontaneous changes of aqueous hemin solutions

Blyumenfeldt's results with protohemin² were, on the whole, confirmed. It was found that the Soret band changes its appearance and that this change follows the kinetics of a first order reaction which is half complete after one to twenty hours after preparing a fresh solution, the half-time depending on the conditions of the experiment. The initial and final spectra from 230 to 490 $m\mu$, the latter extrapolated to infinite time and from a series of experiments, are plotted in Fig. 1.

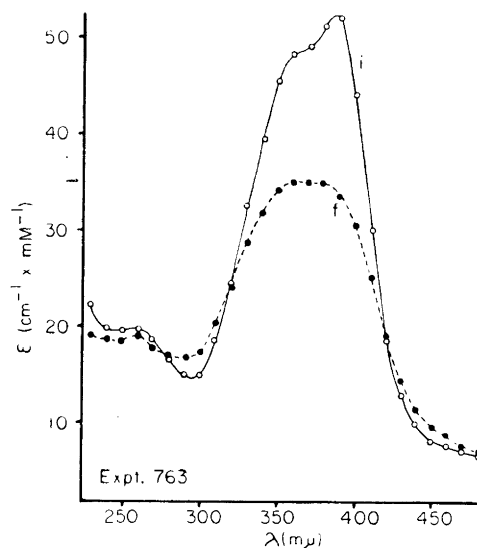


Fig. 1. The spectra of a fresh and an aged solution of protohemin at pH 10.4. Protohemin was dissolved in 0.1 N NaOH and diluted to $1.46 \times 10^{-5} \mu\text{M}$ with 0.01 M phosphate buffer. The resulting pH was 10.4. The spectrum of the solution was measured within a few minutes and the measurements repeated at appropriate intervals for several hours. Extrapolation of the values to infinite time gave the spectrum of the final product. The reaction is of first order, as shown in Fig. 3.

i—○—○— initial spectrum of a fresh solution (2–20 min after dissolving the protohemin).
f—●—●— final spectrum of the aged solution at infinite time.

Shack and Clark¹ had reported that the changes of the visible spectrum were the same, whether the hemin solution was kept under hydrogen or in the air. Joan Keilin⁶, on the other hand, found that hemin solutions in Na_2CO_3 would change several of their properties (formation of a caffeine-CO compound, solubility in ether-acetic acid, crystallizability from acetic acid and NaCl) when standing in air, but not under strictly anaerobic conditions.

We found that the hemin spectrum remained unchanged for more than a week if the solutions were kept and measured in an anaerobic cuvette⁷, which

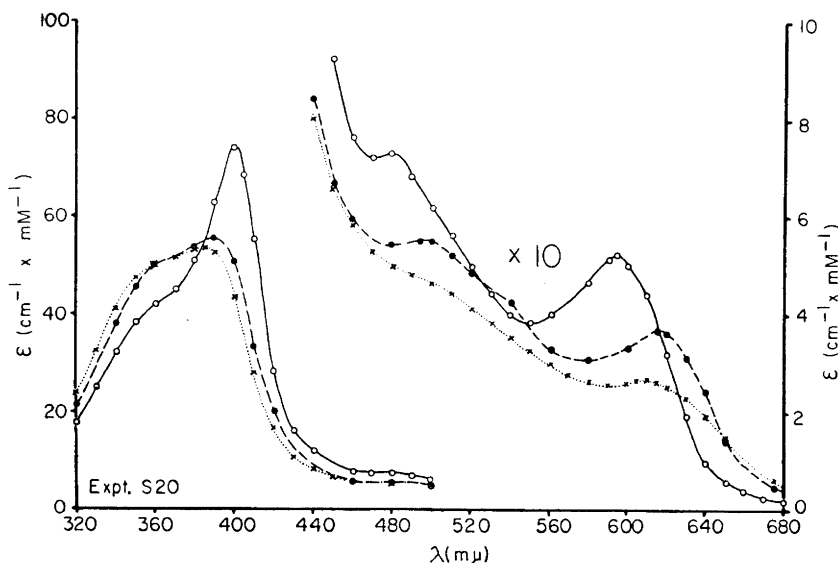


Fig. 2. The spectra of protohemin in water, 50 % ethanol and 30 % sucrose. Phosphate buffer of pH 7.0 was added to give a final concentration of 0.01 M.

- X.....X solvent: water
 O———O solvent: 50 % ethanol
 ●-----● solvent: 30 % sucrose or 30 % glycerol

The final concentration of protohemin was of the order of 10^{-5} M in each case.

was evacuated after some wet $\text{Na}_2\text{S}_2\text{O}_4$ had been placed in one of its sidearms, a procedure recommended by Keilin⁶. The reaction was also slowed down considerably, even in air, if light was excluded. It was further found that the Soret spectrum of aqueous mesohemin and deuterohemin solutions exposed to light and air did not change within three days.

Alcohol has been used by several workers for solubilizing hemin solutions⁸⁻¹⁰. Davies¹¹ suspected the formation of an alcohol compound of hemin from spectroscopic observations. Around 1930, Hamsik¹² crystallized "active hematin" or α -hematin from aqueous acetone or alcohol. He reported spectroscopic differences between hemin dissolved in these solvents and dissolved in water. He found further that the ability of protohemin to couple with free globin from hemoglobin is lost on standing for some time ("nach einiger Zeit") if the hemin is dissolved in aqueous buffer, but is maintained longer if aqueous acetone or alcohol are used as solvents.

Ethanol, glycerol, and sucrose were tried as stabilizing agents in the present studies. Fig. 2 shows the spectra of freshly prepared hemin solutions in phosphate buffer at pH 7 without addition and in the presence of these solvents. It is readily seen that only ethanol has a strong influence on the spectrum, both in the visible and in the Soret regions. The bands at 385, (495) and 610 $m\mu$ of aqueous hemin are sharpened and shifted to 400, 482, and 597 $m\mu$, respectively.

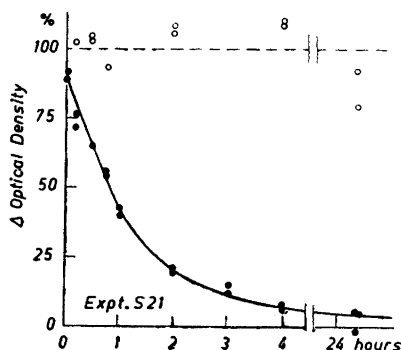


Fig. 3. The change in the Soret spectrum of aqueous and of alcoholic protohemin solutions. The optical density of the Soret band at 385μ of aqueous protohemin (0.01 M phosphate buffer of pH 7.0) was determined as a measure of the spontaneous reaction of protohemin described in the text. The optical density of 50 % alcoholic protohemin (in the presence of the same phosphate buffer as above) at 400μ was also determined. The measurements were repeated as often as practical and the data plotted in percent of the corresponding values at zero time at the ordinate, and time in hours at the abscissa, including one final value after 24 h.

○—○ optical density at 400μ of 50 % alcoholic protohemin
 ○—○ optical density at 385μ of aqueous protohemin. The curve is a theoretical first order reaction curve.

The stability of the hemin solutions was tested in two different ways. One was the change of the Soret spectrum, the other the velocity of the coupling reaction of hemin with apomyoglobin (the protein part of myoglobin after removing the hemin by HCl in acetone) to yield active myoglobin. The completely reversible splitting of myoglobin was first described by Theorell and Åkeson⁴ and some detailed aspects of the coupling reaction will be published later. In Fig. 3, the decrease in the height of the Soret band is plotted *versus* time. Other experiments show that an addition of ethanol preserved these properties of the hemin best whereas glycerol is less satisfactory and sucrose is practically useless for this purpose. The velocity of the coupling reaction with apomyoglobin of aqueous protohemin solutions decreases markedly after some hours whereas 50 % alcoholic solutions of protohemin retain their coupling speed for at least 24 h.

Alcohol compounds of protohemin

The spectral changes of freshly prepared aqueous hemin solutions of pH 7 (0.01 M phosphate buffer) upon the addition of ethanol were measured quantitatively at 400μ and yielded a titration curve with a slope $n = 2$ and $pK = -0.75$ (the concentration of ethanol bringing about one half of the change is 5.6 M). The extinction coefficient of the ethanol compound was computed to be $81 \text{ cm}^{-1} \text{ mM}^{-1}$ (using the fact that 100 % EtOH is 17.2 M) as compared to $\epsilon_{385} = 53 \text{ cm}^{-1} \text{ mM}^{-1}$ for aqueous hemin. The results of such experiments are

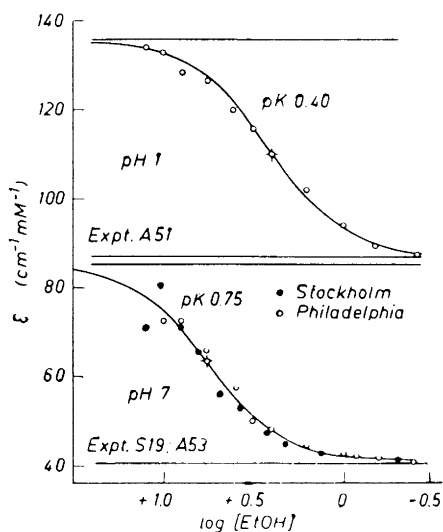


Fig. 4. Spectrophotometric titration of protohemin with ethanol at pH 1 and pH 7. Solutions of 0.1 N H_2SO_4 and 0.02 M phosphate buffer respectively, containing increasing amounts of ethanol were made up. An equal small amount of a concentrated solution of protohemin was added to each sample and the optical density at the maximum of the Soret band ($398 \text{ m}\mu$ at pH 1, and $400 \text{ m}\mu$ at pH 7) was measured. The hemin concentration was 8–11 μM . The millimolar extinction coefficient is plotted versus the logarithm of the ethanol concentration.

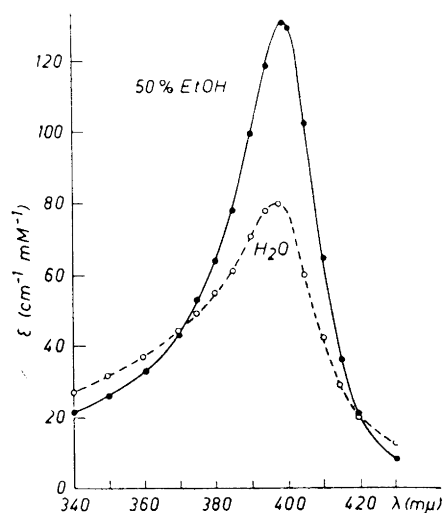


Fig. 5. The Soret spectra of protohemin in water and alcohol at low pH. Protohemin was dissolved in 0.01 N NaOH and added to 0.1 N H_2SO_4 in water and in 50 % ethanol respectively. Final concentration, 5–8 μM hemin.

- aqueous protohemin solution
- alcoholic protohemin solution

presented in Fig. 4. The very high dissociation constants obtained from these plots suggested the possibility that an impurity in the alcohol might be the true reactant. In order to exclude this possibility, three experiments were made. First, especially purified double distilled ethyl alcohol was used. The results obtained with the "ordinary" alcohol were duplicated within the experimental error. Second, Swedish ethanol, and ethanol obtained in Philadelphia gave identical results. Finally, aqueous protohemin solutions were titrated with methanol (absolute, redistilled). This alcohol also forms a hemin compound with a Soret band at $396 \text{ m}\mu$ ($= 81 \text{ cm}^{-1} \text{ mM}^{-1}$ by extrapolation to 100 % or 24.7 M MeOH). The slope of the titration curve was also found to be $n = 2$ (at pH 7, 0.01 M phosphate buffer) and the dissociation constant 10.7 M ($\text{p}K -1.03$), higher than in the case of ethanol.

These results indicate the formation of true alcohol compounds of hemin, with a high tendency to dissociate. Dilution of such alcohol-hemin solutions with aqueous buffer yields the spectrum normally observed with hemin solutions in water, proving the reversibility of the reaction. The readings of optical density at $400 \text{ m}\mu$ in the above mentioned titration experiment with ethanol were repeated after 24 h to test the stability of the alcoholic solutions. At alcohol concentrations above 5 M, no spectral change was noted. Since this corresponds to 23 % (w/v) or 29 % (v/v) ethanol, a 0.01 M phosphate buffer containing 30 % (v/v) ethanol is sufficient to insure the stability of protohemin solutions for at least one day. At this alcohol concentration, each hemin binds on the average, about 1 molecule of ethanol.

At low pH, a different alcohol compound of protohemin is formed. The spectra of protohemin B (*cf.* Ref.³) and of this acid ethanol compound at 50 % or 8.6 M EtOH are represented in Fig. 5. The Soret peak lies in this case at $398 \text{ m}\mu$. Spectrophotometric titration gave the values represented in Fig. 4. The data lie on a theoretical dissociation curve with a $\text{p}K$ of -0.40 , somewhat different from that at pH 7.0. The slope of the curve, here also, is $n = 2$. Extrapolation to 100 % ethanol gives $\epsilon = 134 \text{ cm}^{-1} \text{ mM}^{-1}$ *.

There is an intermediate pH range where the titration values lie on curves with very high n values. At pH 4, n was found to be > 4 . This is the pH region where the alcoholic hemin precipitates even at low concentrations.

Reduction of the alcohol compound at pH 6.8 by sodium dithionite did not change the Soret spectrum but gave a very featureless spectrum in the visible region. The characteristic double bands of hemochromogens were completely absent.

Protohemin in 50 % EtOH showed only the two spectra reproduced above: an acid type (Fig. 5) and an alkaline type (Fig. 2). The pH of conversion lies between 4 and 6 (pH of the buffers before adding the alcohol) but attempts to measure the $\text{p}K$ more accurately failed: spectrophotometric titration gave data which were scattered about a straight line instead of the expected sigmoid curve, and potentiometric titration was made impossible by the low solubility of protohemin between pH 4 and 6 (though the solubility seems to be higher than in the absence of alcohol).

* Sulfuric acid was exclusively used in order to avoid effects of halogen ions which are so dramatically reflected in the Soret spectrum of aqueous acid hemin solutions (*cf.* Ref. ³).

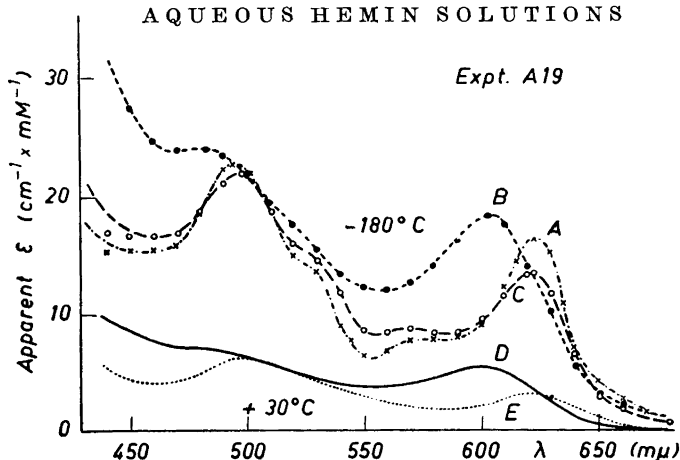


Fig. 6. The effect of temperature upon the absorption spectrum of protohemin. Three solutions were prepared, each containing 50 % ethanol. Solution A had 50 % 0.1 N H_2SO_4 , solution B 0.1 N NaOH, and solution C 0.02 M phosphate buffer of pH 6.8. Small identical amounts of protohemin in 50 % alcoholic 0.05 N NaOH were added and the spectrum was measured using lucite cuvettes of 1 mm lightpath. D is the spectrum of alkaline protohemin-ethanol, and E that of acid protohemin-alcohol at room temperature. The "neutral" compound (in phosphate buffer) also showed spectrum D. The cuvettes were now chilled in liquid air, warmed up to about -55° and chilled again, as described by Estabrook and Mackler¹³. The resulting hemin spectra in solutions A, B, and C are plotted and marked in this order.

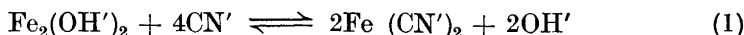
The influence of low temperatures on the spectra of protohemin-alcohol compounds was studied in co-operation with Dr. R. W. Estabrook of the Johnson Foundation*. Lucite cuvettes were filled with the hemin-ethanol solutions (50 % EtOH) and the spectrum recorded on the Yang and Legallais apparatus³. The cuvettes were then cooled in liquid air and the spectra recorded again, at about -180° . The details of this procedure are described elsewhere¹³. Low temperature treatment had two separate effects. One was a 3-fold intensification of the visible bands, without any significant shifts of the peaks as shown in Fig. 6. The other effect was a conversion of the alkaline type of spectrum to the acid type within a certain pH range. At pH 6.8 (0.01 M phosphate) and room temperature, the spectrum of the alkaline protohemin-EtOH compound was found. At the temperature of liquid air, the same solution showed an almost complete spectral conversion into the acid form (curve C in Fig. 6). An investigation of these phenomena (changes of K_w , hydration etc. with temperature) lies outside the scope of these experiments.

Interaction of ethanol, hydroxyl ions, and cyanide ions with protohemin

In alkaline aqueous solutions, one hydroxyl group per iron atom is bound to protohemin, and the resulting compound is known as hematin. Hogness

* Measurements of spectra of biological material at low temperatures have been undertaken by Sinsheimer, Scott, and Loofbourow¹⁴ and, for hemin compounds, by Keilin and Hartree¹⁵ who used visual spectroscopy.

and coworkers¹⁶ showed in 1937 that hematin in aqueous solutions forms dimers * and that cyanide ions are able to dissociate them according to eqn. (1):



If ethanol is bound to the central iron atom, the former would be expected to interact with both CN' and OH' ions. Experiments were designed to study such interactions.

The spectrum of an aqueous solution of hemindicyanide in 25 mM borate buffer (pH 9.2) containing 2.5 μM of protohemin and 20 mM of KCN was measured in the presence and in the absence of ethanol. The effect of 50 % ethanol on the spectrum was very small, resulting in a shift of the Soret band from 425 to 427 $m\mu$, whereas the position of the bands at 355 and 542 $m\mu$ ** remained unchanged. The bands increased in intensity, but this increase was at the most 13 %.

A protohemin solution in 50 % ethanol and 25 mM borate (pH 9.2) was titrated with KCN and the values obtained were found to lie on a theoretical dissociation curve with a dissociation constant of 1.6×10^{-3} and $n = 2$, when calculated for the reacting species, namely CN'. When the dissociation constant for hemin cyanide in aqueous solution is computed from the data of Hogness *et al.*¹⁶ for this particular pH, a value of $K = 0.21 \times 10^{-3}$ is found. The dissociation constant is therefore about 8 times greater for alcoholic hemin cyanide than for hemin cyanide in water.

The extinction coefficient of alcoholic hemin solutions (50 % EtOH) was found to be independent of the protohemin concentration from 0.5 to 5.0 μM hemin at pH 1.0, and from 0.32 to 6.40 μM hemin at pH 7.0: Beer's law is valid within these limits. At pH 13 (0.1 M NaOH) it was found, on the other hand, that the extinction coefficient decreases with the concentration of protohemin, all other experimental conditions being held constant. The possibility was therefore considered that ethanol may depolymerize the hemin dimer molecules in a similar manner as cyanide does. The equilibrium of this reaction is then (*cf.* eqn. (1) and Ref.¹⁶):

$$K = \frac{[\text{Fe}_2(\text{OH})_2][\text{EtOH}]^4}{[\text{Fe}(\text{EtOH})_2]^2[\text{OH}]^2} \quad (2)$$

If the pH and the ethanol concentration are kept constant, we obtain:

$$K^* = \frac{[\text{Fe}_2(\text{OH})_2]}{[\text{Fe}(\text{EtOH})_2]^2}; \text{ equivalent to}$$

$$\log [\text{Fe}(\text{EtOH})_2] = \log [\text{Fe}_2(\text{OH})_2]^{\frac{1}{2}} - \frac{1}{2} \log K^* \quad (3)$$

A plot of $\log [\text{Fe}(\text{EtOH})_2]$ versus $\log [\text{Fe}_2(\text{OH})_2]^{\frac{1}{2}}$ should thus give a straight line with a slope of 1 and an intercept of $-\frac{1}{2} \log K^*$. Fig. 7 shows that the experimental data do lie on such a line.

* The assumption of dimeric hemin molecules is, however, not generally accepted, as will be discussed below.

** Hogness *et al.*¹⁶ report the bands of aqueous hemin cyanide to lie at 365, 422.5, and 545 $m\mu$, respectively.

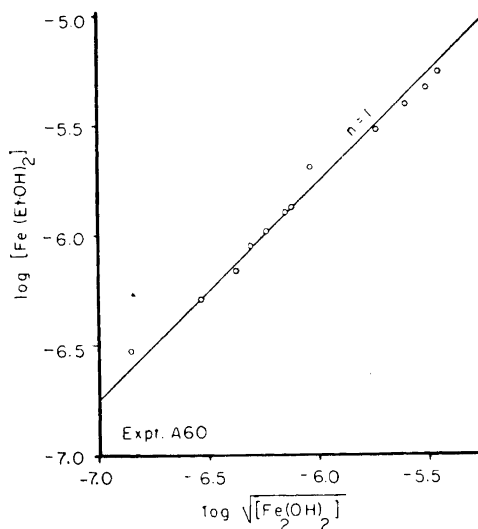


Fig. 7. A plot of the relationship of eqn. (3). While keeping the concentrations of ethanol and hydroxyl ions constant at 5.0 M EtOH and pH 13, the total hemin concentration was varied from 0.338 μM up to 2.35 μM and the resulting optical density measured at 402 $m\mu$ using cuvettes of 5.0 cm optical pathlength. The hemin concentration was further increased from 2.35 μM up to 17.25 μM and D_{402} measured in 1.0 cm cuvettes. From these data, a plot according to eqn. (3) was made. A straight line with the slope of 1.0 was located to give the best fit for the experimental data. The meaning of the formulas is explained in the text.

Magnetic susceptibility

The magnetic susceptibility of free hemins and hematins has been measured by several groups of workers. Hartree¹⁷ has written an excellent review on the subject in 1946 and included magnetic data on hemoproteins. The magnetic measurements reported here were made in cooperation with A. Ehrenberg of the Nobel Medical Institute, using the highly sensitive Gouy balance of Theorell and Ehrenberg⁵. A stock solution of protohemin was prepared by dissolving 6.49 mg of the hemin in 0.60 ml of 0.03 N NaOH and adding 1.40 ml of ethanol to give 1.96 ml total solution of 5.08 mM protohemin. Of this stock solution 0.35 ml was added to 0.80 ml of 0.13 N H_2SO_4 in 67 % EtOH (v/v), and 0.35 ml to 0.80 ml of aqueous 67 % EtOH (v/v). The final concentration of hemin was thus 1.55 mM in each case. The molar magnetic susceptibility in the acid solution was found to be $12\,400 (\pm 250) \times 10^{-6}$ c.g.s. e.m.u. at 20.5°. The alkaline solution gave $13\,450 (\pm 300) \times 10^{-6}$ c.g.s. e.m.u. at 20.5°. These values correspond to 5.41 and 5.64 Bohr magnetons respectively and indicate an essentially ionic bond type with 5 unpaired electrons. The theoretical value for 5 unpaired electrons is 5.92 Bohr magnetons.

Alcohol compounds of deuterohemin

Deuterohemin IX, which differs from protohemin IX by the absence of the two vinyl groups in positions 2 and 4 of the porphyrin ring, gives compounds with ethanol very similar to those of protohemin. Spectrophotometric studies on deuterohemin are facilitated by the stability of its alkaline solutions. The study of alcohol binding was performed at two selected pH regions, pH 1 (0.1 N H_2SO_4) and pH 12 (0.01 N NaOH). The acid and alkaline alcohol compounds formed show distinct spectra, reproduced in Fig. 8. The alcohol compounds at intermediate pH values show either one or the other of these spectra as is the case with protohemin. The pH region at which the acid ethanol compound of deuterohemin changes into the alkaline one has not yet been ascertained because of complications brought about by the pronounced changes of the spectrum of deuterohemin in aqueous solution in the neutral pH region.

The titration data of deuterohemin at pH 1 and pH 12 show that in both cases 2 moles of EtOH are entering the hemin molecule, and that the dissociation constant is nearly the same, namely 0.46 M at pH 12 and 0.57 M at pH 1.

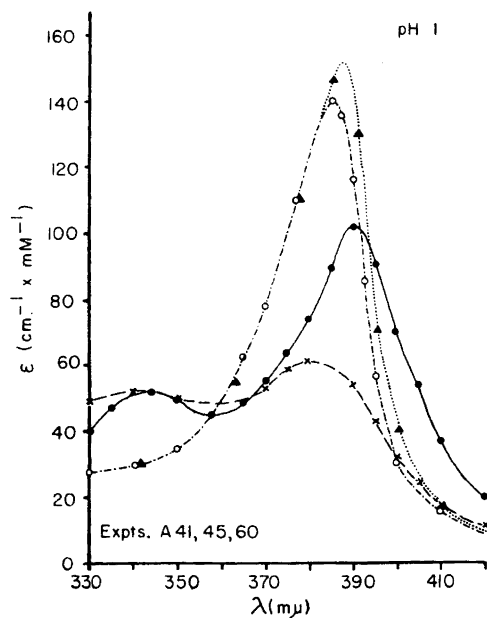


Fig. 8. The Soret spectra of deuterohemin and its alcohol compounds at pH 1 and pH 12. Small amounts of deuterohemin dissolved in 0.01 N NaOH and added to the solutions listed below gave the following spectra plotted in the figure:

- in 0.1 N H_2SO_4 of pH 1
- ▲...▲...▲ in 0.1 N H_2SO_4 containing 50 % EtOH
- X---X---X in 0.01 N NaOH of pH 12
- in 0.01 N NaOH containing 50 % EtOH

The deuterohemin concentrations used were of the order of 10^{-5} M.

Table 1. Spectra and equilibrium constants of hemins in alcohol.

Hemina)	Alcohol ^{b)}	pH ^{c)}	$\lambda(m\mu)$	$\epsilon^d)$	$\lambda(m\mu)$	$\epsilon^d)$	$\lambda(m\mu)$	$\epsilon^d)$	pK ^{e)}
Proto-	EtOH	1.0	398	131	504	6.0	627	2.5	-0.40
Proto-	EtOH	7.0	400	74	482	7.4	597	5.4	-0.75
Deutero-	EtOH	1.0	386	171	—	—	—	—	-0.59
Deutero-	EtOH	12.0	390	96	478	11.7	582	7.8	-0.46
Proto-	MeOH	7.0	396	73	—	—	—	—	-1.03

a) All hemins were of type 9.

b) EtOH stands for ethanol; MeOH for methanol.

c) The pH of the aqueous solution before adding alcohol.

d) All extinction coefficients were calculated from 50 % alcoholic solutions and have the dimension $\text{cm}^{-1}\text{mM}^{-1}$

e) The negative logarithm of the alcohol concentration (in Moles \times liter⁻¹) at which half of the spectral change was observed.

Since the reproducibility of these constants was not better than 0.05 M, this difference can not be regarded as significant.

Table 1 gives a summary of the data on the alcohol compounds of protohemin and deuterohemin presented in this paper.

DISCUSSION *

Evidence for hemin alcohol compounds

The evidence for the formation of true alcohol compounds of hemin can be analyzed as follows: (i) The absorption spectrum of protohemin undergoes a pronounced change on the addition of 50 % ethanol. This suggests the formation of alcohol compounds of the hemin, though a change in the state of aggregation due to the change in solvent can not be excluded. (ii) At all pH values except in a critical neutral range, 2 moles of alcohol are needed to bring about the spectral change. Within the neutral pH range more than 2 alcohol molecules are needed per mole of hemin to convert them into alcohol compounds. Apparently the alcohol compounds can be formed only after some large aggregates of hemin molecules have been dissociated by the alcohol. (iii) The strict adherence to the deviations of Beer's law to a proposed mechanism of dimer splitting suggests that a stoichiometric reaction is involved. (iv) The possibility that an impurity in the alcohol might be the reacting species is excluded by the observation that methanol gives similar, but not identical compounds as ethanol, and that the use of ethanol from different sources gives identical results. (v) The fact that ethanol does not change the spectrum of aqueous hemin-dicyanide appreciably seems to indicate that the CN ions compete for the binding sites of the ethanol molecules. The magnetic data show clearly that the bond type of hematin in ethanol (5.64 B.M.) is different from that measured by Rawlinson¹⁸ (3.62 B.M.) and by Hartree¹⁷ (3.23 B.M.)

* The results of the preceding paper⁸ are included in the discussion.

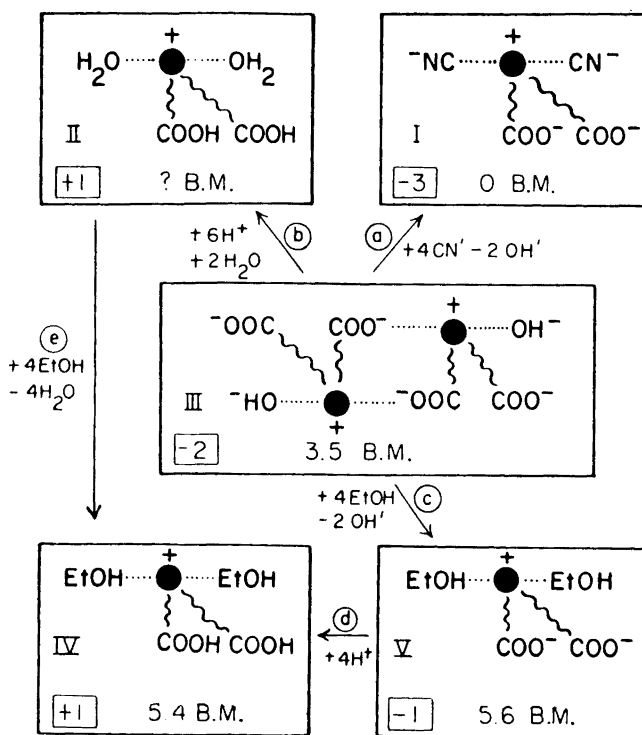


Fig. 9. The proposed reaction mechanism of protohemin with ethanol, cyanide and hydroxyl ions. The compounds are labeled thus (EtOH stands for ethanol):

- I protohemin-dicyanide *
- II protohemin compound B (acid hemin, *cf.* (3)) *
- III protohematin (dimer)
- IV acid protohemin-ethanol *
- V alkaline protohemin-ethanol *

The hemin molecule is not shown as such. Only the central iron atom, marked by a solid circle, 2 of the total of 6 co-ordination places (4 are taken up by the 4 pyrrol nitrogens), and the 2 carboxyl groups of the propionyl sidechains appear in the formulae. The postulated overall charge is shown inside a small box. The paramagnetic susceptibility where known, is given in Bohr Magnetons (B.M.). The individual reactions are marked with small letters inside a circle. The iron remains in the trivalent state throughout.

of protohemin in aqueous NaOH. (vii) The fact that glycerol or sucrose do not change the spectrum of aqueous hemin solutions shows that the alcohol is not merely influencing the hydration of the hemin molecule.

The dimer theory

The alcohol binding data lend further support to the theory that hematin forms a dimer in aqueous solution, since the deviation from Beer's law can be

* Two moles (to fit the stoichiometric relationships).

quantitatively explained on the basis of this assumption. The splitting of the dimer by alcohol is quite akin to the action of cyanide observed by Hogness *et al.*¹⁶ The spectrophotometric observations of Davies¹¹ also fit the dimer theory.

The structure of the dimer as drawn in Fig. 9 (Compound III) was proposed by Lemberg and Legge¹⁹. It is assumed that the iron atom of each hemin molecule interacts with one of the propionyl groups of the other hemin molecule, thus leaving one of the six coordination places of each iron free for the binding of a hydroxyl ion. We constructed models of two hemin molecules, using the correct bond angles and degrees of freedom of the $-\text{CH}_2-\text{CH}_2-\text{CO}-\text{O}$ -chain and found that a strainfree structure linking two molecules in the fashion discussed above could be obtained, with the porphyrin "disks" oriented in parallel planes. An additional bond between the iron atom of one hemin and the second propionyl group of the other hemin is sterically impossible.

The scheme outlined in Fig. 9 is suggested for the reactions of ferric protoporphyrin with ethanol, cyanide ion, and hydroxyl ion, although one must keep in mind that absolute proof for dimerization is still lacking.

The structure of hemin alcohol compounds

The suggested structure of alkaline hemin-ethanol (V of Fig. 9) is subject to some doubt due to the electrophoretic experiments of Haurowitz⁹ which suggest a negative overall charge for the unesterified form of V. In order to account for it one can consider heptacoordination as suggested by Coryell and Stitt²⁰.

The transition from the alkaline to the acid form of hemin-ethanol seems to take place at around pH 5, though accurate measurements could so far not be achieved. The carboxyl ions of the propionyl side chains are the only obvious sites for the reception of hydrogen ions, unless heptacoordination is adhered to. This would also explain why only one spectral change could be observed within the pH range from 1 to 13.

Acid hemin compounds

Fig. 9 shows the formation of an acid hemin compound in aqueous solution (reaction b) where 3 protons and 1 H_2O are taken up by the hemin molecule. This overall reaction is composed of at least 2, but probably 3 or 4, steps. Starting from the alkaline range, the first change of spectrum and redox potential occurs between pH 7 and 8 (Mori²¹ and Shack and Clark¹).

The large spectral change taking place at around pH 1.5 has been described in the preceding paper³, where it was also shown that this reaction does not involve the vinyl groups and that no removal of iron takes place. The compound B formed at very low pH by sulfuric, perchloric, or nitric acid reacts with halogen ions³. It incorporates one Cl' , the equilibrium being independent of the H^+ concentration, at least between pH 1–3³. This reaction differs from that of acid cytochrome c, where Boeri *et al.*²² had found that 2 Cl' are taken up simultaneously and that the dissociation constant increases with

decreasing pH. The authors postulated that the Cl' ions form a "bridge" between the positively charged Fe atom of the hemin and the —NH-groups of the imidazole rings that are believed to participate in the binding of the hemin to the apoprotein of cytochrome c. A similar effect in free hemin would only be possible if polymerization of the hemin molecules with the help of Cl' bridges is assumed. The observed collapse of the high Soret band of acid hemins in the presence of increasing Cl' concentrations makes this assumption not improbable.

The data so far obtained strengthen the hypothesis of Erdman and Corwin²³ that compound B represents the free ionized form of acid hemin, in contrast to "neutral" hemin, where negative ions of small radius (especially F', Cl', Br', I') are thought to move into one of the co-ordination places of the iron and are not dissociated appreciably in aqueous solutions. Erdman and Corwin could show that the spectra of hemin-chloride, -bromide, and -iodide in dioxane are not identical and that these compounds are essentially undissociated*.

There remains one phenomenon that is unexplained. It is not readily evident how the complexing of protohemin with ethanol is able to prevent the spontaneous changes that have been shown to occur in aqueous solutions. The experimental data show that the vinyl groups must be involved in the reaction, since the spectrum of deuterohemin in aqueous solution remains unaltered. It was also demonstrated that the process requires oxygen. It can be postulated that hemin catalyses its own oxydation and that it is the solvent action of alcohol that prevents this process.

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* More recently, U. J. Lewis (*J. Biol. Chem.* **206** (1954) 109) has shown that the spectra of protohemin 9-chloride and -sulfate differ appreciably in acetone.

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