

Acid Compounds of Hemins and Hemoproteins (B-Compounds) *

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Acid compounds of several hemoproteins and free hemins are studied and described, notably in respect to their spectra, equilibrium constants and their reaction with halogen ions. Fluorescent measurements are used to study the possible dissociation of the central iron atom from the porphyrin molecule in acid solutions.

The possible reactions involved are briefly discussed. A detailed discussion will be found in the second communication of these series.

The properties of hemins and hemoproteins in strongly acid aqueous solutions have been relatively little studied. Many hemoproteins, like hemoglobin, myoglobin, and to some extent catalase, are denatured below pH 4. So far only horseradish peroxidase ^{1,2} and cytochrome c ³⁻⁵ have been investigated in strongly acid solutions which did not denature the protein irreversibly.

Iron-porphyrins in their free state have very low solubilities in aqueous systems in the acid range, and it is therefore difficult to study them. In 1929, Hicks and Holden ⁶ measured the absorption of hemoglobin and of protohemin in the Soret region (*ca.* 350—450 m μ) at low pH, and in 1947 Erdman and Corwin ⁷ published a very interesting paper on the acid spectra of mesohemindimethylester in pyridine and dioxane.

The present investigation was begun in order to study the nature of compounds observed by Theorell and Paul ⁸, by B. Chance ⁹ and by this author ¹ on acidifying solutions of horseradish peroxidase (HRP) with acid. At least two such compounds were identified and this author proposed to call them compounds A and B, respectively. Acidification of HRP by H₂SO₄, HNO₃, or HClO₄ yields the acid compound B, where the protein moiety is denatured. With HCl, a hydrochloride, HRP-HCl or compound A is formed, which on further acidification reversibly splits into hemin and undenatured protein.

* Part of the results published here have been reported at a meeting of the Swedish Biochemical Society in Uppsala in December 1953.

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Compound B is only present in small concentrations. HBr, HF, and HCN have effects similar to HCl. B-Compounds can be defined as compounds formed from hemin-containing substances at low pH in the absence of halogen or cyanide ions and are characterized by a high sharp absorption band, somewhere between 380 and 398 $m\mu$ ¹.

METHODS AND MATERIALS

The hemins were prepared by known methods as described in an earlier paper¹⁰ and were crystallized once from acetic acid saturated with sodium chloride. A sample of crystalline horseradish peroxidase (HRP) mentioned in previous work² was used. Its ratio of optical densities at 403 $m\mu$ and 275 $m\mu$ (RZ,*) was 2.68. Hemoglobin (Hb) was prepared from washed human erythrocytes and the dialyzed solution was used without further treatment. Sheep myoglobin (ShMb) was kindly donated by Dr. M. Beznák and had the properties described in her paper¹¹. A sample of human serum albumin was kindly given us by Dr. M. Surgenor (Boston) in the form of fraction V.

Two types of recording spectrophotometers were used in addition to a standard Beckman DU instrument: an apparatus designed by B. Chance¹² recording changes of transmission with respect to time, and a spectrophotometer built by Yang and Legallais¹³ recording absorption spectra (optical density *versus* wavelength). Cuvettes with 1 cm lightpath were used and the temperature was adjusted by circulating thermostated water through »thermospacers». As a rule, measurements of pH were made inside the optical cuvettes using micro-electrodes (Beckman »baby stomach» electrodes) and pH meters of the same firm. The reproducibility of this setup was better than ± 0.1 pH units, which was sufficient for this type of work. The accuracy was considerably higher for the duration of a given experiment.

RESULTS

Spectral data on the acid compounds of HRP. Virtually complete transformation of HRP into HRP-B was accomplished by the addition of sulfuric acid to an aqueous solution of the peroxidase to give a pH of 0.6. The spectrum of the relatively unstable compound B was measured at $+5^\circ$ with the recorder of Yang and Legallais and checked at critical wavelengths with Chance's double-beam spectrophotometer. The formation of B from HRP is most easily observed in the Soret region¹. In 1944, Theorell and Paul⁸ found that the addition of HCl to HRP leads to spectral changes, notably a pronounced increase in optical density at 615 $m\mu$ and a smaller decrease at 655 $m\mu$. They attributed these to pH changes and found, by titrating HRP with HCl, two pK values: 4.0 (measured at 655 $m\mu$) and 5.0 (measured at 615 $m\mu$).

Chance⁹ showed that the negative logarithm of the dissociation constant of the HCl compound of HRP is $pK_{FeH+Cl^-} = 5.5$, but in terms of H^+ ion concentration the pK appears⁹ to lie at 3.4. Sulfuric acid, in contrast to hydrochloric acid, does not influence the spectrum of HRP above pH 3.5 and the pK of compound B by spectrophotometric titration with H_2SO_4 at 397 $m\mu$ was found¹ to be 2.30. Chance and Maehly working at 615 $m\mu$ (*cf.* Ref.⁹) confirmed this value.

* For a definition of this unit see Maehly, A. C. in *Methods in Enzymology*, Vol. II, S. P. Colowick and N. O. Kaplan, eds., Academic Press, New York 1955, p. 801.

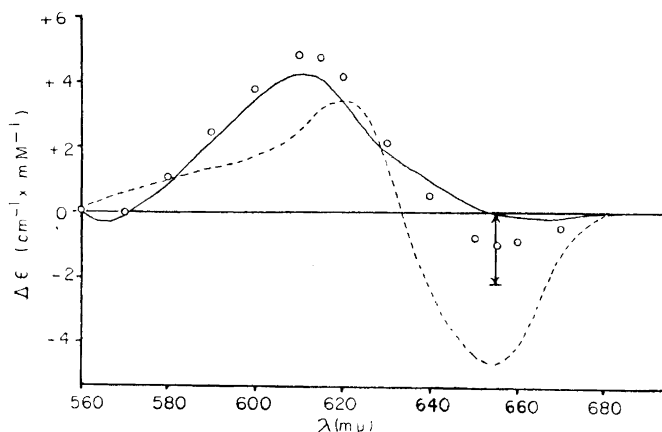


Fig. 1. The spectra of the difference "HRP-B minus HRP" as well as "Theorell and Paul's data"⁸ and "compound A minus HRP".

- HRP-B at pH 0.6 minus neutral HRP (concentration 7.5×10^{-6} M).
- ○ ○ ○ Theorell and Paul's measurements of acidified HRP (extrapolated to pH 2.7) minus neutral HRP (concentration 4.7×10^{-4} M).
- Theorell and Paul's data, corrected for the calculated contribution of HRP-B at pH 2.7. A continuous curve is drawn to fit the calculated points and the resulting spectrum is assumed to represent the difference spectrum of HRP compound A (cf (1)) minus neutral HRP.

The arrow indicates the maximal decrease of the extinction coefficient obtained by Theorell and Paul by addition of HCl to HRP in a separate experiment (pH 5.4).

Addition of HCl to HRP has two separate effects, namely (i) the formation of compound A (HRP · HCl) with absorption bands at 407 and 615 $m\mu$, and (ii) the partial formation of compound B (acid compound) with bands at 397, 500, and 635 $m\mu$. Fig. 1 shows the difference spectrum obtained by subtracting the spectrum of neutral HRP from that of compound B (obtained by addition of H_2SO_4). It also shows some data of Theorell and Paul⁸. These values were arrived at by subtracting the extinction values of neutral HRP from those measured after the addition of HCl. If the "dip" of these data at 655 $m\mu$ is attributed to partial B formation, then the values can be corrected for the contribution of compound B and the difference spectrum of compound A minus neutral HRP obtained (solid line). It shows a peak at 611 $m\mu$. Some implications of these findings have been discussed by Chance⁹.

Acid compounds of other hemoproteins. The formation of acid compounds (B compounds) from HRP and from horseblood catalase (HBC) by sulfuric, perchloric, or nitric acids have been described previously¹. It has since been found that Hb and ShMb also give B compounds under these conditions. Fig. 2 shows the original recordings of their spectra obtained with the recorder of

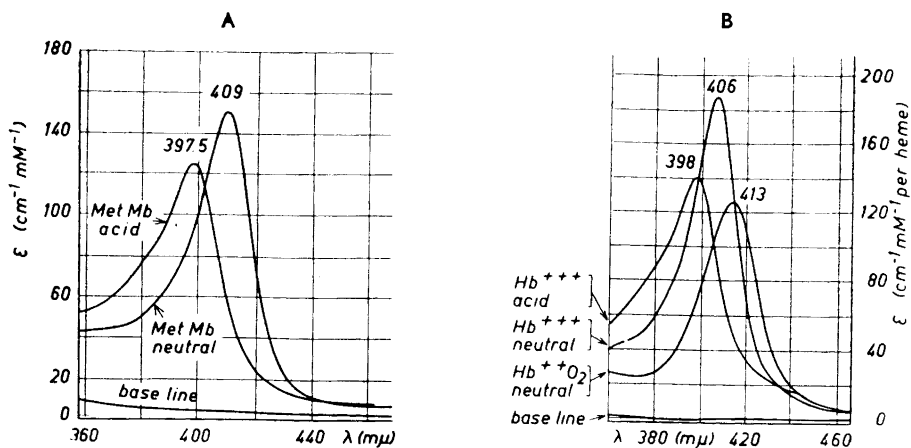


Fig. 2. Original recordings on the apparatus of Yang and Legallais¹³. A: The spectra of sheep metmyoglobin at pH 7.0 (phosphate buffer) and of ShMb-B (H_2SO_4 , pH \sim 1). The concentration of ShMb was $3.8 \mu\text{M}$. B: The spectra of HbO_2 , MetHb (both at pH 7, 0.01 M phosphate buffer) and of Hb-B, formed by the addition of H_2SO_4 to give pH 1. The concentration of hemoglobin was $3.8 \mu\text{M}$.

Yang and Legallais. The peak of the Soret band of B lies at 397 m μ . A typical titration curve for Hb-B is presented in Fig. 3.

Human serum albumin is known to bind hemins, but the resulting "hemalbumins" have not been shown to exhibit any biological activity. Protohemalbumin, studied in some detail by Rosenfeld and Surgenor¹⁴ and others, shows a spectrum that is very similar to that of HRP. When H_2SO_4

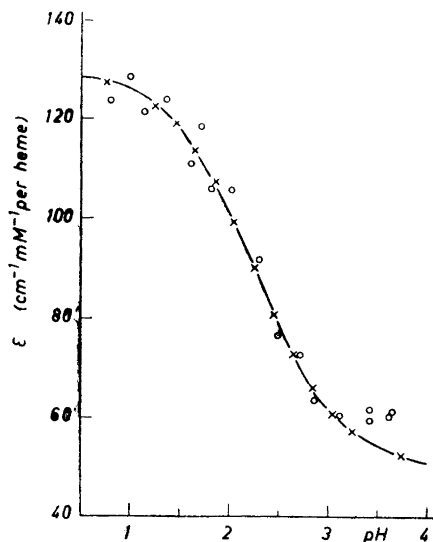


Fig. 3. The titration of hemoglobin with H_2SO_4 . — Each point represents the results of a separate experiment: addition of H_2SO_4 to neutral $2.4 \mu\text{M}$ HbO_2 solution and determination of the maximal optical density at 397 m μ reached during the reaction, as well as of the pH.

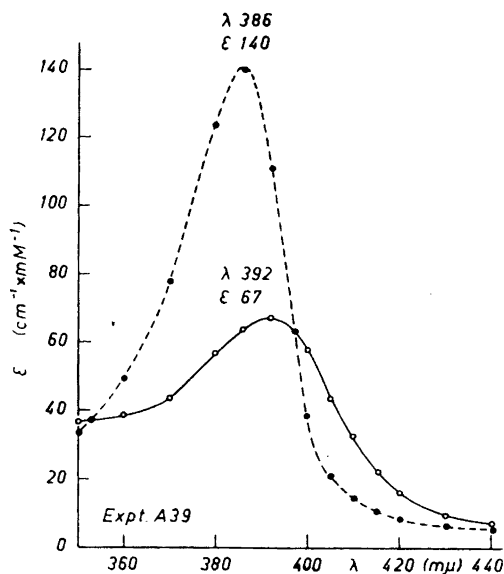


Fig. 4. The Soret spectra of deuterohemalbumin and its B compound.

- deuterohemalbumin, obtained by adding 2.9 μM deuterohemin to 5.8 μM human serum albumin in 0.02 M phosphate buffer, pH 6.8.
- deuterohemalbumin-B, formed by the addition of 0.3 N. H_2SO_4 to the solution described above.

is added, the B compound is formed with a peak at 397 $m\mu$ and $\epsilon = 130 \text{ cm}^{-1}\text{mM}^{-1}$, quite comparable to the compounds of HRP, HBC, ShMb, and Hb. If deuterohemin is added to albumin solutions at pH 6.8 (0.02 M phosphate), deuterohemalbumin is formed. This compound has not been previously reported and its Soret spectrum is therefore rendered in Fig. 4*. When the pH is lowered to about 1 by adding H_2SO_4 , the corresponding B compound is readily formed with a peak at 387 $m\mu$ and $\epsilon = 140 \text{ cm}^{-1}\text{mM}^{-1}$.

Acid compounds of some free hemins. Protohemin is soluble in water only at alkaline pH. Sodium carbonate, or better 0.1 N NaOH, is required to dissolve the compound completely. However, considerable polymerization takes place. This polymerization increases when the pH is lowered, as Shack and Clark¹⁵ have found by experiments involving diffusion, dialysis, and ultracentrifugation. Further acidification leads to a change in spectrum, and the addition of more acid precipitates the hemin from all but the most dilute solutions. At very low pH a further change takes place: compound B is formed, as indicated by the spectrum in the Soret region (Fig. 5). This acid compound

* Compounds formed by a combination of mesohemin with human serum albumin and other proteins have been described by Rosenberg, L. L. and Clark, W. M. *J. Biol. Chem.* 205 (1953) 617.

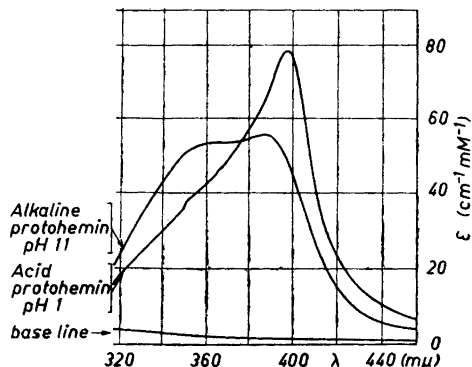


Fig. 5. The Soret spectra of protohemin and protohemin-B. This original recording shows the spectra of protohemin in dilute NaOH (pH 11) and of the B compound obtained from it by adding H_2SO_4 to a final pH of 1.

is also only slightly water-soluble and undergoes further, irreversible changes on standing.

Deutero- and mesohemin lack the two vinyl groups of protohemin and are therefore chemically simpler model compounds. Mesohemin gives a B compound with a Soret band at $380\text{ m}\mu$, deuterohemin forms a similar compound with a peak at $388\text{ m}\mu$. Titration data show that deuterohemin (having no sidechains in 2- and 4-positions) is most readily transformed into its B-form (pK 3.0–3.1). Mesohemin (2 ethyl groups as sidechains) requires more acid

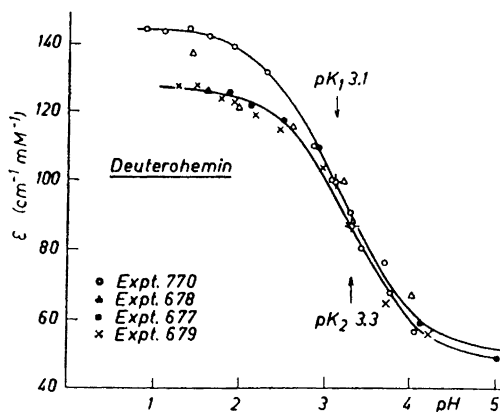


Fig. 6. The reversibility of the formation of the B compound obtained from deuterohemin. A solution of deuterohemin was acidified with H_2SO_4 , acid potassium tartrate was added, and the pH as well as the optical density at $388\text{ m}\mu$ were measured after addition of increasing amounts of NaOH to the sample (Expts. 677–679). The "normal" titration curve from Fig. 8A is represented for comparison (Exp. 779). The concentration of deuterohemin was $5.8\text{ }\mu\text{M}$ when going from high to low pH, and $2.1\text{--}2.4\text{ }\mu\text{M}$ when the pH was raised.

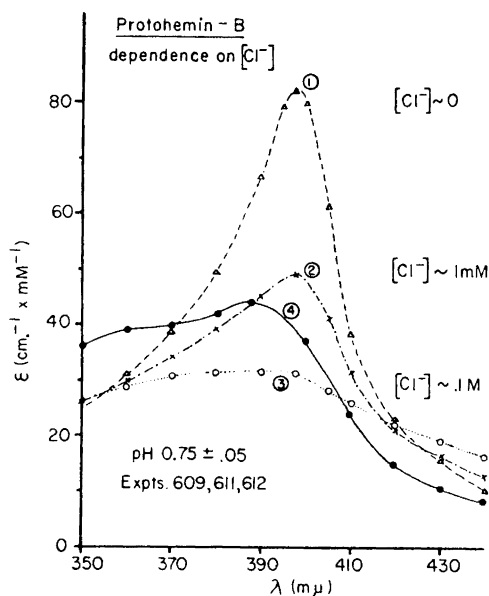


Fig. 7. The influence of the Cl^- ion concentration on the Soret band of protohem-B (PHB). Protohem-B was acidified with H_2SO_4 (pH .75) and its spectrum recorded. The recording was repeated with identical solutions, except for the presence of increasing concentrations of KCl.

- (1) Δ -..... Δ PHB, no KCl
- (2) \times -..... \times PHB, ~ 1 mM KCl
- (3) \circ -..... \circ PHB, $\sim .1$ M KCl
- (4) \bullet -..... \bullet protohem at pH 7 (0.01 M phosphate buffer) shown for comparison.

The protohem concentration varied between 7 and 11 μM .

(pK 2.25), and protohem (2 vinyl groups) still more (pK 1.5). Deuterohem-B appears to be best suited for further studies on the nature of B compounds since it can be obtained under relatively mild conditions. The instability of protohem in aqueous solutions and its tendency to polymerize make it the least suitable of these hemins for such studies.

The relatively high stability of deuterohem-B allowed a measurement of the reversibility of B formation. The technique for obtaining titration data for hemoproteins as well as hemins consisted of adding a definite and increasing amount of acid to each of as many aliquots of the solution of a neutral sample of the compound as there were values needed (*cf.* Ref.¹). The instability of B made a simple reversal of this technique impractical. Some tartrate and increasing amounts of NaOH were added to only one sample of acidified deuterohem (compound B), and the pH as well as the optical density were rapidly

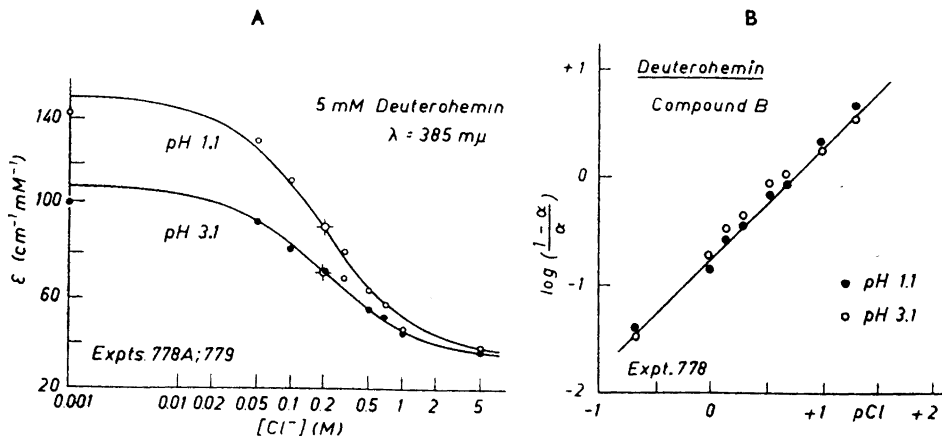


Fig. 8A. The "titration" of deuterohemin-B (DHB) with chloride. Aliquots of a neutral deuterohemin solution were added to solutions of increasing concentrations of KCl, all of them acidified with H_2SO_4 to give either pH 1.1 or 3.1. The optical density of each sample was measured near the peak of the Soret band ($385 m\mu$). The final DHB concentration was $4.4-5.2 \mu M$.

Fig. 8B. A straight line plot of the data represented in Fig. 8A. A logarithmic expression of the law of mass action is used:

$$K = \frac{[DHB] [Cl']^n}{[DHB (Cl')_n]} \quad (1); \quad \alpha = \frac{[DHB]}{[\text{Total deuterohemin}]} \quad (2)$$

$$\frac{1}{K} = \left(\frac{1-\alpha}{\alpha}\right) \frac{1}{[Cl']^n} \quad (3) \quad \log \left(\frac{1-\alpha}{\alpha}\right) = n \log [Cl'] - \log K \quad (4)$$

Abscissa: the negative logarithm of the Cl' ion concentration

Ordinate: $\log \left(\frac{1-\alpha}{\alpha}\right)$

The data lie on a line with the slope $n = 1$ and a $pK_{Cl} = 0.7$.

measured after each addition. The data are plotted in Fig. 6 and show that the titration is essentially reversible. The diminished extinction values are believed to be due to the slow decomposition of B, the shift of the pK from 3.1 to 3.3 may be caused by the presence of high concentrations of tartrate.

The reaction of B compounds with chloride ions was investigated next. In the case of HRP, the addition of chloride at neutral pH has no observable influence. When the solution is acidified, the HCl compound of HRP is formed. If the pH is lowered even more, splitting of the enzyme is brought about and the rate of splitting is accelerated by the presence of chloride¹. In the case of free hemins, increasing concentrations of Cl^- depress the height of the Soret band at $397 m\mu$ upon acidification of the solutions, indicating the formation of a compound of hemin with either Cl^- or with both Cl^- and H^+ . Fig. 7 shows the Soret spectra of protohemin B at concentrations of $<10^{-5}$, 10^{-3} , and 10^{-1} M Cl^- . A complete "titration" of deuterohemin-B with chloride at two different pH values is shown in Fig. 8. At pH 3.1 only half of the

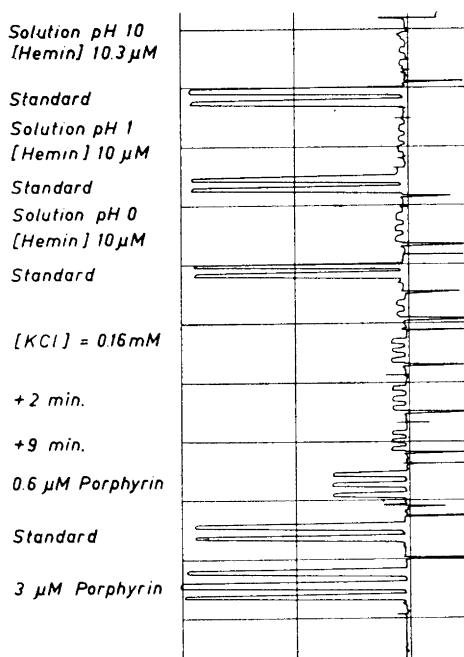


Fig. 9. Original recording of fluorescence measurements on protohemin-B. The apparatus described by Theorell and Nygaard¹⁷ was used to investigate the possible fluorescence of protohemin-B. The experimental procedure is described in the text.

Ordinate: amount of fluorescence.

Abscissa: time sequence. The recorder was started for short time intervals after each addition of reactants. The record should be read from top to bottom.

Standard: A piece of fluorescent plastic was inserted into the apparatus as a reference standard.

hemin is present as B compound, so that the extinction coefficient at $385\text{ m}\mu$ reaches only a value of about $108\text{ cm}^{-1}\text{mM}^{-1}$ at infinitely low Cl^- concentration. At pH 1.1, compound B is almost fully formed at low $[\text{Cl}^-]$. The inflection point, however, lies at 0.2 M Cl^- in both cases ($\text{p}K_{\text{Cl}} = 0.7$).

Fluorescence measurements. It is well known that hemins lose their iron under strongly acid conditions, notably if the iron is kept in the reduced state. The resulting porphyrins have well-defined spectra and show sharp bands in the Soret region not far from $400\text{ m}\mu$. Lemberg and Legge¹⁶ cite from the literature the following Soret bands of porphyrins in strong HCl: Proto-porphyrin: $408\text{ m}\mu$; deuteroporphyrin: $404\text{ m}\mu$; mesoporphyrin: $401\text{ m}\mu$. Since these wavelengths are close to the peaks of the B compounds, it was important to check whether a complete or partial removal of iron could take place on B formation. Professor H. Theorell kindly allowed the author to use his recording fluorescence meter¹⁷.

Free porphyrins fluoresce strongly, whereas hemins do not. The apparatus was therefore used for detecting the possible formation of porphyrin from protohemin by strong acid at room temperature. A 10 μ M solution of protohemin was prepared and its fluorescence measured, as shown in Fig. 9. The pH was lowered to 0 by the addition of sulfuric acid and the fluorescence remeasured. Addition of 1.6×10^{-4} M KCl did not change the result, even after 9 min waiting: the fluorescence remained insignificant, indicating the virtual absence of protoporphyrin. As a check, first 0.6 μ M, then 3.0 μ M protoporphyrindimethylester were now added and gave the expected large deflections.

DISCUSSION

The formation in aqueous solutions of well defined acid compounds is common to hemoproteins and to free hemins as far as they were investigated. These compounds, called B compounds, all show characteristic sharply defined Soret bands with peaks between 380 and 398 $m\mu$ and ϵ between 81 and 163 $\text{cm}^{-1}\text{mM}^{-1}$. Their visible spectra are far less conspicuous, showing relatively flat and low bands. HRP—B has such bands at around 500 $m\mu$ ($\epsilon = 6.4 \text{ cm}^{-1}\text{mM}^{-1}$) and at about 632 $m\mu$ ($\epsilon = 2.3 \text{ cm}^{-1}\text{mM}^{-1}$). The titration of hemin solutions with sulfuric acid yields sigmoid titration curves with $n = 1$ and pK values of between 1.5 and 3.1. Table 1 gives a summary of the spectrophotometric and titrimetric data on B compounds. It is seen that the height and position of the 397 $m\mu$ band is the same for all five protohemin-containing proteins investigated ($\epsilon = 131 \pm 1 \text{ cm}^{-1}\text{mM}^{-1}$ per hemin). Free protohemin-B, while showing the same position of the peak, has a much lower extinction coefficient. The low extinction coefficient of protein-free protohemin-B might well be due to polymerization, which is known to occur in aqueous solutions of this hemin. The addition of protein to protohemin-B increases the Soret band. Alcohols seem to act in a similar way as will be further discussed in the following paper¹⁸. Deuterohemin-B and mesohemin-B do not show abnormally low bands in the Soret region and apparently undergo less polymerization than protohemin*. If this assumption is correct one should not expect any marked intensification of the Soret band of these hemins by the presence of protein, which was indeed observed with the B compound of deuterohemalbumin.

A comparison of the data of Table 1 with the scant values from the literature shows some similarities, even though the experimental conditions were different. Erdman and Corwin's spectrum of acid mesohemindimethylester in dioxane⁷ has a Soret peak at 390 $m\mu$ ($\epsilon = 175 \text{ cm}^{-1}\text{mM}^{-1}$), compared to 380 $m\mu$ and $\epsilon = 163$ for mesohemin-B in water at pH 1 reported here. Hicks and Holden⁶ acidified protohemin with HCl and obtained a peak between 374 and 386 $m\mu$ (emission lines of the iron spark source of their spectrograph) and a minimal extinction coefficient of $45.6 \text{ cm}^{-1}\text{mM}^{-1}$. Comparison with Fig. 7 indicates that the pH must have been about 2, corresponding to a Cl^- concentration of 0.01 M.

* Shack and Clark¹⁵ observed that mesohemin has a much smaller particle weight than protohemin in aqueous solution.

Table 1. Soret bands and pK values of some B compounds.

| Starting material | Magnitudes measured | "pK" a) | cm ⁻¹ mM ⁻¹ per hemin b) | max ^{c)} mμ |
|-------------------|---------------------|---------|--|----------------------|
| Peroxidase | HRP | 2.30 | 132 | 397 |
| Hemoglobin | Hb | 2.25 | 130 | 397 |
| Catalase | HBC | 2.00 | 132 | 397 |
| Myoglobin | ShMb | 2.05 | 131 | 397 |
| Protohemalbumin | PHA | ? | 130 | 397 |
| Protohemin | PH | 1.50 | 81 | 397 |
| Mesoheemin | MH | 2.25 | 163 | 380 |
| Deuteroheemin | DH | 3.05 | 144 | 388 |
| Deuterohemalbumin | DHA | ? | 140 | 387 |

a) accuracy \pm 0.05 pH units. For the meaning of "pK" cf. text.

b) accuracy \pm 1 %

c) accuracy \pm 1 mμ

The "pK" values of Table 1 do not all simply refer to true dissociation constants of the reaction



In the case of deuteroheemin, the reversibility of B formation with a dissociation constant of about 10^{-3} has been demonstrated. The "pK" values of hemoproteins, on the other hand, represent merely the pH at which half of the B compound has been formed, since the breaking of bonds between protein and hemin is also reflected in the data and is thought to be responsible for the variations in "pK" between 2.00 and 2.30 for several protohemin-containing hemoproteins. The abnormally low "pK" of free protohemin suggests that an additional chemical reaction may be required for forming the B compound. This may very well be a partial depolymerization of the hemin.

The results of the fluorescence experiments show that at the most 1 % of the hemin could possibly have been converted to protoporphyrin under the conditions of B formation and also when chloride was added to the solution.

The data presented here make it seem probable that the formation of B compounds involves the configuration around the iron atom of the hemin molecule: The vinyl groups can be excluded as participants, since deutero- and mesoheemin also give B compounds. At pH 1.5 (the "pK" of protohemin B) one should expect the propionyl groups to be present in their undissociated and uncharged form, even though their pK has never been determined directly. A further discussion of the possible structure and the reactions of the B compounds is included in the following paper¹⁸.

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