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ON THE FUNCTION AND MECHANISM OF ACTION OF PEROXIDASES

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CONTENTS

A. INTRODUCTION

One of the requirements for life is the maintenance of molecules in a reduced state even though they are exposed to an oxidizing atmosphere. Another requirement is the generation of energy, commonly accomplished by respiration, in which an essential step is the reduction of oxygen to water

$$O_2 + 4\,H^+ + 4\,e \rightarrow 2\,H_2O$$

This reaction can be catalyzed by a single enzyme, cytochrome oxidase [1]. However, the partial reduction of oxygen is also a widely occurring biological process. Among the inorganic compounds of oxygen in oxidation states intermediate between those of oxygen and water are the superoxide anion, O_2^-, and hydrogen peroxide. These highly reactive species are a threat to life, but they can be removed efficiently by the enzymes superoxide dismutase, catalase and peroxidase. All of the enzymes mentioned above contain iron and/or copper, for which an interesting summary of their possible biochemical evolution has been given [2]. The peroxidases not only remove hydrogen peroxide, but utilize its oxidizing ability in a variety of interesting ways.

Peroxidases are generally hemoproteins and it is only those peroxidases which contain a heme iron group which are discussed in detail here. Hemoproteins range from cytochrome *c* to hemoglobin and myoglobin to peroxidase and catalase. One of the fascinating problems in both chemistry and biology is to decipher how subtle changes in going from one molecule to a closely related one lead to such drastic changes in behavior. One step in solving such a problem is to decipher in detail at the molecular level the structure, function and behavior of each type of molecule. It is the purpose of this review to give a progress report on the function and mechanism of action of peroxidases. After an introductory survey including some related enzymes, details of work on model peroxidase systems and peroxidases themselves will be given. It was found convenient to summarize the literature both on the basis of chemical and physical techniques and resul[4] on individual peroxidases.

(i) Peroxidases

Peroxidases are enzymes catalyzing the oxidation of a variety of organic and inorganic compounds by hydrogen peroxide or related compounds. An extensive list of the sources of peroxidase in plants and animals has been given [3]. All the peroxidases purified so far from plants contain the prosthetic group hemin or ferriprotoporphyrin IX (Fig. 1). Horseradish roots and the sap of fig trees are the richest source of plant peroxidases.

Peroxidases from animal sources which have been studied are thyroid peroxidase, lactoperoxidase, myeloperoxidase and glutathione peroxidase. Lactoperoxidase and myeloperoxidase are known as verdoperoxidases because of their green colour. Neither contain protoporphyrin IX as prosthetic group.

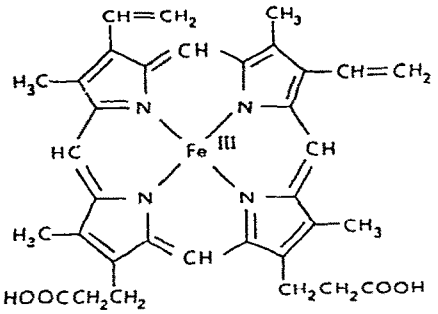


Fig. 1. Ferriprotoporphyrin IX or hemin, also called protoferriheme (1,3,5,8-tetramethyl-2,4-divinylporphine-6,6-dipropionic acid). If the two vinyl side chains are missing the molecule is called ferrideuteroporphyrin IX or deuteroferriheme. If the vinyl side chains are replaced by CH_3CHOH groups, ferrihematoporphyrin IX is obtained.

It has been suggested that lactoperoxidase contains a prosthetic group which is a derivative of mesoheme IX in which two double bonds are in conjugation with the tetrapyrrole nucleus and one or more hydroxyl groups are attached to the side chains [4]. Myeloperoxidase has been found to contain two atoms of porphyrin-bound iron per enzyme molecule [5,6]. The heme may be contained in a formyl type porphyrin and the two heme environments may be different [7]. Glutathione peroxidase is unique among known peroxidases in that it contains one atom of selenium per sub-unit of enzyme [8—10]. Thyroid peroxidase which has been isolated in relatively pure form exhibits spectral characteristics typical of hemoproteins. However, because the spectral details are somewhat different from horseradish peroxidase, it has been suggested [11] that the prosthetic group is more tightly bound or, possibly, is not ferriprotoporphyrin IX. Since the heme can be extracted with 0.2 M hydrochloric acid it is not covalently bound to the protein moiety.

In addition to the plant and animal sources, peroxidases are also found in mold, bacteria and microorganisms. A peroxidase from the mold *Caldariomyces fumago*, chloroperoxidase, has been isolated and characterized. Like the plant peroxidases it has ferriprotoporphyrin IX as the prosthetic group. In many of its chemical and physical properties chloroperoxidase is similar to horseradish peroxidase but it has the unique ability among peroxidases to catalyze the oxidation of chloride ions [12,13]. Cytochrome *c* peroxidase which is obtained from aerobically grown baker's yeast also has ferriprotoporphyrin as a prosthetic group [14,15]. Cytochrome *c* peroxidase catalyzes most typical peroxidase reactions. However its specific activity towards ferrocytochrome *c* is exceedingly high compared to other reducing agents. Although other peroxidases including horseradish peroxidase have been

shown to oxidize ferrocytochrome *c* to ferricytochrome *c* [16,17] they are much less efficient catalysts than cytochrome *c* peroxidase. A cytochrome *c* peroxidase has also been isolated from *Pseudomonas aeruginosa* [18—21]. A flavoprotein with peroxidase activity was isolated from the bacterium *Streptococcus faeolis*. Known as either streptococcus peroxidase or NADH peroxidase (formerly DPNH peroxidase) the enzyme has a relatively high molecular weight when compared with other peroxidases and it contains flavin adenine dinucleotide (FAD) as prosthetic group with no heme or metal ion. Unlike hemoprotein peroxidases it shows a high degree of substrate specificity with nicotinamide adenine dinucleotide (NADH), the only known physiological reductant [22].

Most peroxidases are glycoproteins. (Exceptions are cytochrome *c* peroxidase and myeloperoxidase.) The purpose of the carbohydrate in these enzymes is uncertain. However, horseradish peroxidase has long been known to be a very stable enzyme, even under conditions of elevated temperature, and this property could be due in part to the carbohydrate associated with the enzyme. Table 1 contains a summary of some of the properties of the peroxidases as well as a shorthand notation for each peroxidase which will be used throughout this review.

Horseradish peroxidase (and urease) played an historically important role in the development of the modern concept of the nature of ar ›zyme and the role of metal ions as documented by a famed controversy in .ıe 1920's and 1930's [46,47]. The discovery of the species now known as compound II (HRP-II), formed as a result of the reaction of HRP with H_2O_2, was published in 1937 [48]. Later compound I (HRP-I) was discovered which is formed prior to HRP-II upon addition of H_2O_2 to HRP [49]. The spectra of HRP-I and HRP-II in the 400 mm (Soret) region were measured [50,51] and the kinetics of some HRP reactions were investigated [52,53]. The rate of formation of HRP-I was also measured and demonstrated to be overall second order, first order in H_2O_2 and first order in HRP [52]. The rate of formation of HRP-I when CH_3OOH is substituted for H_2O_2 was also measured [53]. (The —OOH group is essential for the oxidation of HRP by a peroxide.)

It was also demonstrated that HRP-II participates directly in enzymatic reactions, which stands as the first direct proof of the participation of an enzyme compound in a reaction [53,54]. However, the data were interpreted in terms of a modified Michaelis—Menten reaction scheme, despite proof that HRP-II has an immeasurably small dissociation constant [53]. Work on all peroxidases has been hampered by the spontaneous decay of both compound I and compound II, particularly the former, a problem which can be circumvented today only by careful work on pure enzyme samples.

Stoichiometric investigations showed that HRP-II contains only one of the two oxidizing equivalents of H_2O_2 [55,56]. Based partly upon the fact that the HRP-II could not contain OOH^- or the entire H_2O_2 molecule, HRP-II was described as a covalent compound as distinct from a Michaelis—Menten enzyme—substrate complex [56]. Also, since HRP-I decay to HRP-II was

TABLE 1

Some properties of the peroxidases

	M. Wt.	Prosthetic group	Carbohydrate content (%)	Crystallization	Refs.
Horseradish peroxidase (HRP)	40,500 [a] 39,800 [b]	Ferriprotoporphyrin IX	18.4 18	microscopic needles	23–28
Cytochrome *c* peroxidase (CcP)	34,100 [b]	Ferriprotoporphyrin IX	0	long prisms	29–32
Chloroperoxidase (ClP)	40,200 [a] 42,000 [b]	Ferriprotoporphyrin IX	25–30	brown needles	12
Lactoperoxidase (LP)	76,500 [a] 77,500 [b]	Derivative of mesoheme IX?	8	needles	4,33,34
Thyroid peroxidase (ThP)	62,000 [c]	Not ferriprotoporphyrin IX?	—	—	11
Japanese radish peroxidase *a* (JRP*a*)	55,700 [f] 55,500 [d]	Ferriprotoporphyrin IX	28	rhombic prisms	35,36
Japanese radish peroxidase *c* (JRPc)	41,500 [a]	Ferriprotoporphyrin IX		tetragonal prisms	37
Myeloperoxidase (MP)	149,000 [b]	Two atoms of porphyrin bound iron	0	needles	5,6,38–40
NADH peroxidase	12,000 [c]	FAD	—	—	41
Turnip peroxidase (TuP) A_1 A_2 B D	49,000 [d] 45,000 [d] 65,000? [d] 43,000 [d]	Ferriprotoporphyrin IX	—	fine needles	42,43
Glutathione peroxidase	90,000 [d]	One atom of Se per sub-unit	—	—	9,10,44,45

[a] Heme content. [b] Hydrodynamic measurement. [c] Gel filtration. [d] Chemical analysis. [e] FAD content. [f] Osmotic pressure.

accelerated by all substrates capable of reducing HRP-II [53] it was postulated * that the enzymatic cycle is [57]

$$HRP + H_2O_2 \rightarrow HRP\text{-}I \quad (1)$$

$$HRP\text{-}I + AH_2 \rightarrow HRP\text{-}II + \cdot AH \quad (2)$$

$$HRP\text{-}II + AH_2 \rightarrow HRP + \cdot AH \quad (3)$$

It was proved (using CH_3OOH) that the reaction analogous to eqn. (1) occurs with a 1 : 1 stoichiometry and that one mole of one electron reducing agent per mole of HRP-I is required for eqn. (2), in agreement with the above mechanism [58]. Fairly stable preparations of HRP-I were obtained by preincubating the enzyme sample with CH_3OOH, which removed most of the reducing substrate contaminant and hence rate constants for reaction (2) could be measured [59]. Spectral measurements on HRP-I and HRP-II were extended into the visible region [60]. Subsequent papers confirmed the essential correctness of the above mechanism [61,62] and in the case of cytochrome *c* peroxidase showed that compound II has an identical spectrum whether it is formed from H_2O_2 or CH_3OOH [62].

It now appears accepted that the structures of both compound I and compound II are independent of the oxidizing (or reducing) agent used in their preparation and the spectra of either compound I or compound II are not merely similar [63,64] but identical [62] regardless of their method of preparation. This conclusion was not accepted immediately because of the problem of spontaneous decay of the intermediate compounds. (In one or two cases the possibility exists that H_2O_2 is first generated by another oxidizing agent [65,66], but this does not detract from the general applicability of the above statement.)

Kinetic studies led to the conclusion that all of the reactions (1—3) are independent of pH [67,68] but details of the experimental evidence were not presented.

The above summary gives some of the highlights of work on HRP up until 20 years ago, which has had a profound influence on subsequent work on peroxidases. Further details including the limited amount of work on lactoperoxidase and cytochrome *c* peroxidase up to that time are described elsewhere [3,69,70].

(ii) Catalases

Catalases and peroxidases are related enzymes in so far as they are both capable of promoting hydrogen peroxide oxidation by mechanisms which involve similar enzymatic intermediates. The peroxidatic activity of catalases

* The reducing substrate AH_2 in the cycle may be replaced by a one electron donor such as ferrocyanide in eqns. (2) and (3). Thus the older term hydrogen donor is at times inaccurate.

is however inefficient when compared with true peroxidases. The *catalatic* activity of catalases is the highly efficient catalysis of the decomposition of hydrogen peroxide to oxygen and water [71]

$$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$$

Catalase is widely distributed among animals, plants and bacteria. All the catalases isolated so far have similar molecular weight (~25,000) and contain four iron atoms per molecule. The iron is present in four protoporphyrin IX prosthetic groups per molecule in all but the liver catalases where one or more prosthetic groups have been degraded to bile pigment hematins. It has been shown that the ratio of protohematin to bile pigment varies only within narrow limits and that the catalase activity decreases with increased bile pigment hematin content.

(iii) Superoxide dismutase

An enzyme which catalyzed the disproportionation of superoxide free radical anions

$$O_2^{\cdot -} + O_2^{\cdot -} + 2\ H^+ \rightarrow O_2 + H_2O_2$$

was discovered in 1968 and named superoxide dismutase [72]. It has since been found to exist among oxygen-metabolizing organisms but not among anaerobes [73]. It is proposed that superoxide dismutase constitutes an important component of the defenses which have evolved to deal with the toxicity of O_2 [73,74]. Several different kinds of superoxide dismutase have been described so far. The first contains copper and zinc ions and has been isolated from a wide variety of eukarytes including yeast, wheat germ, garden peas, chicken liver and erythrocytes. Proteins containing copper ions which had been isolated from blood, brain and liver tissues many years previously and which were known as *erythrocuprein*, *cerebrocuprein* and *hepatocuprein* were also shown to possess this catalytic property [75].

It has recently been demonstrated that the superoxide anion and more particularly the HO_2 radical are removed with great efficiency by HRP-I [76]. Thus

$$\text{HRP-I} + HO_2 \xrightarrow{k} \text{HRP-II} + O_2 + H^+$$

with $k = 2.2 \times 10^8\ M^{-1}\ s^{-1}$.

B. MODEL SYSTEMS

Perhaps the most relevant model systems for peroxidases are aqueous ferric ion, ferriprotoporphyrin IX and related compounds.

(i) Ferric ion

Ferric complexes polymerize in aqueous solution to form dimeric oxo- and dihydroxo-bridged structures. In the case of the ferric ion, the predomi-

TABLE 2

Stability constants for Fe^{3+}

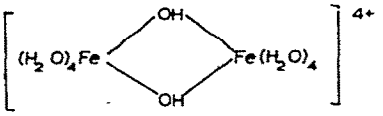
		Ref. 77	Ref. 78	Ref. 79
$2\ Fe^{3+} + 2\ H_2O = Fe_2(OH)_2^{4+} + 2\ H^+$	$\log \beta_{22}$	−2.91	−2.58	−2.96
$Fe^{3+} + H_2O = FeOH^{2+} + H^+$	$\log \beta_{11}$	−3.05	−2.89	−3.05
$Fe^{3+} + 2\ H_2O = Fe(OH)_2^+ + 2\ H^+$	$\log \beta_{21}$	−6.31	—	−6.31
$3\ Fe^{3+} + 4\ H_2O = Fe_3(OH)_4^{5+} + 4\ H^+$	$\log \beta_{43}$	—	—	−5.77
		3 M $NaClO_4$ 25°C titration	3 M $NaClO_4$ 25°C spectrophotometric	

$Fe^{3+} + 3\ OH^- = Fe(OH)_3$, $K_{sp} = 10^{-38.7}\ M^4$ at 25°C 3 M $NaClO_4$ [80]

nant hydrolysis products at moderate concentrations are polynuclear. The stability constants are summarized in Table 2.

The electric field jump method has been used to study the kinetics of the hydrolysis of ferric ion [81]. Kinetic evidence for dimer formation and high molecular weight polymer formation in solutions containing ferric ion has been reviewed [82].

Aqueous solutions of Fe^{III} nitrate, chloride and sulphate have been shown to have magnetic moments lower than that expected for the spin only value of 5.92 Bohr magnetons (BM) for $S = \frac{5}{2}$ systems [83,84]. Magnetic moments in the range expected for $S = \frac{5}{2}$ were obtained by the addition of the corresponding acids. When the pH of an aqueous solution of $Fe(ClO_4)_3$ was increased the magnetic susceptibility was found to decrease. This was attributed to the formation of a dimer with the probable structure [85]



although the magnetic properties of the aquo dimer have not yet been resolved [86,87]. A number of salts of ferric ion react with chelating agents to form dimers, and pK_a values and equilibrium constants for dimerization have been obtained [88,89].

The kinetics of "simple" ligand binding reactions to ferric ion have been well studied [90—95]. These reactions typically proceed by at least two paths, one acid-independent and the other acid-dependent [96]. The acid-independent path may be formulated in two ways. Either the hexaquo ferric ion forms a complex with the anion

$$(H_2O)_5FeH_2O^{3+} + X^- \rightleftharpoons [(H_2O)_5FeOH{-}HX]^{2+} \rightleftharpoons (H_2O)_5FeX^{2+} + H_2O$$

which then hydrolyzes internally prior to ligand exchange [94,97] or the reaction occurs directly between the species

$$(H_2O)_5FeOH^{2+} + HX \rightleftharpoons (H_2O)_4XFeOH^{+} + H^{+}$$

$$(H_2O)_4XFeOH^{+} + H^{+} \rightleftharpoons (H_2O)_5FeX^{2+}$$

Both of these processes are consistent with the experimentally determined rate constant, k_{obsd}, obtained by measuring the rates of changes of $[Fe]_{tot}$ and $[X]_{tot}$, the total concentrations of ferric ion and ligand, regardless of their states of ionization. However when one translates k_{obsd} into rate constants for the first mechanism, these rate constants vary widely for different ligands. This has been explained as being caused by different degrees of hydrolysis within the complex; the greater the basicity of the entering ligand, the greater the extent of hydrolysis [94,97]. On the other hand, if one interprets k_{obsd} in terms of the second mechanism, the rate constants which are obtained are to a large extent independent of the entering ligand, which could favor the latter mechanism [96]. The rate constant for water exchange in the inner coordination sphere of Fe^{3+} is approximately 3×10^2 M^{-1} s^{-1} and for the species $FeOH^{2+}$ one might expect a considerable enhancement in this rate [96]. However, an accurate measurement of the solvent exchange rate in aqueous solutions is a difficult problem because of aging the ferric ion solutions during the time required for the NMR experiments. Despite work in several laboratories, no newer estimates of this rate have been published *. Most of the rate constants for ligand substitution on ferric ion, according to the overall process

$$FeOH^{2+} + HL \rightarrow FeL^{2+} + H_2O$$

are of the order of 1×10^6 M^{-1} s^{-1} [96] which is consistent with the concept that the rate-controlling step is loss of a solvent molecule from $FeOH^{2+}$, according to the crude state of knowledge of the water exchange rate available today. The reciprocal of the average lifetimes of solvent molecules in the inner coordination sphere of ferric ion have been measured as 61 and 28 s^{-1} in dimethyl formamide [98] and 4.4×10^3 s^{-1} in methanol [99].

The reaction of ferric ion with hydrogen peroxide has been used as a model for catalase activity. It was demonstrated by using O^{18} labelling that both oxygen atoms in the evolved oxygen molecule originate from the same hydrogen peroxide molecule [100]. The inhibiting effect on the catalytic decomposition of H_2O_2 by ions capable of entering the inner coordination sphere of the ferric ion has been investigated [101]. Kinetic and spectrophotometric studies led to the mechanism

$$Fe^{3+} + HO_2^{-} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} Fe^{3+}HO_2^{-}$$

* R.B. Jordan, personal communication.

$$Fe^{3+}HO_2^- \xrightarrow{k_3} FeO^{3+} + OH^-$$

$$FeO_3^+ + H_2O_2 \xrightarrow{k_4} Fe^{3+} + O_2 + H_2O$$

It was concluded that the latter reaction occurred by rearrangement within a single complex and that the role of free radicals was not important [102—105]. However, an older scheme [106] involving a redox radical chain has been revived

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + HO_2\cdot + H^+$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^- + HO\cdot$$

$$HO\cdot + H_2O_2 \rightarrow H_2O + HO_2\cdot$$

$$HO_2\cdot + Fe^{3+} \rightarrow H^+ + Fe^{2+} + O_2$$

$$HO_2\cdot + Fe^{2+} \rightarrow HO_2^- + Fe^{3+}$$

on the basis of the retarding effect of a number of organic substrates [107]. It was concluded that the chain length was considerably shorter than originally proposed but that it was highly significant. Furthermore, Fenton's reagent (ferrous ion and H_2O_2) has been revisited [108] and the hydroxyl radical chain reaction proposed earlier [109] has been postulated to be of importance in peroxidase reactions. However, the product distribution is entirely different in peroxidase reactions [3,70]. Furthermore, cyanide inhibition experiments show that neither HRP-I nor HRP-II are capable of binding cyanide (in marked contrast to the native enzyme) even though the enzyme is turning over. Cyanide binding would be expected if hydroxyl radicals were being generated and lost from the active site [110]. Native peroxidase solutions provide stable, monomeric solutions of ferric ion at physiological pH and their reaction pathways appear simpler than those of aqueous ferric or ferrous ion with H_2O_2 and reducing substrate.

(ii) Hemin and related compounds

The molecular and crystal structures of porphyrins [111] and various metalloporphyrins [112] have been determined by X-ray diffraction with a high degree of precision, greater than is possible when the metalloporphyrin is part of the crystal structure of a metalloprotein. The metalloporphyrins have a flexible structure, much diversified, depending on the particular environment in which they are found. This is in accord with their widely diversified biological roles. When the metal ion is zinc, magnesium or high spin iron(II) or (III), it assumes an out-of-plane position so that the metal ion and four pyrrole ring nitrogen atoms are in a square pyramidal (C_{4v}) configuration [112]. This greatly facilitates bonding with a ligand in the fifth coordination position, since interactions of the ligand with the pyrrole nitrogen atoms are minimized. However, it tends to prevent coordination of

a sixth ligand which would have stronger repulsive interactions with the pyrrole nitrogen atoms [112]. Furthermore, the electron density in the anti-bonding orbital tends to concentrate in the sixth coordination position as a "phantom" ligand, an effect greatly enhanced when ferric ion is replaced by ferrous [112]. The out-of-plane distance of a high spin ferric ion is 0.48 Å (when the axial ligand is methoxy) and it is estimated that this distance would be increased to ~0.70—0.80 Å for a ferrous ion [111—113].

The porphyrin ring system is highly aromatic, having a resonance energy of the order of 500 kcal mole^{-1} [114,115]. It may therefore be likened to a copper bar in its ability to conduct electrons. An intensive study of the electronic structure of metalloporphyrins has been conducted from the standpoints of spectral measurements and their theoretical interpretation [116]. (See ref. 117 for the first paper in this series and ref. 118 for one of the latest papers.) The technique of X-ray photoelectron spectroscopy has recently been applied to porphyrins [119,120].

Magnetic [121,122], kinetic [123], polarographic [124], ultracentrifuge [125], spectroscopic and complexation studies [123,126] all suggest that hemin is dimeric in aqueous alkaline solutions. The structure of dimeric hemin has not been established, but infrared evidence [127] suggests that it is an oxo-bridged compound as does the magnetic data [122]. The conditions for formation of the oxo-bridged compound are rather severe however, and this dimeric form may be different from that normally obtained in moderately basic equeous solution [123]. The dimerization constant and pK_a's for protoferriheme and deuteroferriheme in aqueous solution were obtained in a spectrophotometric study [128].

	Protoferriheme	Deuteroferriheme
$2M \overset{K}{\rightleftharpoons} D + H^+$	$K = 4.5$	$K = 1.9 \times 10^{-2}$
$M \overset{K_{a(M)}}{\rightleftharpoons} M' + H^+$	$pK_{a(M)}$ not accessible	$pK_{a(M)} = 7.1$
$D \overset{K_{a(D)}}{\rightleftharpoons} D' + H^+$	$pK_{a(D)} = 7.5$	$pK_{a(D)} = 7.4$

Ultracentrifugal and diffusion studies suggest that the solution contains aggregates of dimers interacting with each other very weakly [129]. The solution chemistry of hemin is further complicated by autooxidation when it is left in contact with the atmosphere over a period of several hours [129]. It has been suggested [127] that the autooxidation involves the vinyl side chain of the porphyrin ligands and this is supported by the lack of this effect with deuterohemin. The oxidation product is called hemin (A), the structure of which has not been determined. The pH dependence of the spectrum of hemin (A) has been studied

Hemin (A) $\rightleftharpoons$ Hematin (A) + H^+ $\quad pK = 8.25$ at 25°C, $\mu = 0.2$

Hemin (A) is also formed by the addition of near stoichiometric concentra-

tions of H_2O_2 [129,130] to freshly prepared hemin and by γ irradiation of hemin solutions [131].

One method of curtailing the dimer formation is to use a mixed solvent such as aqueous ethanol [132] or organic solvents such as dimethylformamide or methanol [98,133]. Another method is to modify the heme in such a way that more hydrophilic groups are attached to the periphery of the molecules [134].

Although high spin heme complexes may be five-coordinate in crystals or in the ferrous form of myoglobin [112,113], diimidazole [135] and dipyridine complexes [136,137] are formed with hemin in solution, indicating that the ferric ion has six coordination positions occupied.

Rate constants for solvent exchange rates with ferric ion in two solvents, and for various metalloporphyrins obtained from NMR measurements are summarized in Table 3. It can be seen that the effect of porphyrin ligand is to enhance greatly the rate of solvent exchange, which is a factor of $\sim 10^3$ faster in methanol and $\sim 10^5$ faster in DMF for the iron—porphyrins compared to hexasolvated Fe^{III}. This labilizing effect should enhance the rates of exchange of ligands other than solvent molecules which occur by either dissociative or ion-pair mechanisms, and such an enhancement of rate has been observed in the case of Co^{III} complexes which are inert in the absence of the porphyrin ligand [140]. Exchange rates of pyridine on ferro- and ferriprotoporphyrin IX in chloroform were recently measured [141].

The kinetics of imidazole binding to ferriprotoporphyrin IX have been studied in aqueous ethanol solution [135] and of imidazole binding to an ethylenediamine-substituted ferriprotoporphyrin IX in aqueous solution [142]. In both cases the binding rate of an imidazole molecule (or ion) was of the order of $10^6\ M^{-1}\ s^{-1}$. In the aqueous ethanol the first imidazole molecule to bind accelerated the binding of the second ligand [135], but for the ethylenediamine substituted porphyrin, the reverse effect was observed

TABLE 3

Rates of solvent exchange with various metal ions and metalloporphyrins at 25°C

Complex	Solvent	τ (s^{-1})	Ref.
$Fe(DMF)_6^{3+}$	DMF [a]	61, 28	98,99
$Fe(TPP)DMF^+$	DMF	5.4×10^6	98
$Fe(CH_3OH)_6^{3+}$	CH_3OH	4.4×10^3	138
$Fe(TPP)CH_3OH^+$	CH_3OH	3.0×10^6	98
$Fe(PPIX)C_2H_5OH$	$C_2H_5OH + H_2O$	3.6×10^6	139 [b]
$Mn(DMPP)DMF^+$	DMF	1.3×10^8	133 [b]
$Mn(DMPP)CH_3OH$	CH_3OH	6.0×10^7	133 [b]

[a] Abbreviations: DMF, dimethylformamide; TPP, tetraphenylporphine; PPIX, protoporphyrin IX; DMPP, protoporphyrin IX dimethylester.
[b] Recalculated from the original reference using a solvation number of one.

[142]. In the binding of cyanide and thiocyanate to Co^{III} and Fe^{III} hematoporphyrin it was assumed on the basis of the simple form of the observed kinetics that the first anion to bind accelerated the binding of a second anion [140], but results obtained using ferriprotoporphyrin IX in aqueous ethanol showed that one cyanide ligand slowed the rate of further substitution by cyanide [143]. The same results for cyanide binding were observed by NMR using DMSO-d_6 as solvent [144]. Therefore the ligand in the fifth coordination position may either accelerate or decelerate the rate of substitution in the sixth position. Dimerization rates of the tetraethylenediamine substituted porphyrin (minus the metal ion) and of deuteroferriheme in water were measured by the temperature jump method [145,146].

In an early study of H_2O_2 decomposition catalyzed by hemin [147] it was demonstrated that plots of $-(d[H_2O_2]/dt)_{initial}$ vs. $[H_2O_2]_{initial}$ had the form of a saturation curve. A re-analysis using Lineweaver—Burk plots [148] showed that for the mechanism

$$E + P \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E{\cdot}P$$

$$E{\cdot}P \xrightarrow{k_3} \text{Products}$$

where E is the model enzyme or hemin, and P hydrogen peroxide the value of $K_M = (k_2 + k_3)/k_1$ was 8×10^{-3} M and k_3 3.5×10^{-2} s^{-1} at 0°C and pH 7. It was implied that k_3 was the rate constant for the process

$$E{\cdot}HO_2^- \rightarrow E{\cdot}O + OH^-$$

analogous to the proposed non-chain mechanism for the ferric ion catalysis. A subsequent faster step

$$E{\cdot}O + H_2O_2 \rightarrow E + O_2 + H_2O$$

leads to the prediction of the decomposition of two molecules of H_2O_2 in each cycle [149].

The pH dependence of the reaction was investigated [150,151] and the possible effect of aging of the hemin upon the kinetics was pointed out [127]. The destructive oxidation of hemin by H_2O_2 was also studied, followed by a reinvestigation of the kinetics of H_2O_2 decomposition catalyzed by aged hemin (hemin A) solutions [151]. An acceleration of rate at high pH was noted which was attributed to the more rapid decomposition of the hematin—peroxide complex compared to the hemin—peroxide complex [151]. Later, the kinetics of dimer and monomer heme reactions with H_2O_2 were sorted out, with the monomer being considerably more reactive [105, 126,152—154].

The rate of monomer reaction with H_2O_2 is inversely proportional to the

hydrogen ion concentration over the pH range 7—11 for deuteroferriheme monomer and (with greater experimental error because of complications from the enhanced dimer content) over the pH range 6—13 for protoferriheme monomer. The plot of log rate constant vs. pH in the latter case intersects the log of the pH independent rate constant for the catalytic activity of catalase at pH 13 [153].

Earlier studies showed the remarkable catalatic activity of triethylenetetramine—Fe^{III} chelate [155,156] and isotopic labelling experiments showed that for the latter catalyst as well as for the catalase and ferric hydroxide, the oxygen molecule is formed from the intact O—O bond in hydrogen peroxide [100]. The kinetics of decomposition of hydrogen peroxide catalyzed by ferric EDTA have been investigated, and the importance of free radical chains has been emphasized [157].

The peroxidatic activity of proto- and deuterohemins have been demonstrated. Ascorbic acid can compete with H_2O_2 for reactions with the primary protohemin—peroxide compound or complex [158]. A compound with an electronic absorption spectrum analogous to compound I of peroxidase was formed from deuterohemin and H_2O_2 and its reactions with a variety of reducing agents were studied at pH 7.4 [159].

Synthetic methods have produced many interesting and valuable porphyrin derivatives [160]. Recently, model iron(II) porphyrins have been synthesized with sterically restricted active sites which can bind oxygen reversibly [161,162]. One of the most spectacular of these compounds is "picket fence" porphyrin [162—164]. These compounds are obvious models for myoglobin, but they are also relevant to HRP compound III.

Models for cytochrome *P*-450 have also been synthesized [165,166]. Mn^{II} porphyrins, isoelectronic with Fe^{III} have been synthesized and their tendency to have five coordination positions occupied has been demonstrated [167].

A recent review summarizes a great deal of interesting work which has been performed on metalloporphyrin π-cation radicals [168]. These may be relevant models for the electronic structures of the compounds of peroxidase and catalase [169] since the spectra of the models and the compounds I are similar. On the other hand, the inability to generate a π-cation radical of an iron porphyrin may possibly be one piece of negative evidence [170]. (See also the discussion of magnetic circular dichroism.)

The various redox potentials of manganese hematoporphyrin IX have been measured in aqueous solution [171]. The rate of oxidation—reduction of the Fe^{II}—Fe^{III}—dicyano complex of hemin has been measured by the coulostatic method at a platinum electrode and was found to be among the fastest of electrode reactions [172].

In summary it can be seen that the porphyrin ligand has profound effects on a metal ion. An obvious function of the protein portion of peroxidases is to make the hemin water soluble but other more subtle roles can be discerned [173].

C. PHYSICAL AND CHEMICAL TECHNIQUES FOR THE PEROXIDASES

(i) Paraperoxidases, isoenzymes, amino acid analyses and assays

Different components of peroxidase may be isolated from the source material. In the case of some preparations of HRP, variable amounts of a species called paraperoxidase could be isolated by electrophoresis from the major component [28], which has major differences in properties [70]. In the case of Japanese radish peroxidase, two major components JRP*a* and JRP*c* are obtained (see Table 1). It has been suggested that the paraperoxidase JRP*c* is carbohydrate-free as is the paraperoxidase of HRP [70] and more recently that the paraperoxidases may exist as low-spin cyanide complexes [174]. Isoenzymes, on the other hand, usually have more subtle differences in properties. There have been several investigations of the multiple components of HRP [24,175–180]. According to one widely quoted classification, the major components of HRP are seven isoenzymes, A1, A2, A3 and B, C, D, E which were isolated and purified by column chromatography from a crude ammonium sulphate preparation [176]. Each isoenzyme was characterized with respect to electrophoretic mobility, sedimentation coefficient, purity number, chromatographic behaviour, amino acid composition and carbohydrate analysis. These isoenzymes were further characterized by their catalytic properties in both a peroxidatic and oxidatic reaction [176]. The acid isoenzymes A1 to A3 are readily separated [181] and have been shown by chromatographic analysis to be absent from two commercial preparations [182]. Another commercial preparation was homogeneous except for microheterogeneity over a limited pH range, attributed to the carbohydrate portion of the molecule [183]. Spectral and circular dichroism studies indicate that isoenzymes A1 and C of HRP have similar active sites [184]. Changes in isoenzyme composition induced by pH changes, which caused no net change in reactivity have been reported [185]. Transient state kinetic studies on a variety of commercial preparations, discussed in detail later, have never shown any indication of multiple components. If two or more significant components of greatly different reactivity were present, biphasic or multiphasic traces rather than pseudo-first order traces would be obtained. It appears that most commercial preparations are predominantly the very similar B and C isoenzymes. However, more work to establish the composition of commercial preparations would be appreciated.

Eighteen isoenzymes were separated from Japanese radish peroxidase upon electrophoresis on polyacrylamide gel [186]. The major components were acidic isoenzymes. Five peroxidase isoenzymes have been isolated and obtained in highly purified form from turnip roots. They all contained hemin as prosthetic group, but in contrast to horseradish peroxidase isoenzymes which possess very similar spectral characteristics, the wavelengths and amplitude of the absorption peaks of turnip peroxidases varied [187]. The crystalline preparation of cytochrome *c* peroxidase is free of isoenzymes

[188]. It has been claimed that lactoperoxidase exists as a number of closely related isoenzymes [189—191]; however, it has been argued that these findings are the result of enzyme degradation during the extensive purification and separation procedures [33].

Preparative disc electrophoretic separation of the myeloperoxidase of normal human leucocytes demonstrated the existence of six isoenzymes [192]. Studies of isolated bands suggested that each isoenzyme was a dimeric molecule arising from three kinds of monomeric subunit A, B and C. The isoenzymes are referred to as AA, AB, AC, BB, BC and CC. The fact that the enzyme can be split into two parts, each containing a heme unit [6] also suggests that the native enzyme consists of more than one subunit.

The amino acid analysis of some peroxidases is summarized in Table 4. The plant peroxidases in common with many plant proteins [194] have a low lysine content. In contrast the lysine content in cytochrome *c* peroxidase and lactoperoxidase is rather high as is the tryptophan and tyrosine content. The amino acid composition of Mann HRP shown in Table 4 is similar to analyses of different sources of this enzyme previously reported in the literature [27,175,195]. The complete sequence of 21 and partial sequences of three tryptic peptides have been determined which account for 203 of the 300 amino acid residues in HRP [183]. Thermolytic peptides which overlap the tryptic peptides in many places accounted for 100 amino acid residues

TABLE 4

Amino acid analysis of some peroxidases

	HRP [a]	JRP[a]	CcP	ClP	LP	MP
Lysine	6	7	21	4	33	11
Histidine	3	4	5	7	14	4
Arginine	20	11	9	6	39	35
Aspartic acid	46	51	42	39	71	51
Threonine	25	27	14	17	28	23
Serine	26	51	14	33	30	21
Glutamic acid	20	26	27	26	60	37
Proline	17	17	15	23	42	31
Glycine	17	6	23	14	41	26
Alanine	23	64	16	24	40	26
Cysteine	8 [b]	10	1	2	12	13 [b]
Valine	18	20	12	1	29	17
Methionine	4	4	6	2	12	12
Isoleucine	12	15	8	9	28	16
Leucine	35	36	23	20	68	43
Tyrosine	6	3	12	10	15	8
Phenylalanine	20	18	16	13	31	17
Hydroxyproline	—	12	—	—	—	—
Tryptophan	—	2	6	—	16	—
Ref.	183	36	193	12	33	39

[a] Abbreviations defined in Table 1. [b] Half cysteine.

[196]. Work on the primary structure of the protein portion of HRP is now nearing completion [197].

A common "assay" for peroxidases is the R.Z. or purity number, the ratio of maximum absorbance in the Soret region to the absorbance of aromatic amino acids at 280 nm. For HRP, R.Z. values of 3 or greater indicate high purity, unless one has a sample of a rare isoenzyme component. Several steady state assay methods have been developed [198—200]. The carcinogenic nature of aromatic amines has been emphasized and an alternative assay proposed [201]. Assays for CcP [188], ClP [13] and LP [202,203] have been described.

(ii) Crystallization

Both apo and reconstituted holoenzymes of cytochrome *c* peroxidase containing natural and unnatural prosthetic groups can be crystallized [204—207] which makes it possible to compare the three dimensional structures of the apoprotein moiety of the enzyme in the absence and presence of the prosthetic group. The crystals obtained from native CcP are orthorhombic with a space group $P_{2_12_12_1}$ and unit cell dimensions a = 107.6 Å, b = 76.8 Å and c = 51.4 Å with four molecules per unit cell [208]. The heavy-atom uranyl and mercury derivatives have been prepared and are isomorphous with the native CcP [209]. Preliminary crystallographic data has also been reported for Japanese radish peroxidase *c*, a paraperoxidase [209]. The space group is P_{422} with unit cell dimensions $a = b$ = 11.2 Å and c = 133.6 Å. One unit cell contains 2 molecules of JRP*c*.

Although many of the peroxidases have been obtained in crystalline form most are not suitable for X-ray analysis (see Table 1). If the difficulties in obtaining satisfactory crystals have been a result of heterogeneity caused by mixtures of isoenzymes, the problem is readily resolved. If they are due to microheterogeneity attributable to the carbohydrate portion of the molecule [183], then satisfactory crystals may be obtained only for carbohydrate-free peroxidases such as CcP.

(iii) Magnetic susceptibility

A difficulty in determining the paramagnetic moment (μ_{eff}) of a hemoprotein lies in the large diamagnetic correction required for the protein. The diamagnetic correction has been found by measuring the difference in the susceptibility of a hemoprotein containing a paramagnetic ion from that of the same hemoprotein with a diamagnetic ion. However it has been shown [210] that this method leads to a small systematic error since it cannot be assumed that the diamagnetism of the protein is the same in both cases and that the 6*d* electrons which are paired in the "diamagnetic" comparison hemoprotein do not make any paramagnetic contribution to the susceptibility.

The predicted μ_{eff} values of pure high and low spin states of heme compounds at 20°C with the iron in various oxidation states are given below [211].

Oxidation state	No. of unpaired electrons	Susceptibility	Predicted μ_{eff} value or range in BM
Fe^{2+}	4	spin + orbital	4.90—5.64
Fe^{2+}	0	diamagnetic	0
Fe^{3+}	5	spin only	5.92
Fe^{3+}	1	spin + orbital	1.73—2.8
Fe^{4+}	4	spin only	4.90
Fe^{4+}	2	spin + orbital	2.5—3.6
Fe^{5+}	3	spin only	3.87

Some ferric hemoproteins have magnetic susceptibilities between the values for 5 and 1 unpaired electrons. It has been suggested that these compounds might be a mixture of two magnetic isomers, one high and the other low spin [212,213]. On the basis of theoretical calculations [214], it was concluded that ferric hemoproteins with intermediate magnetic susceptibilities may be in thermal equilibrium between high and low spin states based on the Boltzmann distribution. Table 5 summarizes the magnetic susceptibility measurements of the peroxidases.

The complexes of horseradish peroxidase with fluoride and cyanide have been shown to obey the Curie law and are in a purely high spin state and low spin state respectively over the temperature range of the experiments (77 K—RT) [215]. For the native enzyme, abnormal temperature and pH dependences of the paramagnetic susceptibility and optical absorption spectra are observed in the frozen state [215]. This is explained on the assumption that there is a pH dependent mixture of two kinds of molecules, probably an alkaline form and an acidic form, and that each of these molecules has high-spin and low-spin states populated in thermal equilibrium.

The paramagnetic susceptibility of the azide complex of horseradish peroxidase has been measured and found to be temperature dependent [217]. The temperature dependence is explained in terms of thermal equilibrium between high- and low-spin states. The paramagnetic susceptibility which was found to be independent of temperature below —100°C was found to be dependent as the concentration of azide. At very high concentrations of azide or in acid solution below pH 4.5 the azide complex did not show a temperature dependence and it is suggested that it is a different kind of azide complex in a purely low-spin state.

The magnetic susceptibility data for HRP-I and HRP-II have been interpreted as Fe^{V} for HRP-I and Fe^{IV} for HRP-II [211].

For cytochrome *c* peroxidase the susceptibility of the enzyme was found to deviate from the Curie law above —100°C [219] whereas the susceptibil-

TABLE 5

Magnetic susceptibility measurements on peroxidases

	pH	Temp (K)	μ (BM)	Ref.
Horseradish peroxidase	4—10.5	77—RT	[a]	217
HRP-CN	—	298	2.67	216
	5.0	77—RT	~2.24	215
HRP-F	—	293	5.92	216
	5.0	77—RT	~5.92	215
HRP-N_3	5.0	77	2.17 [b]	217
			5.19	216
HRP-I	3.43, 8.85	293	3.99 [c]	218
HRP-II	8.85	293	3.53 [c]	218
Cytochrome c peroxidase	5.55	77	5.13	219
		250	4.95	
	6.5	77	3.84	219
		250	4.86	
	7.05	77	3.67	219
		250	4.81	
	7.50	77	3.19	219
		250	4.36	
CcP-CN		77—250	2.13	219
CcP-F		77	5.92	219
		250	5.91	
CcP-N_3		77	2.33	219
		250	2.38	
CcP-I	—	77	4.1 [d]	220
(Complex ES)	7.0	77—268	4.17	221
Ferri-myeloperoxidase	—	293	5.77 [c]	85
Ferri-MP	—	293	3.45 [c]	85
Ferro-MP	—	293	4.46 [c]	85
Ferro-MP	—	293	3.87 [c]	85

[a] μ values of HRP solutions at pH 4, 4.8 decrease with increase in temperature above —100° C, at pH 9.5, 10.5 increase with increase in temperature above —100° C. Below —100° C the values of μ_{eff} for each sample are temperature independent but they are pH dependent.

[b] Independent of temperature below —100° C but dependent on concentration of azide.

[c] μ value of original data has been corrected according to Griffith [210].

[d] When correction is made for one equivalent of free radicals, assuming normal susceptibility of the radicals, the effective magnetic moment of the heme decreases from 4.1 to 3.7 BM [222].

ity of Complex ES (compound I) was found to obey the Curie law rather closely over the temperature range of the experiments (77 K—RT) [219]. The abnormal temperature and pH dependences of the native enzyme are explained on the assumption that the sample is a pH dependent mixture of two kinds of molecule, probably $(Fe^{3+})H_2O$ and $(Fe^{3+})OH^-$ and that both of these molecules have high and low-spin states populated in thermal equilibrium [219,221]. The EPR data for compound I of CcP indicate that one full

equivalent of free radicals is present in the system [220]. The total width of the radical absorption suggests that this radical is situated not too far away from the paramagnetic heme. When correction is made from one equivalent of free radicals, assuming normal susceptibility of the radicals, the effective magnetic moment of the heme of compound I of CcP decreases from 4.1 to 3.7 BM [222].

(iv) Electron paramagnetic resonance spectroscopy

The EPR spectra of high and low spin heme compounds are distinct. High spin compounds have $g_{\parallel} = 2$, $g_{\perp} = 6$ and low spin have three g values $g_x \simeq 1.7$, $g_y \simeq 2.2$ and $g_z \simeq 2.8$. The EPR signals of low spin ferric hemoproteins are often broad and weak even at liquid helium temperatures. In high spin spectra the signals sometimes show evidence of asymmetry or splitting. EPR spectroscopy has been used to detect mixtures of spin states, but because the spectra are measured at low temperatures, (77 K or below) and the position of equilibrium is likely to be temperature dependent, information obtained in this way is not necessarily relevant to the situation at room temperature. Table 6 contains a summary of data obtained from the EPR spectra of some peroxidases.

From a pH dependence of the EPR of native HRP [223] it was concluded that three components were involved in the acid—base transition

HRP(acid)——HRP(I)——HRP(alkaline)
$Fe^{3+}(H_2O)$ $Fe^{3+}(OH^-)$

The EPR absorption of HRP (I) (not to be confused with compound I) was more anisotropic than that of the "alkaline form". The authors suggest that the compound appearing at neutral pH is in thermal equilibrium between two spin states while the alkaline form is in a low spin state over the whole temperature range. The data showed that the two low spin absorptions originated from the pH dependent interconvertible compounds of peroxidase itself and were not due to the coexistence of different peroxidase isoenzymes in the sample. These data are compatible with magnetic susceptibility data described above.

Cytochrome *c* peroxidase at 1.5 K and pH 5.0 exhibits signals which are typical of high spin ferric heme iron while at pH 7 the absorptions showed some formation of the low spin form of heme iron. This was attributed to an equilibrium mixture of high and low spin forms [220,229]. However, magnetic susceptibility data showed this to be incorrect [188] and the data have been reinterpreted as a mixture of high spin CcP ($Fe—H_2O$) and low spin CcP ($Fe—OH^-$).

The ferri-cyanide forms of HRP and CcP both give EPR spectra which are characteristic of low spin heme while that of JRP*a* gives a spectrum at 89 K which corresponds to a mixture of high and low spin forms similar to the

TABLE 6

Electron paramagnetic resonance data on the peroxidases

	pH	Temp. (K)	g values	EPR signature	Ref.
Ferri-HRP (acid) [a]	low	1.4	6.3, 5.4, 2.0	high spin	223
	neutral	1.4	3.18, 2.06, 1.23	low spin	
	high	1.4	2.96, 2.12, 1.66	low spin	
Ferri-HRP (neutral) [a]	low	1.4	6.46, 5.38, 2.00	high spin	223
	neutral	1.4	3.20, 2.05, 1.23	low spin	
	high	1.4	2.94, 2.08, 1.63	low spin	
Ferri-HRP-CN	9.2	1.4	3.05, 2.1, 1.2	low spin	224
Ferro-HRP	7.0	1.4	no signal	none	
Ferro-HRP-CN	9.2	1.4	no signal	none	
Ferro-HRP-CO	7.0	1.4	no signal	none	
Oxyperoxidase (HRP-III)	7.0	1.4	no signal	none	
HRP-II	6.0	1.4	no signal	none	
HRP-I	7.0	1.4	1.995 [b]	radical	225
Ferri-CcP	low	1.4	6.4, 5.6, 2.0	high spin	226
	high	1.4	2.6, 2.2, 1.9		
Ferri-CcP-CN	6.3	1.4	3.0, 2.1	low spin	
Ferro-CcP	—	1.4	no signal	none	
Ferro-CcP-CN	9.2	1.4	no signal	none	
CcP-H_2O_2 product	5.0, 8.4	1.4	2.05, 2.00, 2.00	radical	
CcP-chlorite product	6.0	1.4	2.046, 2.006, 2.006	radical	
CcP-oxygen product	—	1.4	no signal	none	
JRP*a*	3.9	93	$s_m = 5.0, g_m = 2.05$ [c]	high spin	
	6.9	93	$s_m = 6.0, 2.92$	high spin	
			$g_m = 4.3, 2.05$	low spin	
JRP*a*-F	7.0	92	$s_m = 6.1, g_m = 2.07$	high spin	227
JRP*a*-OH	~11.6	92	$s_m = 2.86, 1.67$	low spin	
			$g_m = 2.12$		
JRP*a*-N_3	4.6	92	$s_m = 7.0$ (weak), 2.98	low spin	
			$g_m = 2.16$		
JRP*a*-CN	7.0	92	$s_m = 5.9, 2.86$	high spin	
			$g_m = 2.05$	low spin	
JRP*c*	7.0	89	$s_m = 6.1$ [d]	none	
Ferri-MP	—	169	6.3, 5.3, 4.3, (2.26, 2.08)	high spin	
Ferro-MP	—	77	no signal	none	
Chloroperoxidase	—	—	2.63, 2.26 [e] (7.44, 4.3) [f]	low spin	228
ClP-F	—	—	7.01, 4.80	high spin	
ClP-I	—	—	7.48, 4.17	high spin	
ClP-Cl	—	—	2.66, 2.27 [g] (7.62, 4.05)	low spin	

[a] Acid and neutral isoenzymes as separated [155].

[b] In titration experiment with HRP and H_2O_2, this signal was proportional to concentration of compound I reaching a maximum when equivalent amount of H_2O_2 added.

[c] The authors used the following designations to mark field positions: g_m is a position in terms of g value of maximal absorption, s_m refers to positions calculated as g values of maximal positive or negative slope in the absorption curve or derivative curve.

[d] Authors suggest that this signal might be from denatured JRP*c* produced in the course of deionization and concentration.

[e] The presence of manganese in all samples made the EPR analysis around $g = 2$ difficult. As a result only two g values of each species were obtained with accuracy.

[f] Minority high spin ferric species.

[g] Attributed to protein bound Cu^{2+}.

native enzyme of JRP*a* at neutral pH. None of the ferro derivatives studied gave any signal.

Perhaps the most exciting EPR results have been obtained on the compounds I of CcP and HRP. Complex ES (compound I) of CcP gave an intense narrow signal of a free radical type at $g = 2.00$ [220]. The spin concentrations of this free radical were estimated to be about one equivalent per enzyme hematin unit. It is suggested that the signal is due to the formation of a stable and reversible free radical of an aromatic amino acid residue on the enzyme protein located near the heme iron. Recently a small EPR signal was obtained from HRP-I [225], which had free radical properties and was proportional to the amount of compound I present. It was suggested that there is a free radical present which is relaxed by the near-by iron. It thus appears that HRP-I may be similar to CcP-I (complex ES), the main difference being that the free radical of HRP-I is located so close to the Fe^{IV} that its EPR signal is almost obliterated by the paramagnetic center. No signal was observed for HRP-II.

(v) Nuclear magnetic resonance spectroscopy

NMR methods have been extensively applied to the investigation of biological systems [230,231]. In particular they have been used to obtain information about the environment in the vicinity of the high spin ferric iron of various hemoproteins [232—235] and hemin derivatives [236]. Extremely large chemical shifts are observed for heme protons for which a quantum mechanical model has been developed [237].

Solvent [238,239] and ligand [240] exchange rates can also yield valuable information. It is of great interest to identify the ligands occupying the fifth and sixth coordination positions on the heme iron of HRP. It has been generally assumed, but never proven, that the sixth coordination position is occupied by a water molecule [69,215,241—244] as it is in metmyoglobin and methemoglobin where all normal derivatives are six coordinate [113,245].

A study has been made of the rate of exchange of water with the iron atom of the heme group of HRP at several pH values using a line broadening technique with O^{17} NMR [246]. Although the points in the plots of $\log(1/T_{2p})$ vs. $1/T(K)$ were scattered they did define lines of positive slope. However, it was not possible to decide whether the exchange process was from the inner or outer coordination sphere [247].

From the results of a pH dependence of longitudinal relaxation rates of water protons in HRP [239] the authors concluded that ferric HRP had no water coordinated to its iron and that it was probably a closed crevice structure. This conclusion was based on the fact that the value of the molar relaxivity of neutral ferric peroxidase was closer to that of the low-spin cyanide—HRP complex than that observed for neutral myoglobin [238] with one water molecule bound in the high-spin state or acidic ferricytochrome *c* [233] with two water molecules bound in the high-spin state. Also the molar

relaxivity was found to be constant in the pH range 3—12. A temperature dependence of the relaxation rate would be helpful in checking the presence or absence of water in the sixth coordination position of HRP.

Since ^{35}Cl has a nuclear spin of $\frac{3}{2}$, it has a quadrupole moment which can provide an effective nuclear relaxation mechanism. Thus an NMR halide-ion probe technique can be used to gain information about biological molecules [248—250]. It has been used to detect the binding of Cl^- ions to native and apo-horseradish peroxidase which is probably non-specific in nature. The binding increases markedly as the pH is lowered [251].

In recent studies, NMR has been used to deduce information concerning the orientation of a bound organic molecule to native HRP [252] and to identify amino acids near the heme of both HRP and various isoenzymes of turnip peroxidase [253].

(vi) Mössbauer spectroscopy

Mössbauer spectroscopy is sensitive to the electronic configuration surrounding the iron nuclei. It can give information about spin states, the effect of substrates on heme environment and how changes in formal valence state affect the electronic charge on the iron. Results of Mössbauer studies on various peroxidases are summarized in Table 7.

The Mössbauer spectra of HRP and its peroxide derivatives have been studied [254]. The Mössbauer parameters for HRP-I (pH = 3) and HRP-II (pH = 9) were found to be very similar. This suggests that there is little difference in iron electronic configuration in going from compound I to compound II. The results are consistent with an Fe^{IV} state for both HRP-I and HRP-II. Native horseradish peroxidase has a Mössbauer spectrum which is different to that of its compounds and indicates a change in the iron configuration.

Like the HRP compounds, the Mössbauer parameters for JRP compounds I and II are similar and suggest that the iron electronic configuration in these compounds is alike [258]. The hydroxide, cyanide and azide compounds all have absorptions characteristic of low spin ferric ion.

For native cytochrome *c* peroxidase the spectra indicate the presence of both high and low spin components [259]. It has been reported that the Mössbauer spectrum of the compound formed between ethyl hydrogen peroxide and cytochrome *c* peroxidase is similar to JRP and HRP compounds I and II [260].

Mössbauer studies on chloroperoxidase [254] show that the heme iron of the native enzyme undergoes a spin change from predominantly low spin to predominantly high spin as the temperature is increased from 4.2—245 K. The chloride complex at 4.2 K has a spectrum which is almost identical to that of the native enzyme so it is suggested that chloride is not an a..al ligand in the complex [254].

TABLE 7

Results of Mössbauer spectroscopy studies on peroxidases

	Temp (K)	pH	Quadrupole splitting ($mm\ s^{-1}$)	Isomer shift ($mm\ s^{-1}$)	Ref.
Horseradish peroxidase	205	—	1.87 ± 0.03	+0.18 ± 0.03	254
	77		1.96	+0.25	
	4.6		Too broad to determine		
HRP-II	205	9.3	1.37 ± 0.02	−0.02 ± 0.02	
	77		1.36	+0.03	
	4.6		1.42	+0.03	
HRP-I	77	3.5	1.20 ± 0.02	0.00 ± 0.02	
Chloroperoxidase	4.2	3	Broad spectra characteristic of low spin ferric contains high spin ferric as minority species		255
	194—245		Spectra show that native chloroperoxidase undergoes a spin transition from low spin to high spin as temperature is raised		
ClP-Cl	4.2	3	Like chloroperoxidase at 4.2 K		
	223		Only one quadrupole doublet characteristic of low spin ferric species		
ClP-F	4.2	3	Hyperfine splitting		255
	225		1.00 ± 0.05		
ClP-I	4.2	3	Hyperfine splitting		
	225		0.65 ± 0.07		
Reduced ClP	4.2	—	2.48 ± 0.02	+0.85 ± 0.02	
Reduced ClP-CO	4.2	—	0.52 ± 0.04	+0.29 ± 0.02	
Japanese radish peroxidase *a*	243	—	184 ± 0.06	+0.35 ± 0.06	256,257
	120		2.28	+0.34	
	77		2.49	+0.38	
	4.2		Hyperfine splitting		
JRP*a*-OH	243	—	1.84	+0.27	
	120		2.36	+0.22	
	77		Hyperfine splitting		
JRP*a*-N_3	195	—	2.22	+0.29	
	120		2.22	+0.31	
	77		Hyperfine splitting		
JRP*a*-CN	195	—	1.61	+0.11	
	120		1.71	+0.13	
	77		1.81	+0.15	
JRP*a*-I	195	—	1.38	+0.04	258
	77		1.33	+0.10	
JRP*a*-II	195	—	1.44	+0.07	
	77		1.46	+0.11	

TABLE 7 (continued)

	Temp (K)	pH	Quadrupole splitting (mm s^{-1})	Isomer shift (mm s^{-1})	Ref.
JRP*a*-III	195		2.33	+0.24	
	77		2.37	+0.29	
Cytochrome *c* peroxidase	195	7	2.38	+0.22	259
			1.27	+0.32	
CcP-F	4.2		Hyperfine splitting		

(vii) Electronic absorption spectroscopy

There is considerable interest in the nature of the iron porphyrin prosthetic groups in the native peroxidases and their intermediate compounds. With the increase in techniques based on observing changes in the $\pi \rightarrow \pi^*$ spectrum of the 18π electron porphyrin ring it is apparent that a full assignment of the electronic absorption spectrum in the visible and ultraviolet regions is necessary.

Figure 2 shows the state diagram for the 18π electron porphyrin ring, assuming D_{4h} symmetry for the metal porphyrin system. Absorption in the 15 000—35 000 cm^{-1} region is mainly due to transitions from the A_{1g} ground state to degenerate π^* excited states [106]. The lowest energy transitions comprise a complex set which are identified in the absorption spectrum as the α or Q_{00} band and β for Q_{0i}, Q_{02} etc., the vibrational overtone bands. To higher energy lies the single intense Soret or γ band, with an extinction coefficient of about 10^5 M^{-1} cm^{-1}. At slightly higher energies a further broad band of weaker intensity is often observed especially at low temperatures. It has been suggested that this is a transition to the third π^* state [261].

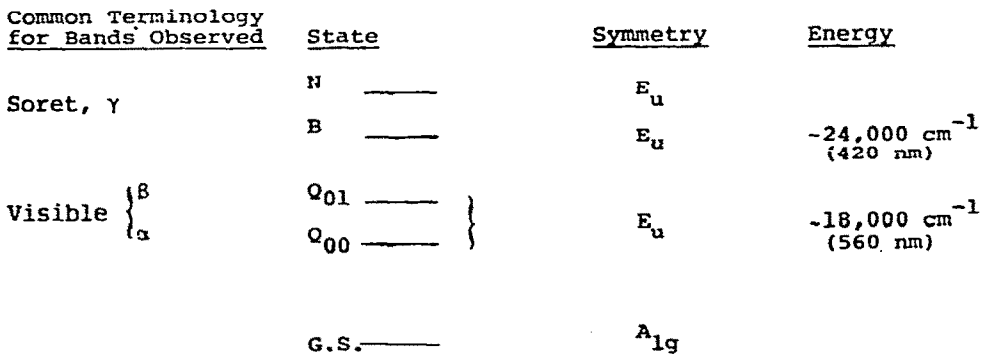


Fig. 2. Schematic energy level diagram for an 18π electron porphyrin ring.

In many cases a series of bands is superimposed on the $\pi \rightarrow \pi^*$ spectrum which arise from charge transfer transitions between the metal and the ring [116,262,263]. These are observed to have extinction coefficients approximately the same as those of the π^* bands. Their presence seriously complicates the problem of assignment of many hemoprotein spectra. The direction of the charge transfer and the energy of the transition depends on the oxidation and spin state of the central iron. For example in high spin Fe^{III} HRP bands are observed at about 10 000 cm^{-1} that can be assigned as charge transfer from the filled ligand π orbitals into the half filled d orbitals of the metal [263—265].

The optical spectra of ferric hemoproteins have been empirically correlated with their magnetic properties as follows [266,267]. For a high spin ferric hemoprotein with five unpaired electrons the magnetic moment will be approximately 5.9 B.M. These compounds have an intense Soret band in the region 400—415 nm and weak α and β bands at about 575 and 535 nm which are often obscured by charge transfer bands lying in the region 450—1000 nm. Low spin ferric hemoproteins with one unpaired electron have magnetic moments of about 2.3 B.M. The Soret band in these compounds lies at about 415—425 nm and they have distinct α and β bands. Charge transfer bands are absent in low spin ferric hemoproteins in this spectral region. Equilibrium mixtures of high and low spin states have spectra which are additive mixtures of the two components [266—268].

Table 8 lists the band maxima observed in the room temperature spectra of the peroxidases and their derivatives.

The pH dependence of the absorption spectrum of cytochrome *c* peroxidase is anomalous. There is little change in the absorption spectrum between pH 5.0—7.0. However above 7.5 it changes to a low spin alkaline type, but no distinct isosbestic point is observed in the transition [271]. At 77 K the spectra of cytochrome *c* peroxidase at pH 5, 6 and 7 become different. The changes indicate a transition of the enzyme to a low spin state upon cooling. There were no isosbestic points among the spectra obtained at different temperatures and on warming the enzyme from 77 K the α and β bands disappeared non-synchronously with the appearance of the charge transfer bands suggesting that the enzyme may exist as a mixture of more than one form. This is supported by magnetic susceptibility measurements of the enzyme at low temperatures [221]. The peroxide compound of cytochrome *c* peroxidase and its cyanide and fluoride compounds were found to be temperature-independent while the azide complex, like the native enzyme showed a temperature dependence, the spectrum at low temperatures resembling that of a low spin hemoprotein [273].

For horseradish peroxidase, its intermediate compounds I and II and many of its ferri- and ferro-derivatives, it has been shown that at low temperature there is no dramatic change in the absorption spectrum, only a general sharpening and resolution of the bands [218,224], (footnote b, Table 8). As the spectra of compound I and compound II of horseradish peroxidase are

TABLE 8

Wavelength in nanometers of band maxima and millimolar extinction coefficients for peroxidases and derivatives

		Soret	Visible	IR	Ref.
Horseradish peroxidase	λ_{max} (nm)	403	498, 640	950, 1065, 1145	264
	ϵ (mM^{-1} cm^{-1})	102	11.25 [b], 3.23 [b]	a a a	
HRP-N_3		416	495 sh, 534, 565, 635		23
		114 [c]	8.2[c], 5.5[c], 1.7[c]		
HRP-CN		422	439, ~580 sh		224
		a	a a		
HRP-F		404	488, 530 sh, 560, 612		23
			7.6[c], 4.8[c], 6.9[c]		
Ferro-HRP		440	~510 sh, 557, ~580 sh		23, 224
		a	a a a		
Ferro-HRP-CO		423	542, 572		23, 224
			a a		
Ferro-HRP-CN		432	536, 566		224
		a	a a		
HRP-I		400	~525 sh, 577, 622, 651		264, 269
		53.8 [d]	a a a a		
HRP-II		420	527, 554		
		105	9.5, 9.65		
HRP-III		416	546, 583, 673		23
		a	a a a		
HRP-OH		416	542, 571, 633		23, 224
Chloroperoxidase	λ_{max} (nm)	403	515, 542, 650		12
		75.3	11.5, 10.8, 4.2		
ClP-N_3	ϵ(mM^{-1} cm^{-1})	432	549, 583, 665		12
		88.5	10.9, 7.8, 3.0		
ClP-CN		437	555		12
		77.8	11.5		

TABLE 8 (continued)

		Soret	Visible	IR	Ref.
Ferro-ClP		409 68.7	420 sh, 450 sh, 550 66.5 38.5 13.7		270
ClP		390 a			
ClP-II		410 a			270
Cytochrome *c* peroxidase	λ_{max} (nm)	408 93	505, ~535 sh, 645 a a a		188, 204, 271
CcP-N_3	ϵ(mM^{-1} cm^{-1})	416 a	535, ~570 sh, 635 a a a		188, 271
CcP-CN		424 a	540, ~570 sh a a		188, 271
CcP-F		407 a	490, 535, 570, 617 a a a a		188, 271
Ferro-CcP		438 a	530, 561, 625 a a a		205
Ferro-CcP-CO		423 a	542, 570 a a		205
CcP-I		420 a	530, 561, 625 a a a		205
Thyroid peroxidase	λ_{max} (nm)	406			272
TP-CN		423	542 broad		272
Ferro-TP		420 (broad)	560 broad		272
Ferro-TP-CO		405—418	542 broad, 562 broad		272
Ferro-TP-CN		428	533, 562		272
Lactoperoxidase	λ_{max} (nm) ϵ (mM^{-1} cm^{-1})	412 a	505, 550, 600, 640 a a a a		273

		Soret	Visible	IR	Ref.
Ferro-LP			557—575, 600, 645		274
LP-I		416			34
LP-II		430	538, 570		34
LP-III			540, 524, 617		34
Myeloperoxidase	λ_{max} (nm)	430	500 sh, 570, 625, 690		275
Ferro-MP		475	550 sh, 585 sh, 637		275
Ferro-MP-CO		468	634		275
MP-I		430			275
MP-II		456	630		275
MP-III		452	574, 625.5		275
Turnip peroxidase					
TP-A_1	λ_{max} (nm) ϵ (mM^{-1} cm^{-1})	402 93.6	494, 643 11.4 2.86		42
TP-A_2		405 106.0	499, 643 10.9, 3.13		42
TP-B		406 100.0	492, 533, 635 10.2, 8.78, 2.50		42
TP-D		404 94.5	497, 642 10.9 2.95		42

TABLE 8 (continued)

	Soret	Visible	IR	Ref.
Reduced TP-A_1	436 98.8	559 13.4		42
Reduced TP-A_2	438 91.5	559 13.1		42
Reduced TP-B	437 91.9	559 13.0		42
Reduced TP-D	438 96.5	557 12.8		42
TP-II	420 —	—		42

[a] Extinction coefficients available from reference diagram.
[b] J.S. Stillman and H.B. Dunford — unpublished results.
[c] Extinction coefficient from table in ref. 224.
[d] Extinction coefficient from ref. 264.

the same at 77 K and 273 K, it was concluded that neither is an equilibrium mixture of species. This disproved a suggestion [276] that compound I represents the sum of the spectra of interconvertible tautomers.

(viii) Circular dichroism, optical rotatory dispersion

The optical activity associated with the heme transitions in hemoproteins is a direct result of the binding of heme to apoprotein since free heme is optically inactive [277]. The CD and ORD techniques are sensitive to many aspects of protein structure such as α-helix [278], β-structure [279—281] and the conformations of certain amino acid residue side chains [282, 283].

The CD spectra in the far ultraviolet region give information about the conformation of the peptide backbone. The CD of native HRP in this region resembles that reported for α-helical polypeptides [278]. It has been estimated that HRP contains about 40% helix [284]. The isoenzymes C and A1 of ferri-HRP have been shown to have very similar absorption and CD spetra in the Soret region [184]. The similarity of the CD bands at 207 nm and 227 nm in HRP and its intermediate compounds I and II suggests that if any conformational change occurs during the peroxidatic reaction then it is restricted to a small region involving only a few amino acid residues [284]. However, this might be the only change necessary for the active site to become more accessible in compounds I and II compared to the native enzyme. (See also the section on photolysis.) In a study of the CD of some derivatives of HRP it was shown [285] that HRP loses most of the heme associated optical activity on reduction, but gains a strong complex CD spectrum on formation of the carbonmonoxy derivative. The authors suggest that this is due to local conformational change with respect to the hemoprotein binding side on reduction from the ferri to the ferro form. The CD of JRP*a* has been used to estimate helical and β-structure [286].

ORD has been used to study the denaturation of HRP [287] and to show that there are no major conformational changes caused by pH changes for either HRP [288] or LP [289].

(ix) Magnetic circular dichroism

The techniques of magnetic optical rotatory dispersion (MORD) and magnetic circular dichroism (MCD) give detailed information about the polarization properties of electronic transitions and have been shown to be of great use in the assignment problem of complex spectra [290,291]. Of these two techniques that of MCD has proved the more fruitful in the characterization of transitions and the calculation of parameters of both the ground and excited states. While there has been considerable work on the porphyrins [291, 292] there is comparatively little data published on heme proteins and to our knowledge there is no published data on catalase or any of the peroxidases. The reviews on manganese porphyrins [293] and the chlorophylls

[261] illustrate well the use of this technique in understanding the nature of the aromatic ring, central metal cation and its axial ligands.

Recent MCD spectra of native HRP and its cyanide derivative * resemble in transition energies, half widths and angular momenta the data obtained for metmyoglobin and its cyanide derivative, supporting observations made previously from the absorption spectra alone [23,241]. The MCD spectra of the intermediate compounds I and II of HRP (Figs. 3 and 4) allow further interpretation of their respective absorption spectra. In both of these figures the absorption spectra recorded at 10 K are shown affording greater resolution of the individual transitions. As with the absorption spectra, it is found that the MCD of HRP-II is considerably better defined than HRP-I and more readily assigned. The HRP-II MCD is characteristic of a low spin iron—porphyrin with little mixing between the iron *d* orbitals and the porphyrin π system. A positive A term at 18000 cm^{-1} identifies the Q_{00} transition, with poorly defined transitions lying in the Q_{01} region to higher energy.

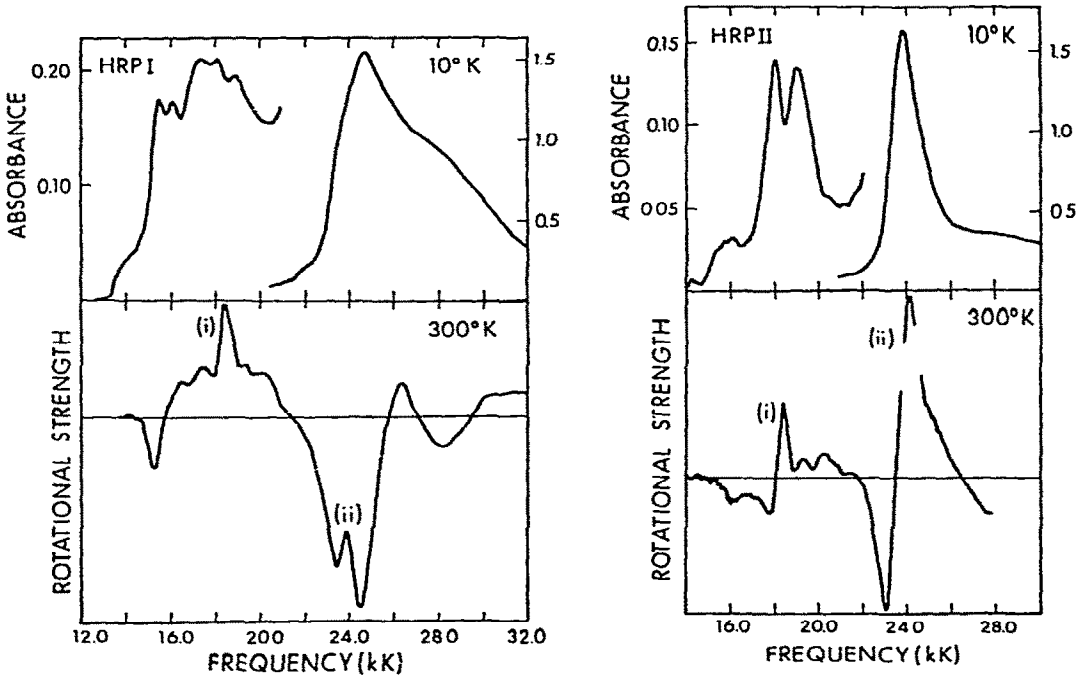


Fig. 3. Magnetic circular dichroism spectrum of HRP-I at room temperature compared to its optical spectrum at 10 K. The optical spectra of HRP-I and HRP-II at 10 K are very similar to the room temperature spectra except that the bands are narrower.

Fig. 4. Magnetic circular dichroism spectrum of HRP-II at room temperature compared to its optical spectrum at 10 K.

* M.J. Stillman, B.R. Hollebone and J.S. Stillman, unpublished results.

The symmetric, positive A term under the Soret band at about 24000 cm^{-1} is assigned as the degenerate B band. Additional features of this spectrum compared with other low spin heme proteins are the bands at about 16000 cm^{-1} and 20000 cm^{-1} in the absorption spectrum. Neither set is clearly resolved in the MCD spectrum.

The HRP-I MCD is not as straightforward to interpret. It is possible, however, to characterize the band in the MCD spectrum at 18000 cm^{-1} (labelled (i) in Fig. 3) as a positive A term displaced from the baseline by the residual positive rotation from other transitions, and to assign this as the Q_{00} of the π to π^* spectrum. Band (ii) in Fig. 3 is then assigned as the porphyrin B band. The basis of this assignment rests upon the correlation of transition energies and relative rotational strengths of bands labelled (i) and (ii) in both the HRP-I and HRP-II MCD spectra.

Some indication of the various electronic structures of the porphyrin ring is perhaps given by the observation that photolysis of HRP-I can occur at 10 K but that neither HRP nor HRP-II are photochemically active at these temperatures under the condition of low power light [294,295]. Additional absorption and MCD transitions observed for HRP-I might arise from charge transfer from ligand to metal.

Of particular importance in the discussion of the nature of the electronic structure of HRP-I is the analysis of the absorption spectra of the positive cation radicals of some porphyrin derivatives [169]. While several different absorption spectra are observed depending upon central metal ion, porphyrin substitution and counterion, the spectrum of $(Co^{III}\ OEP)^{2+}\ 2ClO_4^-$ (where OEP = octaethylporphyrin) appears to resemble that of HRP-I in the visible region. A proper comparison can only be made after full characterization of the Q and B bands in the cation radicals.

(x) Resonance Raman spectroscopy

Resonance Raman spectroscopy permits much lower concentrations of solute to be used since it is much more sensitive than conventional Raman spectroscopy. Metalloporphyrins [296—300], hemoproteins [301] and HRP and its derivatives [302,303] with their strong absorption bands are biological molecules ideally suited for resonance Raman studies. The structure sensitive Raman frequencies and their states of polarization indicate the oxidation and spin state of the derivative [304]. In all cases investigated so far, except for native HRP, the frequencies could be classified according to the known spin states found by other techniques. The data for native HRP were found to be anomalous. Other techniques indicate that it is in a high-spin state in the pH range 7—9, but the Raman marker frequencies did not fall into the high spin grouping but nearer to the low spin. It is suggested that this effect is due to doming of the porphyrin ring and this explanation is related to the facile oxidation of Fe^{III} to Fe^{IV} [303]. The resonance Raman spectra of HRP-I and HRP-II should be most interesting.

(xi) Photolysis

It has been shown recently that HRP-I and HRP-II undergo photochemically induced reductions [294,295]. Table 9 summarizes the reactions which take place at both room temperature and 10 K [294,295]. At room temperature an enhanced rate of decay through the normal enzymatic cycle was observed when a sample of HRP-I was irradiated with light. Similarly when HRP-II was irradiated with light at room temperature an enhanced rate of decay to native enzyme was observed. At low temperatures (10 K, 80 K) in glassy matrices rapid conversion of HRP-I to a product, originally identified as HRP-II, took place. However, with absorption maxima near 540, 575 and 650 nm, the product resembles low spin compounds such as HRP-III or alkaline peroxidase. If HRP-I is represented as R · Fe^{IV}-OH, a possible structure for the product found at low temperatures is R · Fe^{III}-OH, which might be expected to have spectral similarities to alkaline peroxidase and which could be an intermediate preceding HRP-II (Fe^{IV}-OH) formation. * The mechanism by which photolysis occurs appears not to be related to the mechanism of fluorescence [305].

(xii) Heme separation, modification and enzyme reconstitution

Since ferriprotoporphyrin IX is the active site of most peroxidases, the nature of its binding to and interaction with the apoprotein is of vital importance in understanding the mechanism of action of peroxidase and the differences in behavior among various hemoproteins. Early work on the split-

TABLE 9

Horseradish peroxidase reactions catalyzed by light

Temp.	Time sample was illuminated	Reaction	Time required to complete reaction
RT	0 min	HRP-I → HRP-II → HRP	>24 h
RT	60 min	HRP-I → HRP-II → HRP	~76 min [a,b]
10 K, 80 K	1800 s	HRP-I → R · Fe^{III}-OH(?)	1800 s [b]
RT	0 min	HRP-II → HRP	>14 h
RT	60 min	HRP-II → HRP [c]	~76 min [b,d]
RT	150 min	HRP-II → HRP-CN [e]	~184 min [b]
10 K, 80 K	5 h	HRP-II → no reaction	

[a] This time includes the irradiation time.
[b] Dependent on lamp voltage.
[c] HRP-II prepared from HRP-I using *p*-cresol.
[d] Some destruction of HRP takes place.
[e] HRP-II prepared from HRP-I using $K_4Fe(CN)_6$.

* D. Job and W.D. Hewson, personal communication.

ting of the enzyme (separation of the hemin and the apoprotein) has been reviewed [306]. Kinetic studies have been conducted on the splitting reaction and reconstitution of the native enzyme [306—308]. The separation of the hemin occurs readily at extremes of pH. However, in alkaline solution, the splitting is accompanied by irreversible denaturation of the protein so reconstitution of an active enzyme is not possible. In acid solutions with proper precautions it is possible to carry out the reversible splitting of the enzyme. Thus, artificial HRP can be synthesized in which other hemin molecules [309] which may be chemically modified [310] are substituted for the original prosthetic group. It appears that the propionic acid side chains play an essential role in the binding process [311,312]. Chemical properties of the artificial HRP enzymes have been studied [313,314]. Studies have also been conducted on artificial CcP molecules [205,315] including a reconstituted CcP enzyme containing a spin-labelled hemin [316].

(xiii) Studies on proton equilibria; oxidation states

The acid—base titration curves of both native and apo-HRP have been determined. The results are interpreted as showing that a histidine residue is available for titration in the apoperoxidase which is not available in the native enzyme. This suggests that there is a histidine residue which is proximal to the hemin active site. The results also indicate a distal tyrosyl residue [317].

The classic study involving proton-linked oxidation—reduction potential measurements is that for the ferro—ferric HRP couple [243]. The study showed the existence of heme-linked acid ionizations with pK_a values of 10.6 for ferric HRP and 7.6 for ferrous HRP.

There are now five known oxidation states for HRP, ranging from ferrous to compound III, the latter of which, as is discussed in detail later, is the equivalent of a ferrous—molecular oxygen complex [318—320]. The range in oxidation states is therefore from +2 to +6 in unit steps. The intermediate oxidation states of +3, +5 and +4 are represented by HRP, HRP-I and HRP-II, with the conversions in that order representing the path of the normal enzymatic cycle (eqns. 1—3). The direct reduction of HRP-I to the native enzyme [269,321] and ClP-I to the native enzyme have also been demonstrated [322]. Similarly the direct conversion of ferrous HRP to HRP-II has been observed [323]. Recently the proton balance as measured with a pH stat was measured for conversions among the five redox states of HRP [324].

(xiv) Studies in "anti-freeze" solvents

A systematic investigation of physical properties of solvents which remain liquid to −80°C has been made. It is possible to maintain the dielectric constant at a fixed value by varying the solvent composition as the temperature is lowered so that the integrity of the protein structure is maintained. This

elegant technique has been applied to the reactions of HRP where the intermediate compounds can be stabilized for long periods of time so that their properties can be examined at leisure [325—327]. The viscosity of the antifreeze solvent is so high that bimolecular reactions are slowed appreciably. Therefore the method is particularly applicable to enzymes such as HRP which have two substrates, one oxidizing and one reducing, so that every step is bimolecular. A unimolecular step occurring within an intimate enzyme—substrate complex, will also be slowed appreciably by the lower temperature provided it is rate-controlling.

(xv) Product analysis

Because peroxidases tend to be non-specific in their requirements for a reducing substrate, a large number of oxidation reactions can be facilitated by these potent catalysts. The scope of these reactions has been investigated by systematically varying the reducing substrate and analyzing the products which have been formed. Since this approach has been well reviewed elsewhere [3,328] it will not be pursued further here, except to emphasize that the nature of the products is dramatically different from that obtained in the hydroxyl radical redox chain reactions obtained with Fenton's reagent [108]. As is discussed later, a considerable body of knowledge has been accumulated from transient state kinetics on the rate of interconversion of the various peroxidase compounds. However, a physical study, in terms of formulation of rate laws and rate constant measurements, of the subsequent free radical reactions remains a largely untouched research area.

(xvi) Chemical modification

The technique of using chemical reagents to modify residues of a protein molecule has been reviewed [329]. The reactivity of histidine residues in HRP isoenzymes A1 and C was determined by titration with diazonium-1H-tetrazole. No histidine reacted in the native enzyme but all three residues reacted in the apoenzyme [330]. The presence of imidazole in the fifth coordination position of the heme iron is suggested by photooxidation studies of the apo-HRP-protoporphyrin complex [331]. Sodium azide and iodoacetate were shown to inactivate HRP indicating a methionine residue near the active site [197,332]. Periodate oxidation of the porphyrin ring has been studied [333] and reactions of various hydrazines indicate either a carbonyl group from the carbohydrate portion of HRP or an unusual thiol group near the active site [334].

D. HORSERADISH PEROXIDASE

The elucidation of the mechanism of action of an enzyme requires many kinds of quantitative data. Primary structure and crystal structure are re-

quired as is information on structural changes during the course of a reaction. Many physical and chemical techniques must be used to acquire the requisite information. Attempts to correlate structure and function may be made on the basis of equilibrium or thermodynamic data only. The functional nature of an enzyme also requires kinetic information however since it is only through kinetic measurements that one can determine the specificity (or lack of specificity) and obtain a "blow-by-blow" account of the intimate mechanistic details of a reaction. These sections on the more thoroughly studied peroxidases will emphasize kinetic measurements. The ultimate mechanistic picture will arise not from any one logical approach, however, but by a common-sense merger of all pertinent data.

(i) Steady state kinetics

Steady state kinetic measurements can yield accurate turnover numbers. They can also yield accurate rate constants for a rate-controlling step, which in the case of HRP reactions is often the rate of HRP-II conversion to the native enzyme. By operating with a smaller amount of H_2O_2 and a larger excess of reducing substrate it is possible to make HRP-I formation the rate-controlling step. It is nearly always impossible to make the conversion of HRP-I to HRP-II rate-controlling, however, since both compounds are reduced by the same substrate and HRP-I is usually reduced the faster of the two. The steady state equation corresponding to eqns. (1—3) is a modified form of ping-pong kinetics. However, such steady state equations are generally interpreted in terms of two (or more) consecutive Michaelis—Menten complexes. Equations (1—3) contain no hint of reversibility or of complex formation, yet perfectly valid Michaelis—Menten K_M values may be defined for both the oxidizing and reducing substrate. Therefore, a limitation of steady state kinetics is clearly defined. Nevertheless, some interesting steady state studies have been performed on (1) the oxidation of iodide ion [335], (2) the effect of cyanide in inhibition of the normal redox cycle [110], (3) the stimulation of HRP by the nitrogenous compounds ammonia, imidazole and pyridine at higher pH's [336] and (4) the luminescent oxidation of luminol [337—339], pyrogallol and resorcinol [340]. Luminescent reactions involving the latter two reagents are stimulated by non-luminescent reducing agents such as *o*-phenylenediamine. The role of peroxy radicals in organic luminescent reactions has been established [341] and of both free radicals and singlet oxygen in the chemiluminescence of oxygenated ferrous cytochrome *P*-450 [342].

(ii) Transient state kinetics

(a) Ligand binding

The simplest reactions in principle are ligand binding. Equilibrium constants for fluoride binding to native HRP as a function of pH were measured [216,343,344] as were the equilibrium constants for cyanide binding [345,

346]. The kinetics of the binding and dissociation of fluoride [344,346] and the binding [181,345,346] and dissociation [346] of cyanide have been studied as a function of pH. The latest study of the rate of cyanide binding to native HRP was extended from near neutral solutions to pH 2.55 using a pH jump method. The rate of binding decreases with decreasing pH and indicates pK_a values of 2.9 and 3.9 for acid groups which inhibit the binding process [181]. The value of 3.9 probably corresponds to less reliable values of 4.1 and 4.3 obtained from earlier studies in less acid solutions [344,346] whereas the pK_a of 2.9 corresponds to an acid group which has not hitherto been detected in kinetic studies. The two propionic acid side chains of the hemin as well as the imidazole group in the fifth coordination position of the heme iron [262,344,347—349] are possible candidates for these two acid groups [181]. An acid group with a pK_a value in the 6.1—6.4 range was deduced, largely based on the requirements of detailed balancing; otherwise this acid group is not required to account quantitatively for the observed binding kinetics [344,346] so that its influence is small and its existence might be questioned. Finally, in the region of pH 11 large changes in spectral [23,244], kinetic [346] and magnetic properties [23] of the native enzyme occur, which have generally been assumed to correspond to the replacement of water by hydroxide in the sixth coordination position of the heme iron ($pK_a = 10.8$). It was found in pH jump experiments that the spectral changes occur at a finite rate (on a stopped flow time-scale) both in buffered and unbuffered solutions [181,350] which is contrary to the expected rates for proton transfer reactions. (See also the discussion of NMR experiments.) The nature of the transition occurring in native HRP in the high pH region appears to be a topic worthy of further study.

The binding of cyanide to native HRP can be fairly well represented by the overall process

$$\begin{array}{ccc} & H_2P & \\ K_2 & \downarrow\uparrow & \\ & HP\ CN^- & \\ K_1 & \downarrow\uparrow \quad \downarrow\uparrow & K_a \\ & P + HCN \rightleftharpoons P{-}CN + H^+ & \end{array}$$

with a binding rate constant of $1.2 \times 10^5\ M^{-1}s^{-1}$, $pK_1 = 3.9$ and $pK_2 \sim 3.0$ [181]. This scheme ignores the possible effect of an acid group of pK_a about 6.2, which appears to have a small influence on the dissociation rate as a function of pH. In order to account for the near pH independence of the dissociation reaction, the reaction of native HRP with HCN must be extended to include such steps as

$$P + HCN \rightleftharpoons (P \cdot HCN) \rightleftharpoons PHCN \rightleftharpoons PCH + H^+$$

where $(P \cdot HCN)$ is an outer-sphere complex and PHCN an inner-sphere complex. The rate-controlling forward step may be the conversion of the outer-sphere to the inner-sphere complex and the rate-controlling step in the pH-

independent dissociation reaction may be the reverse process. Since the dissociation rate constant is of the order of 0.2 s^{-1} then the dissociation constant for the HRP—cyanide complex is $\sim 2 \times 10^{-6}$ M. The alternative but formally equivalent reaction scheme in which CN^- reacts with protonated enzyme leads to a binding rate constant of 10^{10} $M^{-1}s^{-1}$ which appears too rapid for a diffusion-controlled reaction at a restricted active site [171,181, 351,352].

Fluoride binding to native HRP, on the other hand, may be represented by either of the two formally equivalent schemes

$$\begin{array}{ccc} & HP + F^- & \overset{k_1}{\rightleftharpoons} HPF \\ K_1 \; \Updownarrow & & \Updownarrow K_a \\ & P \quad + \quad HF & \end{array}$$

$$\begin{array}{ccc} & HP \quad F^- & \\ K_1 \; \Updownarrow & \Updownarrow \quad \Updownarrow & K_a \; k_2 \\ & P + HF & \rightleftharpoons PF + H^+ \end{array}$$

The kinetic study was not extended to low enough pH to detect the influence of the second acid group of $pK_a \sim 3.0$ [181,344].

The newer value of $pK_1 = 3.9$ is well within the experimental error of the original determination [181,344], $k_1 = 6 \times 10^6$ $M^{-1}s^{-1}$ and $k_2 = k_1K_a/K_1 = 4.6 \times 10^7$ $M^{-1}s^{-1}$ where $K_a = 10^{-3}$ M is the dissociation constant of HF [353]. Thus the diffusion-controlled-limit rule cannot be used to decide between the two schemes [352] both of which adequately account for the marked pH dependence of the association reaction. If the lower of the two reaction schemes is valid, then it must be extended to account for the near pH independence of the dissociation process ($k \sim 3 \times 10^2$ s^{-1}) in a manner analogous to that described above for the binding and dissociation of cyanide. (Again a small pH dependence for the dissociation reaction and its resulting implications in terms of detailed balancing has been neglected [344,346].)

(b) Compound I formation

In neutral and acid solutions the formation of compound I from H_2O_2 is given by the scheme

$$\begin{array}{ll} & H_2P \\ K_2 \; \Updownarrow & \\ & HP \\ K_1 \; \Updownarrow & \\ & P + H_2O_2 \overset{k}{\rightarrow} \text{Compound I} \end{array}$$

with $pK_1 = 3.9$, $pK_2 \sim 3.0$ and $k = 1.8 \times 10^7$ $M^{-1}s^{-1}$ [171]. Compound I of chloroperoxide has a ferryl structure [354,355] and it would appear that HRP-I also is a ferryl type of compound [110,264]. This means that the

overall process for compound I formation involves several steps such as

$$P + H_2O_2 \rightleftharpoons (P \cdot H_2O_2) \rightleftharpoons PH_2O_2 \rightarrow PO(HRP\text{-}I) + H_2O$$

with the rate-controlling step now perhaps being the formation of the outer coordination sphere complex ($P \cdot H_2O_2$). Some supporting evidence may be provided by the similarity in rates for the formation of a wide variety of heme-protein oxygen-containing compounds [51,356—359]. Addition of a neutral oxygen atom to Fe^{III} would convert it formally to Fe^{V} but an extra electron appears to be supplied from elsewhere in the heme protein so that the end product is Fe^{IV} [254]. Thus the additional oxidizing equivalent of compound I is stored elsewhere in the hemoprotein [220,225]. The overall process which involves formation of the outer- and inner-sphere complexes, the transfer of a hydrogen ion and a hydroxyl ion (or their equivalent), the cleavage of an O—O bond, formation of an Fe—O bond and subsequent rearrangement within the hemoprotein, is amazingly efficient with an activation energy of (3.5 ± 1.0) kcal $mole^{-1}$ [360—362]. Such a low energy barrier would indicate diffusion control, not chemical control of the overall process [363], again in accord with formation of the outer sphere complex as the rate-controlling step. The small pre-activation energy term is in accord with a sterically restricted active site [351]. The rates of formation of HRP-I using methyl, ethyl [53] hydroxymethyl [364,365] and *t*-butyl hydroperoxides [69] are, respectively, 1.5×10^6, 3.6×10^6, 5×10^5 and 0, all with units of $M^{-1}s^{-1}$.

(c) Reactions of compound I

At this point it is essential to discuss the pitfalls in attempting to obtain accurate kinetic data on enzymatic reactions of the peroxidases. If these pitfalls are not fully appreciated, quantitative measurements become impossible and gross errors in interpretation can be the end result. One possible complication is the presence of exogeneous donor, or oxidizable impurities. However, it is also possible to introduce reducible impurities (oxidizing agents) capable of causing spontaneous HRP-I formation. The requirements for the oxidizing agent are rather non-specific provided it is not too bulky a molecule (see previous section). Impurities might be present in detectable amounts in water, in reagent grade salt and buffer preparations and in the enzyme preparation itself. Suitable blank experiments can indicate their presence. The kineticist can also detect their presence or absence by conducting the transient state experiments over a wide range of substrate concentrations under pseudo-first order conditions, plotting the pseudo-first order rate constants vs. substrate concentrations and determining whether a non-zero intercept is obtained. A positive intercept is a measure of the extent of exogeneous donor present and it tends to occur most readily in acid solutions or for very slow reactions when for example, trace amounts of contaminant on the walls of the stopped-flow apparatus have a better chance of competing with the substrate. With suitable precautions the positive inter-

cept disappears or can be kept to a few percent of the bimolecular rate constant determined from the slope of the plot. (A finite intercept does not influence the slope of the plot and hence does not influence the rate constant obtained from an accurate slope determination. If however, the rate were measured at only one substrate concentration then a finite intercept would introduce an error into the bimolecular rate constant measurement.) An alternative approach is to prepare compound I or compound II and study the rate of its spontaneous decay. With very pure preparations, a residual rate of decay always remains [264] indicating the presence of endogeneous donor. In other words the enzyme molecules attack themselves albeit very slowly. (The light used for the spectrophotometric measurements of the enzymatic decay can accelerate the process however [295].) The spontaneous decay of HRP-I is always greater than that of HRP-II.

In practice, the kineticist must contend with the total spontaneous decay rate which even with the greatest precautions can vary from vessel to vessel and will even vary with time within the same vessel. (The state of the interior surface of a stopped flow apparatus is very much a function of the reaction being studied and the extent of the study. This surface can be maintained in a "perfect" condition only by never using it for redox studies.) Another potential source of error occurs if measurements are made under second-order conditions, where determination of the rate constant requires accurate concentration measurements and hence requires accurate molar absorptivity measurements. Spectral constants for HRP have been revised very recently (see Table 8).

Pseudo-first order kinetic measurements do not require any knowledge of either the absolute molar absorptivities or concentrations of any reactants or products of the first order process or of inert species contributing to the total absorbance. A major problem with pseudo-first order measurements is that the required large excess of substrate accelerates the rate of the reaction, so that the initial portion of the reaction may occur too rapidly to be measured. Fortunately, with the large molar absorptivities (10^5 M^{-1} cm^{-1}) of the peroxidases and the large changes in absorbance during reactions, it is still possible to make accurate rate constant measurements when the pseudo-first order rate is curtailed by lowering the enzyme concentration. In the case of studies of HRP-I reactions, with native HRP in one syringe of the stopped-flow apparatus and H_2O_2 and reducing substrate in the other, the formation of HRP-I is essentially complete within the dead time of the instrument and the subsequent reduction of HRP-I is readily followed. By setting the monochromator at 411 nm, an isosbestic point for HRP and HRP-II, any competition of HRP-II, formed from the HRP-I reaction, for the excess of substrate is eliminated as a source of error. Thus all pitfalls can be avoided and transient state measurements can be made for individual steps in the HRP cycle with the usual experimental error expected in rate constant determinations (in the range of ±10% to ±20%).

Of the many reactions of HRP-I which have been studied, two are unique

in that they reduce HRP-I directly to the native enzyme. The substrates possessing this ability are iodide and hydrogen sulfite anions [269,321]. Thus for iodide ion as reducing substrate, the enzymatic cycle becomes

$$HRP + H_2O_2 \rightarrow HRP\text{-}I + H_2O \quad (4)$$

$$HRP\text{-}I + I^- \rightarrow HRP + I^{\cdot} \text{ (or } IO^-) \quad (5)$$

Six pieces of evidence were presented which confirmed the correctness of the two-step cycle. Perhaps the simplest piece was the generation of the HRP-I spectrum in the steady state, which would be impossible if HRP-II were part of the cycle [269]. The same mechanism was proposed for the oxidation of iodide by chloroperoxidase, based on an entirely different experimental approach [322]. The product iodine in a +1 oxidation state perhaps IO^-) reacts rapidly with excess iodide to liberate molecular iodine [269]. In steady state experiments, iodine is slowly incorporated into the enzyme [366] but in the time of a single step single turnover experiment followed by transient state kinetics iodine incorporation does not occur [269] so there is no conflict in the literature data in this regard.

Nitrous acid (or nitrite anion) on the other hand, participates in the usual enzymatic cycle, reducing HRP-I to HRP-II to HRP in one-electron steps [59,321] which appears surprising, since it has been reported that it reduces compound I of catalase directly to the native enzyme [69,367,368]. (The importance of the formation of compound II of catalase and its subsequent reactions has been pointed out [369].)

An acid group with a pK_a of 5.1 has a pronounced effect on the HRP-I-bisulfite kinetics and a similar or identical acid group (measured pK_a 4.6) has a profound influence on the HRP-I-iodide reaction. Log rate constant vs. pH profiles for all HRP-I reactions are summarized in Fig. 5. All of the substrates catalyzing a one-electron reduction of HRP-I appear to be influenced by an acid group of $pK_a \sim 5.1$. Preliminary* results using the excellent substrate *p*-cresol indicate that it may be a basic form of the enzyme which is reactive * whereas with the less reactive substrates ferrocyanide [370,371] and *p*-aminobenzoic acid [372] the acid form of the enzyme is reactive. *N*-methyl-*p*-aminobenzoic acid behaves very similarly to *p*-aminobenzoic acid except that there is an acceleration in rate in accordance with the electron-donating ability of the methyl group **. If nitrous acid and not nitrite anion is reactive it would appear to conform to the same mechanism as ferrocyanide and the benzoic acid derivatives, in which case the rate constant, pH independent at higher pH, should increase if the reaction could be studied when the acidity is increased to below pH 6 [321]. It was suggested that the pK_a value of ~5 for an acid group in the active site of HRP-I could represent either a proximal or distal acid group and that a choice between the two pos-

* W.D. Hewson and H.B. Dunford, unpublished results.
** J.S. Stillman and H.B. Dunford, hitherto unpublished results.

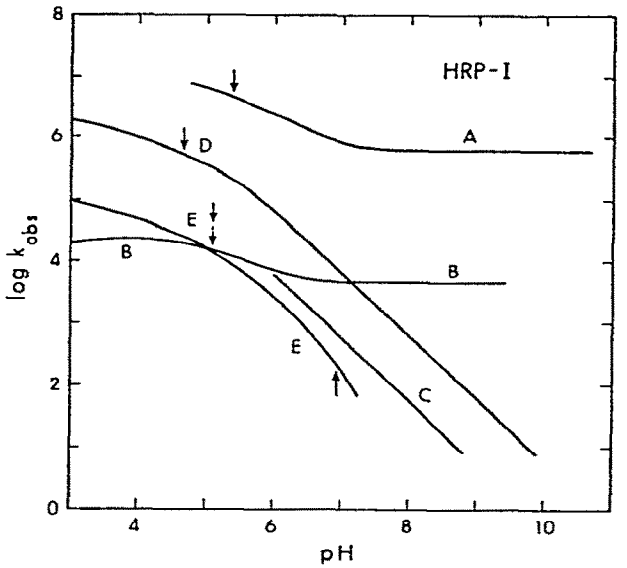


Fig. 5. Log k_{obs} vs. pH for reactions of HRP-I at 25°C and ionic strength 0.11. Units of k_{obs} are M^{-1} s^{-1}. A, ferrocyanide ion [370,371], B, *p*-aminobenzoic acid [372,374], C, nitrite ion or nitrous acid [321], D, iodide ion [269], E, hydrogen sulfite ion [321]. The latter two substances reduce HRP-I directly to the native enzyme. The single slope less than —1 can be accounted for by the influence of the pK_a of 6.9 for the dissociation of HSO_3^-, indicated by ↑. An acid group on HRP-I with a pK_a of ~5.1 appears to be important and is indicated by ↓.

sibilities should be possible on the basis of spectral measurements as a function of pH [373]. These experiments have now been performed. No significant change in spectrum occurs in the critical pH range indicating a distal group *. The study of the reaction of HRP-I with *p*-aminobenzoic acid has been repeated in D_2O. Although the assignment of the isotope effects is complex in acid solution, in the neutral and alkaline region $k_D/k_H = 0.25$ indicating a rate-controlling proton transfer [374].

(d) Reactions of compound II

The precautions necessary for accurate transient state kinetic measurements on HRP-II reactions are of a somewhat different nature from those required for HRP-I studies. An excess of H_2O_2 will ensure the quantitative conversion of native HRP to HRP-I, but with unfavorable consequences, outlined below. The addition of a suitable amount of reducing substrate will then convert the HRP-I to HRP-II. However, as soon as some HRP-II is formed it can compete with the HRP-I for the remaining reducing substrate. At low pH values this competition is very favorable for substrates like *p*-aminobenzoic acid and ferrocyanide with the former actually having a larger rate constant for reaction with HRP-II than HRP-I. Then with some of the

* J.S. Stillman, hitherto unpublished results.

newly formed HRP-II being reduced back to the native enzyme, the cycle is started again by the excess H_2O_2. Therefore it is important to add not more than a stoichiometric amount of H_2O_2 to ensure that no recycling occurs. An accurate assay procedure using HRP to determine concentrations of H_2O_2 has been developed [371]. Depending upon the pH, it may be impossible to convert quantitatively the HRP-I which has been formed to HRP-II. At pH 9 the rate constants are significantly larger for substrates such as ferrocyanide, p-aminobenzoic acid and *p*-cresol reacting with HRP-I than with HRP-II. Therefore with careful work at this pH it is possible to obtain a quantitative yield of HRP-II. The revised spectral data for HRP-II in Table 8 were obtained in this manner. At lower pH values it is convenient to use less than a stoichiometric amount of H_2O_2 and slightly more than a stoichiometric amount of reducing substrate. Then the HRP-I which has been formed is quantitatively converted to HRP-II with a small fraction of the HRP-II being converted back to the native enzyme. A mixture of HRP-II and native HRP is thus obtained but no HRP-I is present. (This can be confirmed by blank experiments conducted at 411 nm, an isosbestic point in the spectra of HRP and HRP-II.) The mixture of HRP and HRP-II can then be placed in one syringe of a stopped flow apparatus and the rate of the HRP-II reaction with whatever reducing substrate has been placed in the other syringe is readily obtained at about 425 nm where maximum change in absorbance occurs for the conversion HRP-II → HRP. Again under pseudo-first order conditions there is no need to know the absolute concentration of enzyme compounds so that quantitative rate measurements are possible. The spontaneous decay of pure HRP-II preparations is very slow whether or not some native HRP is also present.

The aromatic phenols provide an interesting class of reducing substrates, since under suitable conditions one-half mole of substrate can reduce one mole of HRP-I to HRP-II. The titration of HRP-I with guaiacol has been studied in some detail [375].

The substrates which reduce HRP-II to the native enzyme fall into three categories involving two different mechanisms [373,376]. The three categories are for easily oxidized substrates, of which *p*-cresol is the best studied example [377], substrates oxidized with difficulty, (so far only iodide ion fits into this category [378]) and substrates of an intermediate nature, including ferrocyanide [370,371], *p*-aminobenzoic acid [372,374] and *N*-methyl p-aminobenzoic acid *. Log rate constant vs. pH profiles are summarized in Fig. 6. The most striking feature of the profile for the *p*-cresol reaction is the pronounced influence of an acid group with a pK_a value of 8.6. Only the acid form of this group is reactive as shown by studies carried up to pH 11 [377]. Participation of this group is essential for facile reactants. An alternative mechanism of catalysis is applicable to iodide oxidation, where the log rate vs. pH profile has a slope of −1 within small experimental

* J.S. Stillman and H.B. Dunford, hitherto unpublished results.

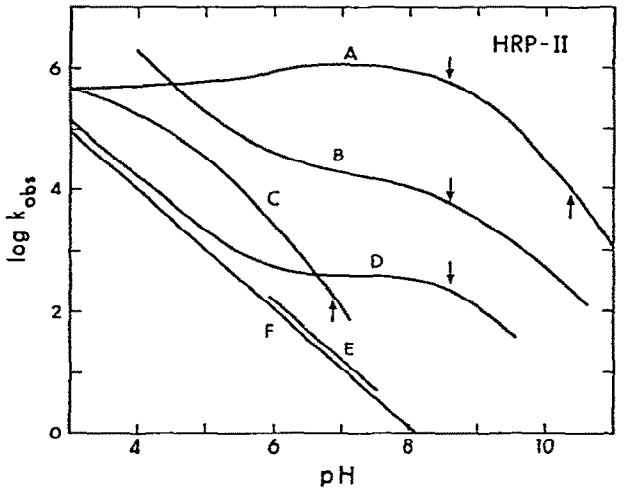


Fig. 6. Log k_{obs} vs. pH for reactions of HRP-II. Units of k_{obs} are M^{-1} s^{-1}. A, *p*-cresol [377]. B, ferrocyanide ion [370,371]. C, hydrogen sulfite ion [321]. D, *p*-aminobenzoic acid [372,374]. E, nitrite anion or nitrous acid [321]. F, iodide ion [378]. The curves for HSO_3^- reacting with both HRP-I (Fig. 5) and HRP-II are similar. Since HSO_3^- reduces HRP-I to native HRP, the possibility exists that HSO_3^- might reduce HRP-II to ferro-HRP. The slopes less than —1 can be accounted for by the influence of the pK_a's of 10.4 for *p*-cresol and 6.9 for HSO_3^- indicated by ↑. Substrates can be classified as (i) readily reduced, e.g. *p*-cresol, (ii) reduced with difficulty, e.g. I^-, NO_2^- and (iii) intermediate in nature, e.g. ferrocyanide ion and *p*-aminobenzoic acid. A pK_a of 8.6 for an acid group on HRP-II is clearly indicated by ↓ for substrates of types (i) and (iii), and a pK_a of ~0 for substrates of types (ii) and (iii) [352,372,373,376].

error over the pH range 2.7 to 9.0 [378]. The participating acid group is clearly on the enzyme and its pK_a value is in the vicinity of 0 [352,378]. By analogy, the reaction of nitrite with HRP-II can be explained by the same mechanism as for the HRP-II—iodide reaction, but because of the much weaker nature of nitrous acid compared to hydriodic acid, it is not possible to prove it using the diffusion-controlled limit [352]. Thus the oxidation of iodide must be described as HE reacting with I^-; the nitrite reaction might be HE + NO_2^- or E + HNO_2.

The reducing substrates of intermediate reactivity follow a combined mechanism in which the catalytic effect of the acid group of pK_a 8.6 is dominant in neutral and alkaline solutions but is superceded by the effect of the acid group of pK_a ~ 0 in acid solutions [373,376] which is in accordance with the Hammond postulate [379]. An equilibrium exists between the solvent and the acid form of the strong acid group during the reaction whereas transfer of the enzyme proton from the acid group of pK_a 8.6 occurs in a rate-controlling step as indicated by k^D/k^H ratios [374,376]. The acid group of pK_a ~ 0 has been tentatively identified [373,374] as the imidazole group in the fifth coordination position of the heme iron. The iron perhaps becomes

five-coordinate with the protonated imidazole group leaving its coordination position [374]. A shift of ~7 pK_a units appears reasonable for an imidazole group coordinated to Fe^{IV} compared to a free imidazole group. The acid catalysis of the HRP-II—ferrocyanide reaction was noted much earlier, with the postulate that the proximal group which becomes protonated is the ferryl oxygen atom [61]. Whichever explanation is correct, the spectra of the HRP-II with the protonated proximal group would be expected to differ markedly from that of the basic form of HRP-II. However, the fraction of HRP-II in this acid form becomes vanishingly small at pH ranges where spectral measurements become feasible. The acid group corresponding to the pK_a value of 8.6, on the other hand, must be a distal group, since spectral measurements are readily made in the vicinity of pH 8.6 and spectral changes are negligibly small as a function of pH [377]. An *N*-terminal amino group or an ϵ-amino group of lysine are potential candidates [373]. The rate-controlling transfer or partial transfer of this proton during catalysis could be to the proximal histidine residue [373,374].

Studies of HRP reactions have led to advances in the theory of acid—base catalysis [380—382].

A feature of the single step, single turnover studies on HRP-II reactions which sometimes occurs involves a modified form of saturation kinetics, for which the simplest reaction schemes are

$$E + S \overset{K_p}{\rightleftharpoons} ES \overset{k_1}{\rightarrow} E' + Prod.$$

$$ES + S \overset{k_2}{\rightarrow} E' + S + Prod.$$

in which productive binding occurs, and

$$E + S \overset{K_u}{\rightleftharpoons} ES$$

$$E + S \overset{k_3}{\rightarrow} E' + Prod.$$

$$ES + S \overset{k_4}{\rightarrow} E' + S + Prod.$$

where unproductive binding occurs. HRP-II is represented by E and native HRP by E'. Both schemes lead to equations of the form

$$k_{obsd} = \frac{k_a[S] + k_b[S]^2}{[S] + K}$$

where $k_a = k_1$ or k_3K_u, $k_b = k_2$ or k_4 and $K = K_p$ or K_u, so that it is impossible to distinguish between them kinetically. Binding was observed in the *p*-cresol reaction with HRP-II at higher pH and it was argued on indirect evidence that it was non-productive [377]. Binding was observed in the reaction of *p*-aminobenzoic acid with both HRP-I and HRP-II at low pH which

was assumed to be non-productive [372]. The stoichiometry and end-product for the reduction of HRP-I by phenols [375] may provide some additional evidence for non-productive binding. The second oxidizing equivalent in HRP-I appears to be located close to the Fe^{IV} of the heme [225]. Hence an intimate productive complex might be expected to reduce HRP-I directly to the native enzyme since it appears that the free radical formed in the initial step is highly reactive [375]. Instead of HRP-I reduction to HRP with a 1 : 1 stoichiometry of HRP-I : phenol the HRP-I is reduced to HRP-II with a 1 : 2 stoichiometry [375]. Although unproductive binding is favored for "simple" reductions of HRP-I and HRP-II, an obvious role for a ternary complex can be seen for iodination reactions.

Reactions of HRP-I and HRP-II are not affected by the oxidizing substrate used in their preparation which is strong evidence that they are not enzyme—substrate complexes [62,371].

(e) Ferroperoxidase; formation and reactions of compound III

As is outlined below there is an intimate connection between ferro-HRP and HRP-III. Ligand binding studies [382—384], and photodissociation studies on ferrous ligand complexes have been performed [382,385] as well as studies on the recombination of liganded heme with the apoperoxidase [386]. HRP-III does not participate in the normal peroxidatic cycle. However, its formation, structure and reactivity are of great interest from several standpoints as outlined below. There appear to be four possible pathways for the formation of HRP-III.

(a) The reaction of the native enzyme with a large excess of hydrogen peroxide. Historically, this represents the first synthesis of HRP-III [48].

(b) Reduction of the native enzyme which contains ferric iron to ferroperoxidase followed by oxygen addition. The role of peroxidase as an oxidase was first outlined in 1939 [387,388]. Oxygen is consumed and dihydroxyfumarate is oxidized in the presence of peroxidase, with the reaction accelerated by the presence of Mn^{2+}. Addition of H_2O_2 initiates the reaction. However, certain substrates such as quinoles and indoleacetic acid can reduce the enzyme directly to the ferrous state which then forms compound III [389—391]. The earlier literature which contains a lively discussion as to the importance of the reduction of the ferriperoxidase and indeed as to whether reduction does occur prior to HRP-III formation is well reviewed [70,392—396]. However, the following experiments appear to confirm that HRP-III is formed by the addition of oxygen to ferroperoxidase.

The reduction of ferri- to ferroperoxidase by hydrosulfite (dithionite) is neither a rapid nor a clean reaction. However, when the reaction has gone to completion and excess hydrosulfite is removed oxygen addition results in HRP-III formation [397,398]. Photolysis of the ferroperoxidase—carbon monoxide complex in the presence of oxygen also leads to formation of compound III [244,399].

(c) The reaction of H_2O_2 with HRP-II yields HRP-III as product [60,400].

(d) The addition of superoxide anion to ferriperoxidase also produces HRP-III [401].

All of these experiments are compatible with an oxygen—ferroperoxidase compound as the structure of HRP-III [321] or its isoelectronic equivalent, a superoxide—ferriperoxide compound. That HRP-III has a similar structure to the oxygen—myoglobin compound is supported by the similarity of their spectra [400]. Titration results show that HRP-III contains four oxidizing equivalents in excess of the ferrous state [398,399]. Infrared evidence indicates that the O_2 bound by myoglobin and hemoglobin is held by more than a single chemical bond, which may be applicable to the structure of HRP-III [402,403].

In general reactions of HRP-III are much slower than those of HRP-I and HRP-II [404—409]. However, an exception is the rapid reaction of the plant hormone indoleacetic acid with HRP-III, suggesting a physiological role for the reaction [410—416]. The decomposition of HRP-III is accelerated by excess ferroperoxidase, leading to the proposal that the reaction is a suitable model for a terminal oxidase [417]. Of great interest is the possible role of HRP-III in reactions in which hydroxylation of aromatic molecules has been observed [418—421].

Further compounds can be formed by exposure of HRP to excess H_2O_2 [422].

(f) Oscillatory kinetics

Consider the coupled reaction scheme shown in Fig. 7. The faster the cycles involving X, Y and Z turn over, the faster the cycle in V turns over which produces 2V for each entering V. V in turn produces W faster which in turn makes the cycles in X, Y and Z go faster. Conversely the slower the process, the rate of production of V is slowed even more. The scheme is one to which steady state kinetics may not apply, in which case there may be sustained oscillations, damped oscillations or exponential growth in plots of concentration vs. time. Such phenomena have long been known in both chemistry and biology, but interest has been greatly enhanced recently because of progress being made in understanding the processes on a quantitative basis [423—425]. At the level of a single enzyme, reactions of com-

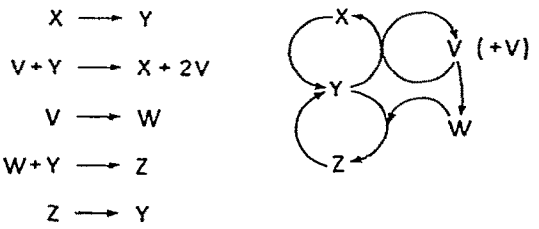
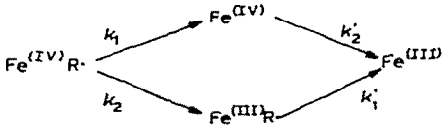


Fig. 7. Two alternative representations of a reaction scheme which might lead to oscillatory kinetics.

pound III of HRP may provide the first biological example of oscillations [426—428], where the interconversion of ferroperoxide compound III oscillates in phase with the oscillatory consumption of O_2. Sustained oscillations have been observed in lactoperoxidase compound III reactions [429]. A theory involving an intermediate species has been developed for both steady state and oscillatory reactions in the photochemical behavior of hemoproteins [430].

E. CYTOCHROME *c* PEROXIDASE

This enzyme, discovered in yeast in 1940 [30] has been purified, crystallized and its properties studied. Work up to 1969 which includes studies by Ellfolk and Yonetani and coworkers has been reviewed [188]. It was shown that with a pure preparation of CcP one mole of H_2O_2 reacts with one mole of CcP to form a compound (called complex ES) which contains both oxidizing equivalents of the H_2O_2 [431]. Complex ES of CcP has an optical spectrum similar to that of HRP-II. A free radical signal in complex ES, detected in EPR studies, is apparently contained in an amino acid residue close to, but not immediately adjacent to, the heme iron [220]. The Mössbauer spectrum of complex ES indicates that the heme iron is in an oxidation state of +4 [260] so that $Fe^{IV}R\cdot$ provides a descriptive schematic representation. Subsequent titration and kinetic experiments indicated that there are two intermediate compounds with one less oxidizing equivalent than complex ES, one of which, $Fe^{III}R\cdot$, has an optical spectrum similar to that of the native enzyme, and the other of which, Fe^{IV}, has an optical spectrum similar to that of complex ES [432]. The observations are compatible with a reaction scheme



(6)

in which k_1 and k_1' are rate constants for reaction of reducing substrate with the radical site and k_2 and k_2' for reaction with the Fe^{IV} site [432]. The data are also compatible with an equilibrium mechanism

$$Fe^{IV}\,R\cdot \xrightarrow{k_1} \begin{bmatrix} Fe^{IV} \\ \Updownarrow\ K \\ Fe^{III}\,R\cdot \end{bmatrix} \xrightarrow{k_2} Fe^{III} \qquad (7)$$

which does not specify the reactive enzyme species in the reaction with rate constant k_2. The latter scheme is favored because fluoride ion, which does not bind to $Fe^{IV}R\cdot$ (indicating the sixth coordination position is blocked in Fe^{IV}) enhances the free radical signal in a mixture of $Fe^{III}R\cdot$ and Fe^{IV}. Values of K change as a function of pH [432].

The kinetics of fluoride and cyanide binding by CcP have been thoroughly investigated by the stopped flow method [433]. The native enzyme is stable over the pH range 4 to 7.5 but this range can safely be extended to pH's from 3.5 to 9 by using a pH-jump method in the stopped flow experiments [433]. The apparent association rate constants can be described using either of the schemes

$$\begin{array}{ccc} & HE + L & \xrightarrow{k_1} EL + H^+ \\ K_E \Updownarrow & \Updownarrow & \Updownarrow K_L \\ & E \quad HL & \end{array}$$

$$\begin{array}{ccc} & HE \quad L & \\ K_E \Updownarrow & \Updownarrow & \Updownarrow K_L k_2 \\ & E + HL & \longrightarrow EL + H^+ \end{array}$$

For fluoride binding, $k_1 = 5.1 \times 10^7\ M^{-1}\ s^{-1}$, $k_2 = 1.6 \times 10^5\ M^{-1}\ s^{-1}$, $K_L = 10^{-3}$ M and $pK_E = 5.5$. For cyanide binding $k_1 = 1.1 \times 10^5\ M^{-1}\ s^{-1}$, $k_2 = 4.4 \times 10^3\ M^{-1}\ s^{-1}$, $K_L = 1 \times 10^{-9}$ M and $pK_E = 5.4$. The pK_E value clearly refers to an acid group on the enzyme, K_L refers to the dissociation constant of either HF or HCN, and k_1 and k_2 are interrelated rate constants. The two schemes are kinetically indistinguishable since for both cyanide and fluoride, neither k_1 nor k_2 exceeds the diffusion-controlled limit [352]. However, reasons for favoring the species HF and HCN as participants in the rate-controlling step are well discussed [433]. The rates of dissociation of both the cyanide and fluoride complexes show small pH dependencies, which when interpreted in terms of the influence of acid–base groups considerably complicate a comprehensive kinetic scheme describing both the association and dissociation reactions [433]. There appears to be substantial agreement both in behavior and interpretation of ligand binding reactions for HRP and CcP. The reaction of cyanide with CcP is complicated by isomerization reactions in alkaline solution [433] an effect which does not occur in the glycoperoxidases HRP and LP [181,346,434].

The kinetics of the reaction of CcP with hydrogen peroxide to form complex ES, have been investigated [435]. At 25°C and ionic strength 0.1, the true bimolecular rate constant is $4.5 \times 10^7\ M^{-1}\ s^{-1}$ for the reaction of neutral H_2O_2 with a basic form of the enzyme. The pK_a of the acid group on the enzyme is 5.5 in agreement with the results from cyanide and fluoride binding [435].

The first intensive studies of the kinetics of reductions of oxidized CcP have recently been published [436,437]. In these studies the names compound I (for complex ES) and compound II (for the mixture of Fe^{IV} and $Fe^{III}R\cdot$) are used, which is consistent with the terminology for other peroxidases. In all cases the transient state kinetics were monitored near 424 nm, the position of maximum difference in absorbance between the Fe^{IV} and Fe^{III} species. With an excess of substrate, biphasic first order kinetics are observed. If the equilibrium mechanism, described above, is valid, then the

initial (faster) phase represents the reduction of CcP-I to CcP-II and the later (slower) phase the reduction of CcP-II to native CcP. The rate constants for the two processes differ by a factor of about three. Since the amplitude of the absorbance change is much smaller for the first step, the rate constants for the reduction of CcP-II to CcP are obtained with greater precision. When the reducing substrate is the neutral molecule dicyano-bis(1,10-phenanthroline) iron(II) a rate constant of 2.9×10^5 M^{-1} s^{-1} at 25°C and ionic strength 0.1 is obtained in acid solution [437]. An ionizable group on the enzyme with a pK_a of 4.5 strongly influences the reaction rate. The basic form of the enzyme is unreactive over the pH range 3.5—6 [437].

When the reducing substrate is ferrocyanide [436] the most striking feature of the transient state results is that the log k vs. pH plot, containing over 20 experimental points, is linear with a slope of —1 over the pH range 5 to 8 for the reaction of CcP-II indicating that the acid catalysis mechanism valid for the phenanthroline compound is also valid for ferrocyanide [437]. Therefore, the original explanation of the ferrocyanide results [436] which can be criticized on several grounds [374,438] is subject to reinterpretation [435].

The nature of CcP-I has been investigated using substrate analogs of hydrogen peroxide [439]. Complex formation between cytochrome *c* and native CcP has been intensively investigated [440—442].

F. CHLOROPEROXIDASE, CYTOCHROME *P*-450

The detection of a peroxidase in the mold *Caldariomyces fumago* was reported in 1961 [443]. It was subsequently isolated, obtained in crystalline form and its properties have been studied [12]. This peroxidase appears to be unique in three interesting ways. It possesses the unique ability to oxidize chloride ions, hence the name chloroperoxidase [13]. Furthermore, the product distribution in the chlorination of anisole corresponds to attack by an electrophilic reagent, (Cl^+?) when the reaction is catalyzed by chloroperoxidase, in contrast to a free radical mechanism when sulfuryl chloride, benzoyl peroxide and light are used to generate chlorine atoms [322]. Thus halogenation reactions involve the direct conversion of compound I back to the native enzyme with the acceptance of two electrons from the halide ion for both ClP [270,322,444] and HRP [269].

The second unique property possessed by ClP is its ability to catalyze reactions formerly thought to be unique to one heme-containing enzyme, catalase [26,71]. Thus, ethyl alcohol is oxidized to acetaldehyde by ClP. Similarly the catalatic reaction $2\ H_2O_2 \rightarrow 2\ H_2O + O_2$ is catalyzed by ClP. In addition, ClP can catalyze the evolution of oxygen from ethyl hydrogen peroxide and *m*-chloroperbenzoic acid, a reaction not catalyzed by either catalase or HRP [322]. ClP is also able to catalyze the typical peroxidatic reactions in two successive one-electron steps. It is not as efficient as catalase in reactions

catalyzed by the latter enzyme, nor as efficient as HRP in the typical peroxidatic reactions.

Finally, ClP when reduced to the ferrous state has properties very similar to cytochrome *P*-450 [445]. The latter enzyme obtained its name because of the unique position of the center of the absorption band for its carbon monoxide complex, 450 nm as is also found for chloroperoxidase [445], in contrast to the 420 nm found for most ferrous heme protein—CO complexes. Cytochrome *P*-450 was first detected in rat liver microsomes [446,447]. It possesses the ability to hydroxylate a variety of substrates [448,449] and appears to be involved in the metabolism of a variety of compounds including drugs [450—452]. Studies of cytochrome *P*-450 have been hampered by the inability to obtain homogeneous solutions in the absence of detergents or other solubilizing agents, indicating that it is usually a membrane-bound enzyme. However, cytochrome *P*-450_{cam}, isolated from *Pseudomonas putida*, can be obtained in a soluble form in the absence of solubilizing agents [453—456] which has facilitated the direct comparison with reduced chloroperoxidase [445]. Cytochrome *P*-450_{cam} is so named because it readily hydroxylates camphor. The hydroxylation reactions catalyzed by cytochrome *P*-450 and ClP may be initiated by the electrophilic reagent OH^+ [457,458] which may originate from the ferrous oxygen compound. Chemiluminescence of oxygenated ferrous cytochrome *P*-450 has been observed [342].

The evolution of oxygen from *m*-chloroperbenzoic acid, uniquely catalyzed by ClP, has been utilized in ^{18}O double-labelling experiments which are the most definitive to date in the elucidation of the structure of compound I [354,355]. The isotopic compositions of the evolved oxygen indicated clearly that a scrambling mechanism had occurred, in contrast to a retention mechanism. The results are consistent with a ferryl type of structure for compound I, Fe—O, containing one oxygen atom. Oxygen evolution occurs via the formation of a trioxide complex so that the chloroperoxidase reverts to its native state and a *m*-chlorobenzoate ion is formed simultaneously with the oxygen evolution. When the reaction was initiated in H_2O^{18} with unlabelled *m*-chloroperbenzoic acid no ^{18}O incorporation into the evolved oxygen or the benzoic acid product occurred, which eliminates reaction of the intermediate with the solvent. If compound I contained no oxygen, it appears impossible to write a plausible mechanism other than

$$R{-}\overset{\overset{O}{\|}}{C}{-}OOH \xrightarrow{\text{Compound I}} R{-}C^{+}{=}O + O_2 + H^{+}$$

which is eliminated by the isotopic scrambling. If compound I contained the acyl moiety, then *m*-chloroperbenzoate anion could not be released quantitatively in the presence of excess enzyme, which is contrary to observations [264,355]. Furthermore, it is known that reactions of HRP-I occur at identical rates regardless of the type of peroxide used in its generation [371].

It has recently been found that chlorite reacts with both ClP and HRP to form an intermediate compound capable of chlorinating various substrates [459]. It was found that one mole of chlorite produced two moles of compound I in agreement with complete use of the 4 oxidizing equivalents contained in chlorite. The reaction appears to proceed by the mechanism

$$E + H_2O + ClO_2^- \rightarrow E{-}O{-}Cl + OOH^-$$

$$E + OOH^- \rightarrow \text{Compound I}$$

The E—O—Cl species which catalyzes the chlorination reactions has an absorption maximum at 417 nm and rapidly decomposes to compound I. Thus HRP can be utilized as a chlorinating agent, although HRP-I is not a good enough oxidant to oxidize chloride ion, unlike ClP-I [459a].

The halogenation reactions and halogen-dependent reactions catalyzed by ClP-I have a marked pH dependence, being faster in acid solutions [270]. A quantitative measure of the pK_a's of the relevant acid groups has yet to be made. Transient state kinetic studies of chloroperoxidase reactions have been limited to two measurements of the rate of compound I formation, from H_2O_2 at pH 2.8 where $k = 1.5 \times 10^6$ M^{-1} s^{-1} [444] and from *m*-chloroperbenzoic acid at pH 6.0 where $k = 9.9 \times 10^6$ M^{-1} s^{-1} [354].

G. OTHER PEROXIDASES, HALOGENATION REACTIONS

Among other plant peroxidases, most work has been done on Japanese radish peroxidase (see ref. 209 and earlier references) with work on the turnip peroxidases being performed at an accelerating rate [460—463]. Among mammalian peroxidases, lactoperoxidase has received the most attention (see refs. 464 and 465 as references to considerable earlier work on LP). Unfortunately available preparations of LP are of considerably lower purity than those of HRP. Thus it was not possible to study LP-I formation by transient state kinetics since the LP-I decayed spontaneously almost as fast as it was formed. However, it was possible to make LP-I formation rate-controlling in a steady state study [356]. The different pH-rate profiles for cyanide formation compared to compound I formation from LP indicates a fundamental difference in the rate-controlling step for the two processes [434].

It may be that mammalian peroxidases tend to be bound rather tightly to membranes which would account for difficulties in isolation and purification. Because of the established physiological importance of thyroid peroxidases it is unfortunate that pure preparations have not been obtained. Halogenation reactions catalyzed by peroxidases are being reviewed elsewhere [466]. (See also the discussion of HRP and ClP.) One interesting aspect of reactions involving iodine is that nature may make use of the heavy atom to facilitate reactions which would otherwise be spin forbidden [467,468].

H. SUMMARY

By use of a wide variety of techniques, great advances have been made in our understanding of the peroxidases. A few conclusions follow. At a qualitative level similarities between the peroxidases appear to outnumber the differences. Thus complex ES (compound I) of cytochrome *c* peroxidase appears to differ from other compounds I mainly in that the free radical portion (contained on an amino acid residue) is somewhat farther removed from the heme iron. The compounds I of other peroxidases may contain heme group π-cation free radicals. Alternatively, they may be similar to complex ES of CcP except that the free radical is so close to the heme group that some of the electronic absorption bands of the heme are seriously perturbed. Which of the two possibilities is correct should be settled shortly. It now appears established that compound I of chloroperoxidase is a ferryl type of structure containing Fe^{IV} and this is likely to apply to compound I and compound II of all heme-containing peroxidases. Compound I formation occurs at close to the diffusion-controlled limit for a bimolecular process at a restricted active site, which is remarkable considering the complexity of the process. Transient state kinetic studies show that there are two mechanisms of acid catalysis involving three classes of substrates for the reduction of HRP-II to native HRP. At least one of two acid groups of $pK_a \sim 0$ and 8.6 is important for all substrates. Again rate constants are at, or approach, the diffusion-controlled limit. However, when the more acid group is effective a vanishingly small portion of the total amount of HRP-II is catalytically active at physiological pH. Certain reducing substrates are two electron donors to compound I. A variety of techniques indicates that the imidazole group of a histidine residue occupies the fifth coordination position of the heme iron of HRP. This group appears to play an important role in the catalytic mechanism.

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