Reactivities of Hydroxylamine and Sodium Bisulphite with Carbonyl-Containing Haems and with the Prosthetic Groups of the Erythrocyte Green Haemoproteins

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(Received 18 August 1978)

The reactivities of alkaline NH_2OH and neutral NaHSO_3 with carbonyl and olefinic groups conjugated with the tetrapyrrole nucleus of haems were studied. The reactions were carried out with 2–3 nmol of haem *a*, spirographis haem, isospirographis haem, 2,4-diacetyldeuterohaem and protohaem. Vinyl side chains were found to be insensitive to the chemical action of both alkaline NH_2OH and neutral NaHSO_3 . The formyl-containing haems reacted rapidly with both reagents at room temperature, as evidenced by sizable hypsochromic shifts of the reduced pyridine haemochrome spectrum. In less alkaline solution, the reactions of these formyl-containing haems with NH_2OH were much slower. 2,4-Diacetyldeuterohaem reacted with alkaline NH_2OH , but not with neutral NaHSO_3 . These rapid, simple and straightforward tests are readily usable in differentiating among formyl, acetyl and other electron-withdrawing side chains conjugated with the tetrapyrrole ring of haems. We applied these observations to an investigation of the two unique prosthetic groups of the bovine erythrocyte green haemoproteins. The prosthetic groups of these two proteins were isolated and spectrally characterized. Under the conditions used, the haems did not react with either NH_2OH or NaHSO_3 , but were altered by dithionite, suggesting that the previous interpretation that a formyl group was present [Hultquist, Dean & Reed (1976) *J. Biol. Chem.* **251**, 3927–3932] may have been premature. These studies also provide evidence that the α -hydroxyfarnesylethyl side chain of haem *a* affects the α -band maximum, but not the β - or Soret bands of the reduced pyridine haemochrome spectrum of haem *a*.

The reaction of NH_2OH with carbonyl groups to give the corresponding oxime derivative has classically been used as a test for the detection of carbonyl-containing porphyrins and, less frequently, carbonyl-containing haems (Rawlinson & Hale, 1949; Lemberg & Falk, 1951; Oliver & Rawlinson, 1955; Connelly *et al.*, 1958; Parker, 1959; Morrison *et al.*, 1960; Clezy & Barrett, 1961; Clezy *et al.*, 1964). The magnitude of the blue-shift of the near-u.v.–visible absorption spectrum that accompanies oxime formation is much larger for formyl-containing tetrapyrroles than for acetyl-containing tetrapyrroles. Oxime formation results in a 17–22 nm shift of the α -peak of the reduced pyridine haemochrome of haems with a formyl group in conjugation with the tetrapyrrole nucleus, but gives only a 1–2 nm shift with haems containing a conjugated acetyl group (Lemberg & Falk, 1951). This difference has been used to distinguish between formyl and acetyl substitution on the periphery of the porphyrin nucleus.

A blue-shift of a porphyrin spectrum on reaction with NaHSO_3 has likewise been cited as evidence for

the presence of a formyl group in conjugation with a tetrapyrrole nucleus, since the acetyl-containing porphyrins, cryptoporphyrins *p*, are reported not to undergo bisulphite-adduct formation (Clezy *et al.*, 1964).

The unique prosthetic group of a human erythrocyte green haemoprotein (Hultquist *et al.*, 1976) was found to undergo reactions with NH_2OH and NaHSO_3 under the conditions that have been used with other haems; characterization of this prosthetic group and its derivatives distinguished it from all other naturally occurring prosthetic groups and suggested that it is a complex haem containing both a formyl group and polar acetylable functional groups. Similarly, we have studied two bovine erythrocyte green haemoproteins and shown that these proteins differ in terms of the spectral and chemical properties of their prosthetic groups (DeFilippi & Hultquist, 1978*a,b*).

In attempting to identify unambiguously the side chains of these haems we further studied the reactions of NH_2OH and NaHSO_3 with model compounds. We discovered that haem *a*, which has been shown to possess a formyl group as one of its substituents (Lemberg & Falk, 1951; Lemberg, 1953; Connelly

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et al., 1958; Caughey *et al.*, 1975), does not rapidly undergo oxime formation under the neutral or mildly alkaline conditions at which the reaction was believed to occur. However, we found that the reaction proceeds rapidly in the strongly alkaline conditions used in pyridine haemochrome formation, a procedure that was believed to be simply a process to assess visually the extent of the reaction. Moreover, we realized that the reaction of formyl-containing haems with NaHSO_3 has received relatively little attention in the literature (Orie & Washio, 1977; Kitagawa *et al.*, 1977). Reactivity of porphyrins (but not haems) with HSO_3^- was apparently first described by Parker (1959) as yielding alteration in the absorption spectrum of cryptoporphyrin *a* in dilute cold pyridine; reference was made to unpublished work by R. Lemberg.

In the present paper we report the reactivities of haems with alkaline NH_2OH and neutral NaHSO_3 . These reactivities constitute a rapid and straightforward means of differentiating among formyl, acetyl and other electron-withdrawing side chains of haems and have allowed us to re-examine the question of whether a formyl group is present on the prosthetic groups of the erythrocyte green haemoproteins.

Experimental

Materials

The outdated human erythrocytes were obtained from the University of Michigan Medical Center

Blood Bank. Ox heart was purchased from Kappler Packing Co., Ann Arbor, MI, U.S.A.

Pyridine for spectrophotometry was dried over KOH pellets and distilled from ninhydrin. Purified pyridine was stored over 4A Linde molecular sieves (Union Carbide Corp., New York, NY, U.S.A.) and KOH pellets. All other chemicals were reagent grade and were not further purified.

Silica gel 60 F-254 t.l.c. plates were obtained from EM Laboratories (Elmsford, NY, U.S.A.); alumina (Woelm, neutral activity, grade I, used for column chromatography) was obtained from Alupharm Chemicals (New Orleans, LA, U.S.A.); polyamide CC6 for column chromatography was obtained from Brinkmann Instruments (Westbury, NY, U.S.A.).

Preparation of model haems

The structures of the various haems used in this paper are shown in Fig. 1. Protohaemin IX was obtained from packed out-dated erythrocytes by using the acetic acid method of Fischer (1955). Protoporphyrin IX dimethyl ester was prepared from protohaemin IX by the method of Caughey *et al.* (1966). Deuterohaemin IX was prepared and purified as described by Falk (1964). 2,4-Diacetyldeuterohaemin IX was prepared from deuterohaemin IX by the method of Fischer & Orth (1937) and was purified (Lamson *et al.*, 1973) on polyamide columns (2 cm $\times$ 20 cm) by using 2.5% (v/v) acetic acid in methanol.

Spirographis haemin (2-formyl-4-vinyldeuterohaemin IX) and isospirographis haemin (4-formyl-

Fig. 1. Schematic representation of the model haems used in this study

Haem	R	R'	R''
Deuterohaem IX	—H	—H	—CH ₃
Protohaem IX	—CH=CH ₂	—CH=CH ₂	—CH ₃
Spirographis haem	—CHO	—CH=CH ₂	—CH ₃
Isospirographis haem	—CH=CH ₂	—CHO	—CH ₃
Haem <i>a</i>	α -Hydroxy-farnesylethyl	—CH=CH ₂	—CHO
2,4-Diacetyldeuterohaem IX	—COCH ₃	—COCH ₃	—CH ₃

Fig. 2. Spectra of derivatives of haemin I of bovine erythrocyte green haemoprotein

Reduced pyridine haemochrome spectra are shown for freshly prepared haemin I at 20°C . —, Haemin I in a solution of 0.025 M-potassium phosphate buffer, pH 6.00, containing 20% (v/v) pyridine and 2 M- NaHSO_3 , but no $\text{Na}_2\text{S}_2\text{O}_4$. The haem is fully reduced under these conditions and possesses maxima at 579.5, 543 and 433 nm. - - -, Haemin I after 10 min in a solution of 0.025 M-potassium phosphate buffer, pH 6.00, containing 20% pyridine and 0.3 mM- $\text{Na}_2\text{S}_2\text{O}_4$, but no NaHSO_3 . The derivative shows maxima at 556, 524 and 418.5 nm. ····, Same sample after 2 h of reaction with $\text{Na}_2\text{S}_2\text{O}_4$; absorbance maxima are at 553, 522 and 415.5 nm.

2-vinyldeuterohaemin IX) were prepared as follows. The porphyrin dimethyl esters corresponding to these haemins were prepared and purified by the method of Caughey *et al.* (1966). The esters of spirographis and isospirographis porphyrins were separated by using silica-gel t.l.c. with unwashed chloroform as the solvent (Sono & Asakura, 1974). Each of the porphyrin esters was hydrolysed to the free porphyrin at 20°C in 6 M-HCl; paper chromatography with lutidine/water (10:7, v/v) in an atmosphere saturated with aq. 7 M- NH_3 (Chu & Chu, 1955) was used to monitor the conversion. Haemins were prepared from each of the porphyrin isomers by the method of Morell *et al.* (1961), and were extracted into ethyl acetate by the method of Asakura & Sono (1974). The haemins were purified on polyamide columns (1 cm $\times$ 6 cm) by using 2.5% (v/v) acetic acid in methanol (Lamson *et al.*, 1973).

Haem *a* was isolated from fresh ox hearts as the

Fig. 3. Spectra of derivatives of haemin II of bovine erythrocyte green haemoprotein

Reduced pyridine haemochrome spectra of freshly prepared haemin II in aq. 20% pyridine containing 0.3 mM- $\text{Na}_2\text{S}_2\text{O}_4$ and 0.29 M- NaHSO_3 at 20°C . —, Zero time; absorbance maxima are at 571, 537 and 430 nm. The spectrum is the same as that obtained in alkaline solution in the presence of $\text{Na}_2\text{S}_2\text{O}_4$, but the absence of NaHSO_3 . ····, After 1.7 h; - - -, after 11.5 h; absorbance maxima are at 552, 521 and 414 nm. For clarity, only one intermediate spectrum is shown. Other intermediate spectra show the general maintenance of isobesticity.

low-spin dipyrindine ferrous complex by the method of York *et al.* (1967). All haem samples were evaporated to dryness *in vacuo* at 35°C or below and the dried samples were stored under desiccation at -20°C .

Isolation of the prosthetic groups of the bovine erythrocyte green haemoproteins

The prosthetic groups were isolated from the bovine erythrocyte green haemoproteins by electrophoresis on polyacrylamide gels as previously described (DeFilippi & Hultquist, 1975). Cyanide (40 mM) was included in the upper electrophoresis buffer as well as in the protein sample. Haemin samples were freeze-dried and stored at -20°C . Storage of the samples for many weeks resulted in the appearance of a degradation product with a marked absorbance peak at 533 nm. This impurity could be removed from the haemin by chromatography on a column of Sephadex G-25 (2.2 cm $\times$ 45 cm) with 15% (v/v) pyridine in water as eluent. The reduced pyridine haemochromes of the isolated haems (Figs. 2 and 3) are spectrally indistinguishable from the reduced pyridine haemochromes of the haemoproteins (DeFilippi & Hultquist, 1978*a,b*) from which the prosthetic groups were isolated. Haemin I, the prosthetic group of green haemoprotein form I, shows maxima at 579.5, 541 and 433.5 nm; haemin II, the prosthetic

group of form II, shows maxima at 571, 537, and 430 nm.

General procedures

Reduced pyridine haemochrome spectra (Paul *et al.*, 1953) were used to quantify the haems and to calculate the absorption coefficients of the haem derivatives. The haemochrome of haem *a* was prepared in pyridine/water (9:1, v/v) rather than in pure pyridine. As reported previously (DeFilippi & Hultquist, 1977), the spectra in pyridine/water and in pure pyridine are identical. An absorption coefficient of $32.7 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ (Caughey *et al.*, 1975) was used for the α -peak of haem *a*. The haemochromes of spirographis and isospirographis haems were quantified by using an absorption coefficient of $138 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ for the Soret peak (Sono & Asakura, 1974). In all experiments conservative amounts of $\text{Na}_2\text{S}_2\text{O}_4$ (approx. 0.3 mM) were used as the reductant so as to avoid reductive alterations of the type observed by Vanderkooi & Stotz (1965). All spectra were recorded at 20°C with a Cary model 17 spectrophotometer.

Reactions of haems with hydroxylamine were carried out both in aqueous pyridine/ Na_2CO_3 and in aqueous pyridine/ NaOH . To examine the reaction of haem *a* with NH_2OH in aqueous pyridine/ Na_2CO_3 by the method of Lemberg & Falk (1951), haem *a* was dissolved in aq. 25% (v/v) pyridine in a 2 ml stoppered quartz cuvette to give a concentration of $3.25 \mu\text{M}$. An equimolar mixture of $\text{NH}_2\text{OH} \cdot \text{HCl}$ and Na_2CO_3 (1 mg/ml) was added and spectra were recorded at intervals to assess the extent of the reaction. In accord with the conditions of Lemberg & Falk (1951), $\text{Na}_2\text{S}_2\text{O}_4$ was absent in this experiment. To perform the reactions in more alkaline solution, 1 ml of freshly prepared 20% (v/v) pyridine in 0.5M- NaOH was added to a sample of haemin in the cuvette. Approx. 0.1 mg of solid $\text{Na}_2\text{S}_2\text{O}_4$ was added and dissolved; 1 mg of $\text{NH}_2\text{OH} \cdot \text{HCl}$ was then added and spectra were recorded every few minutes until the reaction was complete.

The effect of the concentration of NH_2OH on the rate of oxime formation was studied by using haem *a*. The reactions were carried out in the cuvette by using freshly prepared 20% (v/v) pyridine in 0.5M- NaOH . Appropriate amounts of $\text{NH}_2\text{OH} \cdot \text{HCl}$ and $\text{Na}_2\text{S}_2\text{O}_4$ (final concn. approx. 0.3 mM) were dissolved in 1.5 ml of the pyridine/alkali solution, and 10 μl of a pyridine solution of haem *a* was added. The final concentration of haem was $3.1 \mu\text{M}$. The reaction was monitored by measuring the decrease in A_{594} as a function of time. Spectra were recorded at the end of the reaction to ensure that the oxime had indeed been formed.

The effect of pH on the rate of oxime formation of haem *a* was monitored in a similar manner.

$\text{NH}_2\text{OH} \cdot \text{HCl}$ was added to 20% (v/v) pyridine in water. The pH of the solution was adjusted with conc. HCl of 20% (v/v) pyridine in 0.5M- NaOH . The solution was then diluted to give a final NH_2OH concentration of 10 mg/ml. After addition of $\text{Na}_2\text{S}_2\text{O}_4$ (final concn. approx. 0.3 mM) to 1.5 ml of the solution in a cuvette, 10 μl of haem *a* in pyridine was added to give a final haem concentration of $3.1 \mu\text{M}$. Even though the haem iron was reduced in the above solution in the absence of $\text{Na}_2\text{S}_2\text{O}_4$, this reductant was included to minimize the bleaching of haem that was observed when haem *a* was incubated in the presence of Na_2CO_3 and NH_2OH .

Bisulphite addition reactions were performed in aqueous pyridine at pH 6.00. Fresh stock solutions containing 1.9M- NaHSO_3 and 0.025M-phosphate were prepared daily by dissolving NaHSO_3 and KH_2PO_4 in water/pyridine (81:19, v/v) and adjusting the mixture to pH 6.00 with 12M- HCl at 20°C. A small sample of haem or haemin, dissolved in pyridine or pyridine/water (4:1, v/v), was transferred to the bottom of the cuvette and evaporated to dryness with a stream of N_2 . NaHSO_3 solution (1 ml) was added and the haemin dissolved by mild agitation. Spectra were recorded immediately and then monitored for any slow reactions that might be occurring. As with the oxime, conservative amounts of $\text{Na}_2\text{S}_2\text{O}_4$ (not more than $0.3 \mu\text{mol/ml}$) were added to ensure complete reduction of the haem iron.

For the titration of haem *a* with NaHSO_3 , fresh samples of haem and NaHSO_3 were used for each concentration of NaHSO_3 . In these experiments, samples of the stock solution of NaHSO_3 were diluted with 0.025M- KH_2PO_4 /pyridine (4:1, v/v), pH 6.0, as described above. All bisulphite concentrations refer to the total concentration of HSO_3^- and SO_3^{2-} . The oxygen partial pressure in the solution in the cuvettes was lowered before the addition of $\text{Na}_2\text{S}_2\text{O}_4$ by bubbling a thin stream of N_2 through the solutions for 2–3 min.

Results and Discussion

Oxime formation from model haems

Haem *a* reacted rapidly with NH_2OH in the presence of strong alkali (0.5M- NaOH), as demonstrated by a marked blue-shift in the absorbance maxima of the reduced pyridine haemochrome (Fig. 4 and Table 1).

In contrast, the formation of the oxime of haem *a* was not observed when the reaction was carried out by the method of Lemberg & Falk (1951) in the presence of Na_2CO_3 (in place of NaOH) with essentially the same concentration of $\text{NH}_2\text{OH} \cdot \text{HCl}$ (0.4 mg/ml instead of 0.6 mg/ml). The positions of the absorption maxima did not change when a $3.25 \mu\text{M}$ solution of haem *a* was incubated for 2 h; the only spectral change that occurred during this period was

Fig. 4. Reduced pyridine haemochrome spectra of oximes of formyl-substituted haems

Millimolar absorptions were calculated as described in the Experimental section. Spectra were recorded after 15 min of reaction of haem with 1 mg of NH_2OH /ml of 0.5M-NaOH/pyridine (4:1, v/v) in the presence of $\text{Na}_2\text{S}_2\text{O}_4$. (a) Spirographis haem oxime; (b) isospirographis haem oxime; (c) haem *a* oxime.

reacted with NH_2OH to give a 17nm blue-shift in the α -peak of the reduced pyridine haemochrome spectrum.

This difference between our findings and those of Lemberg & Falk (1951) might be explained in one or more of the following ways: (1) NaOH might be necessary to reveal the oxime formation; (2) an inhibitor of oxime formation might be present in our reaction mixture; (3) the extent of the reaction might be highly dependent on NH_2OH concentration; (4) strongly alkaline conditions might be necessary for the reaction to proceed at an appreciable rate.

The last of these explanations was shown to be correct by the following experiments. Incubation of haem *a* with NH_2OH resulted in no spectral shift when Na_2CO_3 was eliminated from the reaction mixture or when sodium acetate (Morrison *et al.*, 1960) was substituted for the Na_2CO_3 . However, as shown in Fig. 4(c) the oxime was formed when 0.5M-NaOH was used in place of the Na_2CO_3 . When a mixture of haem *a* and NH_2OH , which had failed to react in the presence of Na_2CO_3 , was then made 0.25M in NaOH, a spectral change indicative of oxime formation was observed. After 1 min, double α -peaks were observed (587 and 569nm); within 15min, oxime formation was complete. The extent of the reaction at various times was approximately the same as when the reaction was carried out in NaOH without prior incubation in the presence of Na_2CO_3 . This finding rules out the possibility that oxime formation occurred in the carbonate solution, but that alkali was required to convert it into an anionic form that is blue-shifted. It also rules out the possibility that an inhibitor is present in the reaction mixture.

Measurement of the effect of NH_2OH concentration on the rate of haem *a* oxime formation in 20% pyridine in 0.5M-NaOH showed that the reaction rate (but not the extent) was indeed concentration-dependent. NH_2OH concentrations of 0.0144, 0.0719 and 0.144M gave observed rate constants of 0.35, 1.36 and 2.5min⁻¹ respectively. The second-order rate constant calculated from these values is 50mmol⁻¹·min⁻¹. This concentration-dependency is not sufficiently large, however, to explain the difference between our findings and those of Lemberg & Falk (1951).

The anomaly can be explained by the effect of pH on the rate of the reaction. The pH-versus-rate profile is shown in Fig. 5. The rate of oxime formation in 0.5M-NaOH (calculated pH of 13.7) is 40–150 times the observed rates in the pH range 8.0–11.0. The pH of the carbonate/ NH_2OH solution, calculated to be similar to that used by Lemberg & Falk (1951), is in the range where the rate is lowest (pH 8–11), thus explaining our failure to observe oxime formation. We believe that in previous studies, where the reaction

a general loss of absorption (an 85% decrease in the absorbance at the α -peak). Addition of $\text{Na}_2\text{S}_2\text{O}_4$ (0.3mm) subsequent to the incubation did not alter the spectrum. Lemberg & Falk (1951) reported that after 30min under similar conditions haem *a* had

Table 1. Absorbance maxima of reduced pyridine haemochromes

Final concentrations are: *20% (v/v) pyridine, 0.5M-NaOH, †19% pyridine, 1.9M-NaHSO₃; ‡25% pyridine, 0.5M-NaOH; §90% pyridine.

Haem	Derivative	Absorbance maxima (nm)		
		Soret	β	α
Haem <i>a</i>	Free*	431	—	587
	Oxime*	418	524	567
	Bisulphite adduct†	417	522	559
Spirographis haem	Free*	434	538	583
	Oxime*	419	526	558
	Bisulphite adduct†	416	521	553
Isospirographis haem	Free*	434	536	581
	Oxime*	419	526	558
	Bisulphite adduct†	416	522	553
2,4-Diacetyldeuterohaem	Free‡	426	—	581
	Free§	442	540	577
	Oxime*	421	—	573
	Bisulphite adduct†	No reaction

Fig. 5. Rate of formation of haem *a* oxime versus pH

Reactions were carried out at 27°C in aq. 20% (v/v) pyridine with final concentrations of 10mg of NH₂OH.HCl/ml and 3.1 μ M-haem *a*. The pH was adjusted with either dilute HCl or 20% (v/v) pyridine in 0.5M-NaOH. After addition of a few crystals of Na₂S₂O₄ to 1.5 ml of NH₂OH solution, 10 μ l of a pyridine solution of haem *a* was added and the reaction monitored at 594nm. Apparent rate constants (k_{obs}) were calculated from the slope of the plots of $\log(A_{594}$ at time t - final A_{594}) versus time.

was carried out at neutral or slightly alkaline pH, little haem oxime formation occurred until NaOH was added for the purpose of obtaining the reduced pyridine haemochrome by the method of Paul *et al.* (1953).

Having explained this anomaly, we can now present a rapid straightforward method for oxime formation with formyl-containing haems. The reaction conditions are: NH₂OH.HCl (>1 mg/ml) in

pyridine/aq. 0.5M-NaOH (1:4, v/v). Under these conditions the reaction proceeds equally well in both the presence and the absence of Na₂S₂O₄, but this compound is normally included in order both to minimize the bleaching of the haem and to ensure complete reduction of the haem iron. The rate of formation of the oxime is independent of Na₂S₂O₄ at the low concentrations tested.

Under these conditions spirographis haem and isospirographis haems, like haem *a*, react readily with NH₂OH (see Figs. 4a and 4b and Table 1) Diacetyldeuterohaem IX reacts with NH₂OH to give a small shift in the absorbance maxima of the reduced pyridine haemochrome derivative. In contrast with the blue-shift of 20–25 nm and 13–15 nm observed for the α - and Soret peaks respectively of the formyl-containing haems, reaction of diacetyldeuterohaem IX with NH₂OH gives 8 nm and 5 nm blue-shifts for the α - and Soret peaks respectively. Small shifts had previously been reported to result from reaction of acetyl-containing porphyrins with NH₂OH (Lemberg & Falk, 1951). Exposure of protohaem IX to this reagent resulted in no significant spectral change in the reduced pyridine haemochrome. Conjugated vinyl groups of haems are thus unreactive towards alkaline NH₂OH.

Bisulphite adduct formation from model haems

Haem *a* was found to react readily with 2M-NaHSO₃ in pyridine/0.025M-potassium phosphate buffer (1:4, v/v), pH 6.00. Initiation of the reaction resulted in an immediate decrease in absorbance at 587 and 431 nm concomitant with the appearance of an α -peak at 560 nm, a β -peak at 522 nm and a Soret peak at 416.5 nm (see Fig. 6). The addition of Na₂S₂O₄ did not alter the spectrum, except to give a slight sharpening of the absorption maxima.

Fig. 6. Titration of haem *a* with NaHSO_3

Solutions containing 0, 0.161 M-, 0.322 M-, 0.643 M-, 1.29 M- and 1.93 M- NaHSO_3 were prepared by mixing a solution containing 19.2% (v/v) pyridine, 1.93 M- NaHSO_3 and 0.025 M-potassium phosphate, pH 6.0, with a similar solution containing no NaHSO_3 . Dithionite (final concn. approx. 0.3 mM) was included to ensure complete reduction. Spectra were recorded immediately after mixing. The arrows show the change in spectra with the increasing concentrations. Inset (a): reduced pyridine haemochrome spectra (500–700 nm) in the pyridine/phosphate buffer used above. ----, Unchanged haem *a*; —, the bisulphite adduct prepared with 1.93 M- NaHSO_3 . The minor peak at 587 nm in the presence of NaHSO_3 is due to the unchanged haem *a*. Inset (b): inverse plot of change of absorbance with change in NaHSO_3 concentration. Change was normalized to the value in 1.93 M- NaHSO_3 . The slope from this plot divided by the *y* intercept gave a value of 0.97 M for the dissociation constant of the bisulphite adduct of haem *a*.

In contrast, virtually no spectral change was observed when haem *a* was incubated with a saturating concentration of NaHSO_3 in pyridine by the method used by Lemberg [as communicated by Parker (1959)] for the formation of bisulphite adducts of formyl-containing porphyrins. Under these conditions very little NaHSO_3 dissolves. Thus it appears that the lower concentrations of NaHSO_3 , which have been thought to be useful for forming adducts of porphyrins, are not suitable for derivative formation of haem *a*.

The stability of the haem *a*-bisulphite complex was studied by titrating the haem with NaHSO_3 . In-

cremental increases in NaHSO_3 concentration resulted in a less than proportional change in absorbance values, indicative of a reversible equilibrium. Analysis of the spectral changes by the method of Benesi & Hildebrand (1949) yielded a dissociation constant of 0.97 M (see Fig. 6, inset b). At each stage of titration, the spectrum was rather stable; the absorbance of an approximately equimolar mixture of the adduct and the free haem fell by 15% over a period of 3 h at 20°C.

Spirographis and isospirographis haems, like haem *a*, also formed bisulphite adducts at pH 6.00 in the presence of 1.9 M- NaHSO_3 . The differences

Fig. 7. Reduced pyridine haemochrome spectra of bisulphite adducts of formyl-substituted haems

—, Reduced pyridine haemochrome spectra were recorded immediately after solution of haem in 0.025M-potassium phosphate, pH6.0, containing 19% (v/v) pyridine, 1.9M-NaHSO₃ and 0.3mM-Na₂S₂O₄. ----, Pyridine ferrihaemochrome spectra formed by the solution of the haem in 0.025M-potassium phosphate, pH6.0, containing 19% (v/v) pyridine. The millimolar absorption scale at the left ordinate applies to wavelengths shorter than 500nm and that on the right to wavelengths longer than 490nm. (a) Spirographis haem; (b) isospirographis haem.

between the pyridine haemochrome spectra of the haems and their adducts are shown in Fig. 7 and Table 1. In contrast with the formyl-containing haems, the addition of 2M-NaHSO₃ to a solution of 2,4-diacetyldeuterohaem IX or protohaem IX did not result in any spectral alteration of the reduced pyridine haemochrome, demonstrating the specificity of the reaction conditions for a formyl group. The lack of reactivity of the acetyl-substituted haem is undoubtedly due to the well-known resistance of hindered carbonyl groups towards addition of NaHSO₃ (Steward, 1905). Preliminary results with 2,4-diacrylyldeuterohaem dimethyl ester indicate that acrylyl groups, in conjugation with the tetrapyrrole nucleus, are also insensitive to the action of NH₂OH and NaHSO₃.

Spectral comparisons of model haem derivatives

The oximes of spirographis and isospirographis haems possess very similar reduced pyridine haemochrome absorbance spectra. The positions of the absorbance maxima of these two compounds are indistinguishable, and are similar to those reported by Lemberg & Falk (1951) for the oxime of spirographis haem. The absorption of the α -peak of the spirographis haem derivative was observed to be slightly greater than that of the isospirographis haem derivative. The bisulphite adducts of these two formyl-containing haems were virtually identical.

However, the spectra of the oximes and the bisulphite adducts of spirographis and isospirographis haems are distinct from the corresponding spectra of haem *a* derivatives. Comparison of the pyridine haemochromes of the bisulphite adducts shows β - and Soret peaks at essentially the same wavelengths, but the α -peak for the haem *a* adduct is at a longer wavelength (559nm) than are the α -peaks for the synthetic monoformylmonovinylhaem adducts (553nm). Likewise, the α -peak of the pyridine haemochrome of haem *a* oxime is found at a longer wavelength than the α -peaks of the oximes of the synthetic monoformylmonovinylhaems, but the positions of the β - and Soret peaks are very similar. Parallel but less definitive findings can be discerned by inspection of the spectra of the dipyrindine complexes of the free haems (Table 1).

Differences in the spectra of haem *a* and the synthetic monoformylmonovinylhaems are not unexpected, since the symmetries of the electron-withdrawing substituents are different. However, if the formyl and vinyl groups are considered to be the only electron-withdrawing groups of haem *a*, spectral differences of the oximes of these haems would have been predicted to be much smaller, since oxime formation greatly decreases the electron-withdrawing capacity of a formyl group. Moreover, through symmetry considerations, the bisulphite adducts would have been predicted to be spectrally indistinguishable, since reaction with NaHSO₃ obliterates the effect of a formyl group on the spectrum. [For example, the spectral similarity of the reduced pyridine haemochromes of the adduct of 2-formyl-4-vinyldeuterohaem (α -peak at 553nm) and 4(2)-hydroxyethyl-2(4)-vinyldeuterohaem (α -peak at 552nm) demonstrates that a bisulphite adduct of a formyl group affects the spectrum to the same degree as a hydroxyethyl group, which in turn is similar to the effect of an ethyl group (Porra & Jones, 1963).]

Thus it must be concluded that the α -hydroxyfarnesylethyl group of haem *a* has a considerable effect on the spectrum. This perturbation may be a result of simple electron-withdrawing ability. Such an explanation is weakened, however, by the failure of the α -hydroxyfarnesylethyl group to act as a rhodo-

fyng group in the porphyrin spectrum (Lemberg & Falk, 1951) and the failure of acetylation of the hydroxy group to perturb the reduced pyridine haemochrome spectrum of haem *a* dimethyl ester (DeFilippi & Hultquist, 1977). Alternatively, the perturbation may result from effects on the solvation of the porphyrin system, or, most likely, from interaction of the double bonds of this side chain with the porphyrin π -electron system as has been suggested by Caughey *et al.* (1975).

The absorption spectrum of the reduced pyridine haemochrome of 2,4-diacetyldeuterohaem IX was found to be quite solvent (v/v) sensitive. In 90% pyridine (with a small amount of $\text{Na}_2\text{S}_2\text{O}_4$ as reductant), the absorption maxima (and relative absorptions in parentheses) are 442 nm (1.00), 540 nm (0.081) and 577 nm (0.123). In 50% (v/v) pyridine the Soret absorption fell relative to the α - and β -peaks and the trough between the α - and β -peaks became more shallow, the absorption maxima (and relative absorptions) now being 447 nm (1.00), 544 nm (0.10) and 581 nm (0.148). Lowering the pyridine concentration to 14.3% (v/v) almost obliterated the trough between the α - and β -peaks. The presence of 0.01 M-NaOH along with either 14.3% (v/v) pyridine or 50% (v/v) pyridine eliminated the β -peak, the maxima (and relative absorptions) now being 443 nm (1.00) and 582 nm (0.15). Unusual solvent-dependence has been noted before and has been attributed to direct interaction of pyridine with the acetyl groups (O'Keeffe, 1974). A strong solvent-dependence explains the differences between our values and those reported in the literature (O'Keeffe, 1974; Lemberg & Falk, 1951).

Reactivities of the haems of bovine erythrocyte green haemoproteins towards hydroxylamine, bisulphite and dithionite

Neither of the haem prosthetic groups of the bovine erythrocyte green haemoproteins yielded an oxime. Treatment with NH_2OH over a wide range of alkalinity and solvent composition resulted in no spectral change that could be interpreted as the formation of a derivative of a formyl group. However, both haems were unstable in aqueous alkali/pyridine; general loss of absorbance was observed with the conditions used by Lemberg & Falk (1951), with 17% (v/v) pyridine in 0.05 M-NaOH, with 30% (v/v) pyridine in 0.25 M-NaOH and with 25% pyridine in 0.5 M-NaOH, all in the presence of 0.3 mM- $\text{Na}_2\text{S}_2\text{O}_4$.

Likewise, neither of the haems formed a bisulphite adduct. Both haems were stable for at least 1 h in a solution of 2 M-NaHSO₃, 20% pyridine and 0.025 M-phosphate buffer, pH 6.0, as evidenced by the maintenance of the reduced pyridine haemochrome spectrum (Fig. 2). These conditions yielded the ferrous form of these haems (as well as other haems

studied), and thus no dithionite was added in this particular reaction.

Whereas these haems did not react with NH_2OH or NaHSO_3 under conditions which formed derivatives of formyl groups at the 2-, 4- and 8-positions of the tetrapyrrole ring, they did react readily at pH 6.0 with $\text{Na}_2\text{S}_2\text{O}_4$ under conditions that did not alter haem *a*, spirographis haem or isospirographis haem. The addition of $\text{Na}_2\text{S}_2\text{O}_4$ (final concn. 0.3 mM) to a solution of haemin I in pyridine/phosphate buffer, pH 6.0, resulted in the formation (within 10 min) of a derivative with a sharp α -peak at 556 nm, a β -peak at 524 nm and a Soret peak at 418.5 nm, with relative absorbances of 0.16, 0.104 and 1.00 respectively. A slight shoulder at 580 nm suggested the presence of a small amount of unchanged haem. This spectrum slowly changed to one with peaks at 553, 522 and 415.5 nm, with relative absorbances of 0.14, 0.11 and 1.00 respectively. Haem I, as well as the other haems in this study, showed little reactivity with $\text{Na}_2\text{S}_2\text{O}_4$ under alkaline conditions.

When $\text{Na}_2\text{S}_2\text{O}_4$ was added to a solution of haem I already containing 2 M-NaHSO₃, incubation for 10 min yielded a spectrum with maxima at 551, 521 and 415 nm, and shoulders (indicating the presence of unchanged haem) at 577 and 430 nm. Within 2 h the conversion was complete, the 551, 521 and 415 nm maxima having relative absorbances of 0.16, 0.11 and 1.00 respectively. It would appear that the initial derivative formed by reaction of $\text{Na}_2\text{S}_2\text{O}_4$ with haem I is different from that when NaHSO₃ is also present, but the final reaction products in these two cases are spectrally identical.

Haem II also reacts with $\text{Na}_2\text{S}_2\text{O}_4$ at pH 6.0. However, unlike haem I, haem II does not form an intermediate compound, but goes directly to a derivative with maxima at 552, 521 and 415 nm. The same spectral species results from the reaction of haem II in the presence of NaHSO₃ plus $\text{Na}_2\text{S}_2\text{O}_4$ (Fig. 3). The spectrum of this derivative is essentially identical with that of the final product derived from haem I and is also quite similar to the spectrum of 4(2)-hydroxyethyl-2(4)-vinyldeuterohaem (Hultquist *et al.*, 1976; Porra & Jones, 1963).

The reductive conversion of haems I and II into the same spectral species indicates a structural relation between the two haems. Haemin I undergoes reductive conversion into the monovinylhaem-like species through a protohaem-like spectral intermediate. In contrast, haem II, which has side chains of lesser electron-withdrawing capacity, is converted into the final product without evidence of an intermediate. Taken together, these observations suggest that: (1) there are two reducible functional groups in conjugation with the tetrapyrrole nucleus of haem I; (2) haem II differs from haem I in that one of these two groups is already in the reduced form; (3) the

unaltered functional group common to both haems is reduced preferentially on reaction of haem I with $\text{Na}_2\text{S}_2\text{O}_4$, accounting for the two-stage reduction of this haem.

We conclude from these findings that the haems of the bovine erythrocyte green haemoproteins contain electron-withdrawing side chains, which can be distinguished from the formyl, acetyl and acrylyl groups of other haems by their ease of reduction with $\text{Na}_2\text{S}_2\text{O}_4$ and their lack of reactivity with NH_2OH and NaHSO_3 . These conclusions contrast with those for the isolated prosthetic group of human erythrocyte green haemoprotein (Hultquist *et al.*, 1976), which were based on the results of the classical reactions with NH_2OH and NaHSO_3 . Since the spectral properties of haem I from the bovine protein agree well with those of the isolated haem from the human protein, the present findings bring into question the structure proposed for the haem of human green haemoprotein. The nature of the reducible electron-withdrawing side chains of these haems remains to be elucidated.

This study was supported by Research Grant AM-09250 and Training Grant GM-00187 from the U.S. Public Health Service. We gratefully acknowledge Dr. Vincent Massey for the use of his laboratory facilities and Dr. Richard Lawton for helpful suggestions.

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