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Spectral Studies of Iron Coordination in Oxidized Compounds of Hemoproteins. Difference Spectroscopy below 250 mμ*

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ABSTRACT: The feasibility of observing the spectral behavior of protein groups in the coordination sphere of the iron in hemoproteins has recently been demonstrated. Application of the method, difference spectroscopy below 250 mμ, to oxidized compounds of hemoproteins is reported in this paper. The absolute absorption spectrum (250–450 mμ) of bacterial catalase compound I is displayed. Ultraviolet difference spectra (210–280 mμ) of compounds III of metmyoglobin and methemoglobin and compounds I and II of bacterial catalase and horseradish peroxidase *vs.* the free hemoproteins are shown. Compounds III of metmyoglobin and methemoglobin, and compounds II of bacterial catalase and horseradish peroxidase have one difference band which peaks in the region 237–247 mμ. Absorption in this region is assigned, in part, to a transition involving histidine in the fifth coordination position, and in the other part to a charge transfer transition involving porphyrin. Compound I of bacterial catalase has two difference bands (235 and 224 mμ), while I of horseradish peroxidase has a broad band which is probably the sum of two bands. The spectral properties of compounds I are like those of ferric hemoprotein complexes. The spectral properties of compounds II and III support the other evidence that these are ferryl structures.

Several years ago, in the course of investigating possible involvement of tyrosine in the formation of compound I of catalase, we recorded difference spectra *vs.* free enzyme down to a wavelength of 250 mμ and noted that the absorbance rose just as this wavelength was approached. We were thereby prompted to look deeper into the ultraviolet region, and found the bands described in this paper. In checking possible explanations of these bands, we were led to try more fundamental experiments with hemoprotein complexes (Brill and Sandberg, 1968). In the latter research, the groups involved have been identified on the basis of data from simple ligands. In this paper, the difference spectra of peroxide compounds *vs.* free proteins are reported and used to describe with more assurance than previously the coordination spheres of the iron in the oxidized compounds of MetMb,[1] MetHb, BMC, and HRP.

Only one spectroscopically distinct compound (denoted in the literature as "compound III") has been observed upon reaction of MetMb with any of the substrates H_2O_2, methyl hydroperoxide, or ethyl hydroperoxide. The stoichiometry of reduction of compound III by ferrocyanide indicates that this compound has one oxidizing equivalent above free MetMb (George and Irvine, 1952, 1953). Since the peroxide substrate has two oxidizing equivalents, one of the equivalents is not retained by the heme group.

Similarly, only one spectroscopically distinct compound (also called "III") has been observed for the reaction of MetHb with H_2O_2 (Keilin and Hartree, 1951; Dalziel and O'Brien, 1954). The oxidation state of MetHb III has not yet been determined. It is unlikely to differ from that of MetMb III.

The reaction of HRP with either H_2O_2 (concentration less than 1 mM so that a third compound of peroxidase does not form) or alkyl hydroperoxides produces two spectroscopically distinct compounds, labeled "I" and "II" on the basis of order of appearance. The stoichiom-

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[1] Abbreviations used: MetMb, horse heart ferrimyoglobin; MetHb, horse ferrihemoglobin; BMC, bacterial micrococcus catalase; HRP, horseradish peroxidase; HBC, horse blood catalase; suffixes I, II, and III, compounds I, II, and III.

etry of reduction by ferrocyanide indicates that compound I has one oxidizing equivalent above compound II (Chance, 1952a), and that compound II has one oxidizing equivalent above free enzyme (George, 1952, 1953a).

In the catalatic reaction of catalase, only one spectroscopically distinct compound is observed with the direct addition of H_2O_2 (Chance, 1949). With alkyl hydroperoxides, two spectroscopically distinct compounds are observed (Chance, 1952c), denoted "I" and "II" according to order of appearance. Reduction by ferrocyanide indicates that I has an oxidizing equivalent above II, and reduction of II by nitrite shows that II has an equivalent above free enzyme (Nicholls, 1959).

The considerable data and proposed structures for the oxidized compounds of peroxidases and catalase have been reviewed several times in recent years (Nicholls and Schonbaum, 1963; Paul, 1963; Saunders *et al.*, 1964; Brill, 1966), and the reader is referred to these treatments for further information.

Materials

Hemoproteins. BMC was prepared in this laboratory from *Micrococcus lysodeitkicus* (American Type Culture Collection no. 4698) grown in submerged culture (Beers, 1955). The enzyme was extracted by the method of Herbert and Pinsent (1948) and purified by $(NH_4)_2SO_4$ fractionation, acetone precipitation from neutral 0.01 M phosphate buffer, and gel filtration. All preparations used had purity values (defined for BMC as the ratio $A_{406\ m\mu}/A_{280\ m\mu}$) greater than 0.90. At pH 7 we determined the Soret absorptivity (λ_{max} 406 mμ) to be 102.(7) mM^{-1} cm^{-1} (heme concentration) from parallel measurements of absorbance, iron concentration (as *o*-phenanthroline complex), and heme concentration (as pyridine hemochromogen), and thereafter computed the enzyme concentration from the Soret absorbance.

HRP was obtained from Worthington Biochemical Corp., Freehold, N. J. The purity value ($A_{403\ m\mu}/A_{275\ m\mu}$ for HRP) was greater than 2.9. At pH 5.6 the Soret absorptivity (λ_{max} 403 mμ) is 91 mM^{-1} cm^{-1} (Keilin and Hartree, 1951) and was used to determine concentration.

MetMb was obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, and used without further purification. A slight molar excess of potassium ferricyanide was added to each stock solution to ensure complete formation of the ferric form, and then the stock solution was dialyzed against the desired buffer. The ratio $A_{409\ m\mu}/A_{280\ m\mu}$ was 5.0 at pH 6.8. At pH 6.4 the Soret absorptivity is 188 mM^{-1} cm^{-1} (Scheler *et al.*, 1957) and was used to determine concentration.

MetHb was obtained from Mann Research Laboratories, Inc., New York, N. Y., and was used without further purification. (Commercially available hemoglobin is known to be chromatographically heterogeneous (Sober and Peterson, 1960). The differences reflected by this separation method would not be expected to influence the experiments reported here.) Stock solutions were treated with ferricyanide as in the case of MetMb. The ratio $A_{405\ m\mu}/A_{275\ m\mu}$ was greater than 4.1. At pH 6.4 the Soret absorptivity is 179 mM^{-1} cm^{-1} (heme) (Scheler *et al.*, 1957) and was used to determine concentration.

Peroxides. H_2O_2, analytical reagent grade, was obtained from Mallinckrodt Chemical Works. The concentration of H_2O_2 was determined from the absorbance at 230 mμ ($\epsilon_{230\ m\mu}$ 72.4 M^{-1} cm^{-1}; George, 1953a).

Ethyl hydroperoxide was synthesized without mishap by the method, with some modification, of Minkoff (1954) and Harris (1939). The product, obtained from vacuum distillation, had a refractive index, n_D^{20}, of 1.3808 (Bausch & Lomb Abbe-3L Refractometer). Refractive index values in the literature are 1.3810 (Minkoff, 1954) and 1.3800 (Weast, 1966). The concentration of undiluted ethyl hydroperoxide is 15 M as computed from the density, 0.9332 g/cc (Weast, 1966). A 2 M stock solution was obtained by diluting the pure ethyl hydroperoxide with glass-distilled water to a final volume 7.5 times the original volume, and stored in a brown bottle. From the absorbance at 230 mμ of the 2 M solution, an absorptivity of 42 M^{-1} cm^{-1} was calculated. This value is to be compared with the published value of Reiche (1931), 43 M^{-1} cm^{-1}.

Methods

A Cary Model 14 recording spectrophotometer was used throughout these studies. The absorption cells were produced by Pyrocell Manufacturing Co., Westwood, N. J., with windows of far-ultraviolet (S18-260) silica, in sets of four matched to within 2% transmission at 220 mμ.

Because the peroxide compounds are somewhat labile reaction intermediates, it is difficult to obtain the "true" spectrum of a particular compound without contributions from free hemoprotein and other compounds. If the maximum concentration (under conditions when this approaches the total heme concentration) of a compound is reached *after* the first 10 sec of the reaction, then peroxide can be introduced on a stirring rod into a standard open cell and a rapid flow system is not required (see, *e.g.*, Brill and Williams, 1961). The choice of ethyl hydroperoxide as substrate for the heme enzymes made the use of the open-cell method possible in the experiments reported below. If the steady-state maximum persists for a time which is significant (*e.g.*, 30 sec) for scanning wavelength, then a region of the absorption spectrum of the compound can be charted with each addition of peroxide. For shorter-lived intermediates, the spectra are synthesized from the stationary values of the kinetic curves obtained at a discrete set of specified wavelengths. Both time courses and rapid scans were used to obtain the various data reported in this paper.

The optimal conditions (peroxide-to-heme ratio and pH) for the maximization of the steady-state concentration of a particular compound differ among the hemoproteins and, therefore, had to be found. In each case the time interval of the steady state was determined from the behavior of the Soret absorbance. For BMC

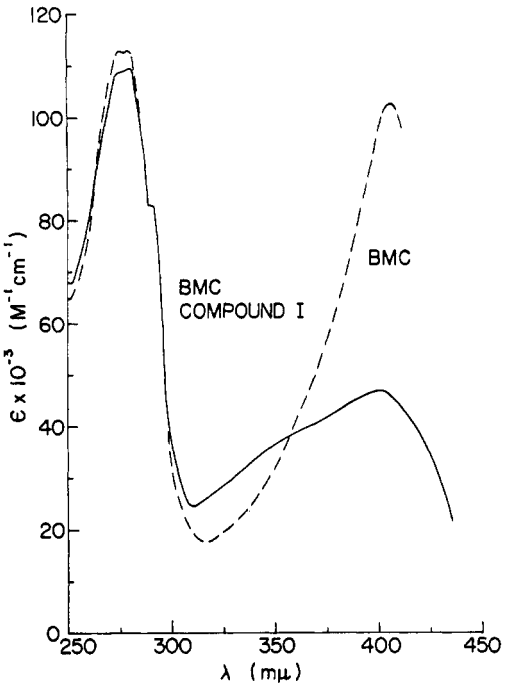

FIGURE 1: Absorption spectra of free BMC and BMC compound I (250–450 mμ). The dashed curve is the spectrum of free BMC. The solid curve is the spectrum (from absorbance values recorded at the time of maximum formation) of BMC compound I. At pH 7, the maximum formation of compound I (as determined by $A_{656\ m\mu}$) from 7.3 μM (heme) BMC is achieved at the ratio [ethyl hydroperoxide]/[heme] = 117 and occurs 40 sec after the addition of ethyl hydroperoxide.

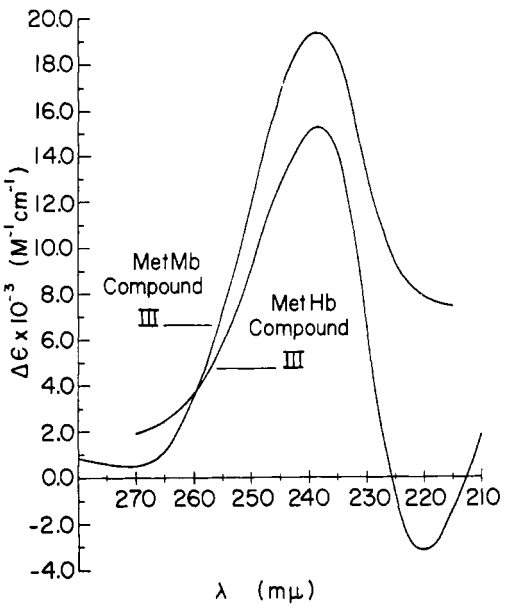

FIGURE 2: Difference spectra of MetHb and MetMb compounds III (210–280 mμ). The optimum formation of MetHb compound III (as determined by $A_{424\ m\mu}$) occurred at pH 7.4. The maximum absorbance at 424 mμ and the maximum difference absorbance at 238 mμ were observed 9 min after 10 μl of 4.3 mM H_2O_2 was mixed with 2.0 ml of 4.0 μM (heme) MetHb. The observed difference spectrum was corrected for the dilution attendant the H_2O_2 addition. Between pH 8 and 9, MetMb compound III is formed with minimal side reactions and is stable (George and Irvine, 1952). The maximum absorbance at 426 mμ was observed 45 min after 20 μl of 2.2 mM H_2O_2 was mixed with 2.0 ml of 4.9 μM MetMb. Before recording the difference spectrum, 20 μl of buffer was added to the 2.0 ml of MetMb solution in the reference cell to balance the dilution of the sample.

and HRP, the spectrum was repeatedly recorded over the wavelength region which includes the Soret peaks of the free hemoprotein and compounds I and II, the elapsed time from the initiation of the reaction being noted. For MetHb and MetMb, the time for the maximum formation of compound III was determined from a time course at the wavelength in the Soret region where there is a maximum difference in absorbance between free hemoprotein and the compound.

The concentration of ethyl hydroperoxide which is needed for maximal formation of BMC I exceeds 100 times the heme concentration. This concentration of ethyl hydroperoxide produces an absorbance which cannot be neglected but which can be accounted for quantitatively. (We assume here that only one molecule of ethyl hydroperoxide is ultimately bound to the hemoprotein, and that very few are used up in reaction with endogenous donor.) Similarly, the concentration of ethyl hydroperoxide which is needed for maximal formation of HRP I is about 40 times the heme concentration, cannot be neglected, but can be corrected for. For compounds III of MetHb and MetMb, only a five to ten times excess of peroxide is required, the absorbance of which can be ignored. Thus the difference spectra of all the compounds were recorded with the usual single-cell configuration rather than the tandem cell arrangement (Brill and Sandberg, 1968).

The following conditions were common to all measurements, unless otherwise stated. The temperature of the sample and reference cell compartments was thermostated at 25 ± 1°. All solutions were equilibrated at room temperature before use. A slow scan rate of 5 Å/sec was employed to ensure an averaging of fluctuations due to the rapid changes in the Servo-controlled slit width. The absorbance range (slide wire 1480360) was 0–1.0. The sample and reference beam cells were each of 1-cm path lengths and received 2.0 ml of the hemoprotein solution.

Results

In order to extend the work of Brill and Williams (1961), the absolute spectrum of BMC compound I was recorded in the ultraviolet region under conditions indicated in the legend of Figure 1. From the absorbances at 656 mμ for a sequence of increasing ethyl hydroperoxide/heme ratios, the concentration of I formed under the conditions of the experiment was determined to be greater than 95% of the limiting value, and hence the conversion of all of the BMC into compound I is approximated (Brill and Williams, 1961; Sandberg, 1967).

MetMb Compound III. The difference spectrum was recorded during the time interval of maximum (in this case, full) formation indicated by the kinetic course followed at 426 mμ. The Soret absorptivity, $\epsilon_{422\ m\mu}$

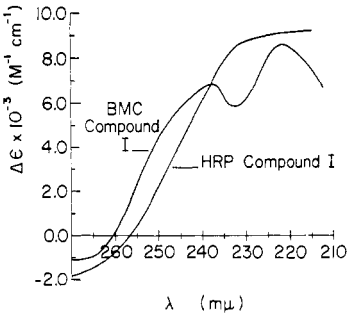

FIGURE 3: Difference spectra of BMC and HRP compounds I (210–270 mμ). In order to record the difference spectrum of BMC compound I, 2.0 ml of 1.5 μM (heme) BMC (pH 6.8) was pipetted into both sample and reference cells. The maximum formation of compound I occurred between 100 and 300 sec after 20 μl of 20 mM ethyl hydroperoxide was mixed in the sample cell. The observed difference spectrum was corrected for the absorbance of ethyl hydroperoxide and the dilution in the sample cell. In order to record the difference spectrum of HRP compound I, 2.0 ml of 4.8 μM HRP (pH 6.8) was pipetted into both sample and reference cells. The maximum formation of compound I occurred between 30 and 100 sec after 20 μl of 8.4 mM ethyl hydroperoxide was mixed in the sample cell. The difference spectrum was synthesized from absorbance values recorded at the optimum time and was corrected for the absorbance of ethyl hydroperoxide and for the dilution.

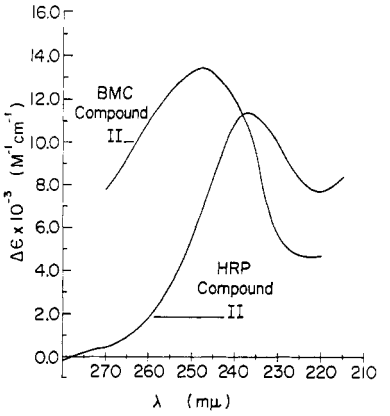

FIGURE 4: Difference spectra of BMC and HRP compounds II (210–280 mμ). In order to record the difference spectrum of BMC compound II, 2.0 ml of 3.7 μM (heme) BMC (pH 6.5) was pipetted into both sample and reference cells. The maximum formation of compound II occurred 1 hr after 20 μl of 37 mM ethyl hydroperoxide was mixed in the sample cell. The observed difference spectrum was corrected for the absorbance of ethyl hydroperoxide and for the dilution. In order to record the difference spectrum of HRP compound II, 2.0 ml of 4.7 μM HRP (pH 8.4) was pipetted into both sample and reference cells. The maximum formation of compound II occurred between 17 and 27 min after 20 μl of 37 mM ethyl hydroperoxide was mixed in the sample cell. The observed difference spectrum was corrected for the absorbance of ethyl hydroperoxide and for the dilution.

117 mM^{-1} cm^{-1}, of compound III agrees with earlier data (George and Irvine, 1952) when the latter is normalized to the value of Soret absorptivity of free MetMb used in this paper. The difference spectrum of compound III produced with H_2O_2 is identical with that produced with ethyl hydroperoxide. A typical difference spectrum is shown in Figure 2. The average difference absorptivity at 238 mμ is 19.3 ± 0.4 mM^{-1} cm^{-1}.

MetHb Compound III. The difference spectrum was recorded during the time interval of maximum formation indicated by the time course of the reaction with H_2O_2 followed at 424 mμ. However, the absorption spectrum in the Soret region, recorded at optimum time and pH, is a broad band. The broadness suggests a mixture of free MetHb and III. The data of Dalziel and O'Brien (1954) for *human* MetHb III is not directly applicable to horse MetHb, and we are unable at the present time to compute the fraction of III formation. A typical difference spectrum is shown in Figure 2. The average difference absorptivity at 238 mμ is 15.1 (+1.5, − 0.9) mM^{-1} cm^{-1} referred to total heme.

BMC Compound I. A typical difference spectrum is shown in Figure 3 where compound I formation is in excess of 90%. The average difference absorptivity at 240 mμ is 6.8 (+0.4, −0.2) mM^{-1} cm^{-1}.

HRP Compound I. Compared with pH 4.4 and 8.5, at pH 6.8 the lowest absorbance at the shortest wavelength peak is found for HRP compound I. At pH 6.8 the absorptivity of the peak (401 mμ) is 49 mM^{-1} cm^{-1}, somewhat lower than values in the literature (Chance, 1952b). Hence the experimental conditions for the difference spectrum of Figure 3 achieve a greater extent of compound I formation than previously obtained. The maximum formation occurs at about 30 sec after initiation of the reaction and ends at about 100 sec an interval of 1 min. The formation of compound II can be noted during the latter half of this time interval. Because of the limited time and slow scanning rate, the difference spectrum was synthesized by the repeated scan technique in four overlapping subsections of the wavelength region 210–270 mμ. Absorbance values at the time of optimum formation were used. A single first scan of the same region yields a record essentially identical with the composite difference spectrum. The average difference absorptivity at 230 mμ is 9.0 ± 0.2 mM^{-1} cm^{-1}.

BMC Compound II. A typical difference spectrum under conditions where compound II is fully formed (according to the absorptivity at 428 mμ (Maehly, 1961)) is shown in Figure 4. The average difference absorptivity at 247.5 mμ is 12.6 (+1.1, −1.7) mM^{-1} cm^{-1}.

HRP Compound II. In the absence of a hydrogen donor (*e.g.*, *p*-cresol) full formation of HRP compound II cannot be achieved (George, 1953b). Since it is impossible to correct accurately the ultraviolet difference spectra for the changing absorbance of reacting hydrogen donor, and since the concentration of II rapidly decreases in the presence of such a reductant, hydrogen donor cannot be used in these experiments to induce full formation. On the basis of parallel measurements in the Soret region, with *p*-cresol present and absent, we estimate the fraction of full formation to be 83% in the typical difference spectrum of Figure 4. The average difference absorptivity at 237.5 mμ is 11.6 (+0.1, −0.2) mM^{-1} cm^{-1} referred to total heme.

TABLE I: Wavelength and Absorptivity of Absorption Maxima[a] in the Visible and Near-Ultraviolet Spectra, and Magnetic Moments of Hemoprotein Compounds.

Hemoprotein	Absorption Bands: λ (mμ), ε (mM^{-1} cm^{-1}) CT1	α	β	CT2	Soret	Magnetic Moment (Bohr Magnetons)
Free Hemoprotein						
BMC (pH 7.0)[b]	627.5		537	504	406	5.7 (HBC)[c]
	7.0		9.7	11.1	102.(7)	
HRP (pH 5.6)[d]	641	(~580)	(~530)	497	403	5.4
	2.8			10.0	91	
MetMb (pH 6.4)[e]	630	(580)	(550)	502	408	5.8
	3.9			10.2	188	
MetHb (pH 6.4)[f]	631	(575)	(540)	500	404.5	5.7
	4.4			10	179	
Compound I						
BMC[g]	662	(583)			398	3.7
	9.4				44	
HRP[h]	650	570			400	4.0
	5.5	6.7			52	
Compound II						
BMC[i]		568	530		428	3.0 (HBC)[j]
		18	12.5		77	
HRP[h]		555	527		418	3.6
		9	9		98	
Compound III						
MetMb[k]		(580)	550		423	2.9
			10.9		118	
MetHb[l] (human)		(575)	545		418	No data
			10.5		105	

[a] Shoulders in parentheses. [b] Brill and Sandberg (1968). [c] Deutsch and Ehrenberg (1952). [d] Keilin and Hartree (1951); Hartree (1946); Theorell and Ehrenberg (1952). [e] Scheler *et al.* (1957); Theorell and Ehrenberg (1951); Beetlestone and George (1964); Taylor (1939). [f] Scheler *et al.* (1957); Havemann and Haberditzl (1958). [g] Brill and Williams (1961). [h] Values based upon $\epsilon_{403\ m\mu}$ 91 mM^{-1} cm^{-1} for free HRP; Chance (1952c); Theorell and Ehrenberg (1952). [i] Maehly (1961). [j] Theorell and Ehrenberg (1952). [k] Values based upon $\epsilon_{408\ m\mu}$ 188 mM^{-1} cm^{-1} for free MetMb; George and Irvine (1952); Brill *et al.* (1960); Theorell and Ehrenberg (1952). [l] Dalziel and O'Brien (1954).

Discussion

Porphyrins have characteristic absorption bands in wavelength regions which have been experimentally accessible for some time, the Soret (390–430 mμ) and the visible between 500 and 700 mμ. Much valuable information has been obtained from studies of the influence upon these bands of chemical changes at position 6 of the coordination sphere of the iron in hemoproteins (positions 1–4 occupied by the pyrrole nitrogens of protoporphyrin and position 5, by a ligand atom from the protein). However, because the orbitals involved are predominantly porphyrin, the Soret and visible transitions reflect only indirectly the electronic interaction between the metal ion and the protein ligand. In the region below 250 mμ, where protein groups which are potential ligands have characteristic bands, there are experimental difficulties. These have been discussed in detail elsewhere (Brill and Sandberg, 1968), and the feasibility of observing the spectral behavior of such groups has been demonstrated. In the interpretation of the difference spectra obtained in these studies, we have assumed as a first approximation that the changes in absorption by the amino acid residues other than the one involved in metal binding are negligible, and that changes in peptide-bond absorption are also of secondary importance. This assumption is supported by the appearance of spectral features which can be correlated with independently measured magnetic and optical differences. Confirmation of the assignments given in this series of papers depends upon additional observations of the same spectral effects in model systems, and upon observations of spectral variations in hemoprotein deriva-

tives (*e.g.*, ferrous hemoproteins) which are predicted from these assignments.

Table I summarizes the salient features of the visible and Soret absorption spectra of hemoprotein compounds from previous studies. The bands of compounds I of BMC and HRP peak at about the same wavelength, but differ in strength. This correspondence has been expressed in terms of composite spectra, one component (POR) having normal Soret intensity and a weak band in the red, and the other component (ROX) having a converse relation for these intensities (Brill and Williams, 1961). In this picture BMC I is about one-third POR and two-thirds ROX, and HRP I is about two-thirds POR and one-third ROX. The reduction in the absorptivity of the Soret band of BMC compound I is shown in Figure 1. Compounds I of other catalases and peroxidases would have other proportions of POR and ROX, there being no apparent reason to correlate this feature with catalytic differences. Compounds II of BMC and HRP also have spectroscopic similarities, but in the catalase compound, the bands are more toward the red. The peroxide compounds of MetMb and MetHb have been labeled "III" because of their spectral resemblance to compounds III of catalase and peroxidase.

In Table I the magnetic moments are also given. The moments of compounds I of BMC and HRP are very close to that corresponding to the "spin-only" value for three unpaired electrons. Several possibilities exist for this unusual moment, most of them involving some degree of covalency (states of low spin): (1) A mixture of low- and high-spin ferric states could exhibit any intermediate magnetic susceptibility; (2) a low-spin ferric compound plus a biradical in the porphyrin or protein would contribute susceptibilities which could sum to the appropriate value; (3) a low-spin quadrivalent iron compound plus a free radical would be as in 2; (4) pentavalent iron would most likely have a spin of $^3/_2$; (5) a ferric compound with lower and upper orbital singlets and an orbital triplet of intermediate energy would also have a spin of $^3/_2$.

The moments of compounds II of BMC and HRP approximate the "spin-only" value for two unpaired electrons. When the observed magnetic susceptibility of HRP II (Theorell and Ehrenberg, 1952) is corrected for incomplete (80%) formation in the absence of a hydrogen donor (George, 1953b), the magnetic moments of HRP II and HBC II agree within experimental error. All available data support the view that these are ferryl compounds containing low-spin quadrivalent iron (George, 1953b). Compound III of MetMb is magnetically like the latter compounds, and all compounds III are generally believed to have low-spin iron.

Since MetMb and MetHb compounds III are low spin, the "imidazole" difference bands described in the preceding papers (Brill and Sandberg, 1967, 1968) are expected to appear and do so. The difference spectra of the low-spin cyanide and azide complexes (of ferric hemoproteins) also show difference bands which were assigned to charge transfer transitions involving porphyrin (pyrrole) orbitals and the ligand in the sixth coordination position. Because of the more positive charge on the metal, a charge transfer transition from ligand to iron would be expected to shift to a longer wavelength in ferryl compounds. We interpret the spectra of Figure 2 as having a porphyrin to metal component (shifted from about 225 mμ in low-spin ferric to about 235 mμ in ferryl) and an imidazole to metal component (shifted from about 235 mμ to about 245 mμ). There is no component involving the ferryl oxygen corresponding to the band at 213 mμ involving cyanide or the one at 280 mμ involving azide in the MetMb and MetHb complexes. The difference spectrum of MetHb III has a trough at 220 mμ which is indicative of the absence in this compound of a transition which is present in free MetHb. Histidine in aqueous solution (Sussman and Gratzer, cited in Wetlaufer, 1962) and hemin in methanol (A. S. Brill and P. A. Turley, 1968, unpublished data) have absorption bands at 221 and 220 mμ, respectively. Both of these transitions could contribute to this 220-mμ band in free MetHb.

The difference spectrum of BMC compound I has two difference peaks, at 238 mμ (the "imidazole" band) and at 222 mμ, while the difference spectrum of HRP compound I has one very broad difference band. We suggest that the latter is the sum of two difference bands of which the long-wavelength band ("imidazole") is located at about 230–235 mμ, and the short one at about 220 mμ. The spectra of Figure 3 are similar to those of ferric hemoprotein complexes with low-spin character (Brill and Sandberg, 1968), except that the charge transfer band associated with cyanide or azide is not (and would not be expected to be) present. The intensities of the "imidazole bands" in compounds I are low, indicating that all of the iron is not in the low-spin state, in agreement with interpretation 1 of the magnetic susceptibility measurements taken at room temperatures. The absorptivity (6.8 mM^{-1} cm^{-1}) of BMC compound I at 238 mμ is about 0.6 of the absorptivity (12.0 mM^{-1} cm^{-1}) of BMC–CN at 236 mμ, a ratio much the same as those found for bands in the Soret and visible regions which led to the proposal that compound I of BMC is about two-thirds low spin (Brill and Williams, 1961).

The magnetic moments of compounds II of BMC and HRP require that these be essentially of low spin regardless of the oxidation state of the iron. The appearance of the positive difference absorption in the spectra of Figure 4 is therefore expected. The high intensities of these bands and the absence of bands below 230 mμ are properties shared by the spectra of compounds III of MetMb and MetHb (Figure 2) and we interpret all four spectra in the same way. These data support the picture of compounds II and III as ferryl structures in which there is a strong covalent bond between the metal and a histidine residue. Some structural differences are present among these compounds since neither the ultraviolet difference spectra nor the absolute visible spectra are identical.

The ultraviolet difference spectrum of cytochrome c peroxidase complex ES has bands at 225 and 241 mμ (Yonetani *et al.*, 1966), similar to the spectra of compounds I of HRP and, especially, BMC. Complex ES also contains two oxidizing equivalents above free

enzyme and about the same magnetic moment as compounds I (Yonetani and Schleyer, 1966). In spite of the facts that the Soret and visible bands of complex ES are not typical of compounds I and complex ES shows an electron spin resonance absorption centered at $g = 2.0$, we suggest, on the basis of the ultraviolet difference spectrum, that it is a ferric compound in the low-spin state. The differences between complex ES and compounds I of BMC and HRP are explained on the basis of the location of the oxidizing equivalents. In complex ES the porphyrin ring does not suffer an oxidative attack.

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