Cytochrome P$_{450}$: Substrate and Prosthetic-Group Free Radicals Generated During the Enzymatic Cycle

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Cytochrome P_{450}: substrate and prosthetic-group free radicals generated during the enzymatic cycle

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During the enzymatic cycle of the cytochromes P_{450}, dioxygen binds to the ferrous háemprotein when the resting ferric haemprotein has undergone a one-electron oxidation after substrate binding. A further one-electron reduction generates an intermediate that is isoelectronic with a peroxide dianion coordinated to a ferric iron. Heterolytic cleavage of the O—O bond generates water and a species which is formally an oxene (oxygen atom) coordinated by iron(III). However, on the basis of model reactions and by analogy to the catalases and peroxidases, this active oxidizing intermediate is formulated as an oxo–Fe^{IV} porphyrin π-cation radical. The radical is stabilized by delocalization on the porphyrin macrocycle and the high oxidation state is achieved by oxidizing both the metal and the porphyrin ring of the haemprotein.

Hydrogen atom abstraction from a saturated hydrocarbon substrate generates a substrate free radical, constrained by the protein binding site, and the equivalent of a hydroxyl radical bound to iron(III). Coupling of the 'hydroxy' and substrate radicals generates hydroxylated product and resting protein.
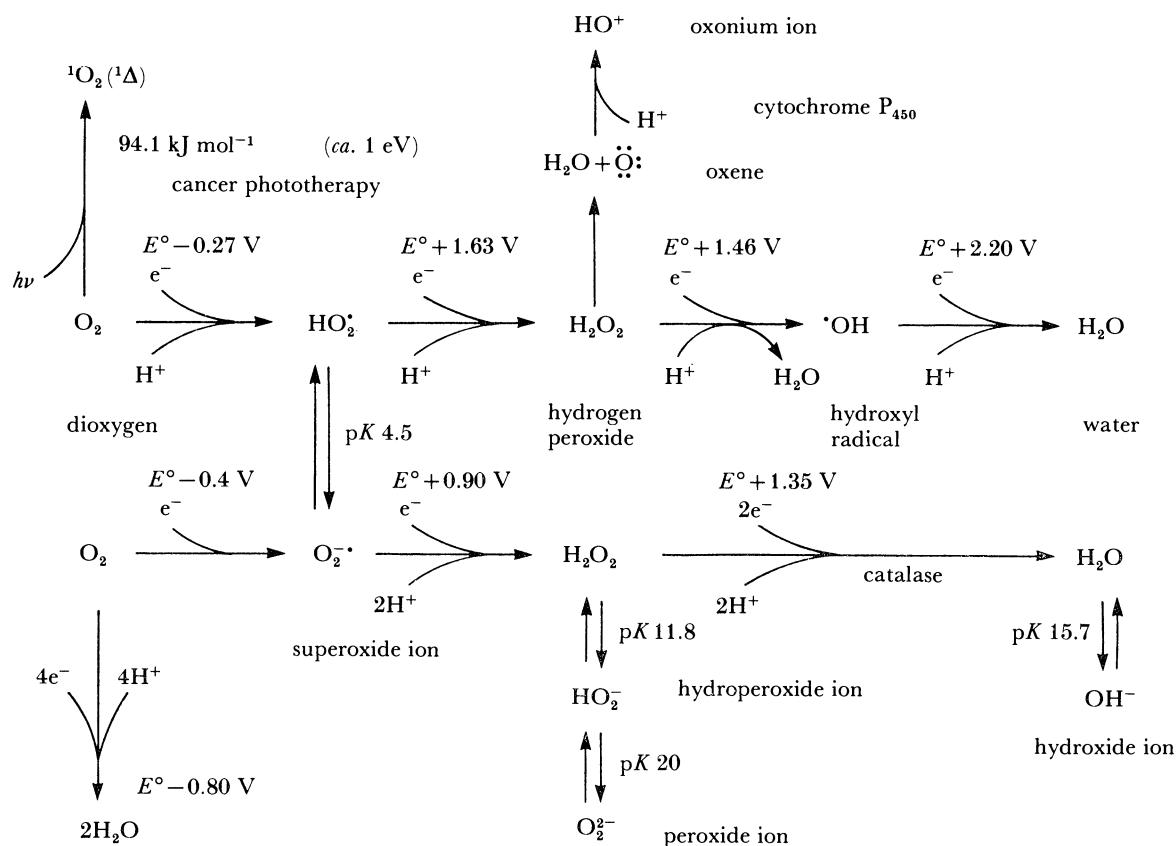
For olefins an initial electron transfer to oxidized haemprotein gives a substrate cation radical. Further reaction of this radical can give the epoxide, the principal product; an aldehyde or ketone by rearrangement; or an alkylated haemprotein resulting in suicide inhibition.

Two classes of radicals occur in Nature: those which are destructive and unwelcome in living cells and for which complex and elegant protective mechanisms exist and those which are generated and controlled enzymatically (Boschke 1983). Many examples of both types occur in the reductive chemistry of the naturally occurring stable free radical, triplet dioxygen. Scheme 1 outlines some of the radical species generated from the reduction and subsequent reactions of dioxygen.

Much of the chemistry of dioxygen and its reduction products is controlled, naturally, by haemproteins. The classic example is the catalase-catalysed destruction of hydrogen peroxide (Hewson & Hager 1979). As we shall see, this decomposition of hydrogen peroxide occurs without formation of hydroxyl radicals; however, stable radicals are generated on the haem moiety (Dolphin & Felton 1974). A closely related family of enzymes, the peroxidases, also use hydrogen peroxide as an oxidant but then generate substrate (aromatic amines, phenols) free radicals which may have biological significance (D. H. R. Barton, this symposium).

We plan to show in this paper that the cytochromes P_{450}, currently the most widely studied of all enzymes, function in a manner analogous to the catalases and peroxidases and generate both haem and substrate radicals under carefully controlled conditions.

Interest in these haemproteins arose from our studies on the redox properties of metallo-porphyrins containing a redox-inert metal (Fajer *et al.* 1970). Thus both zinc and magnesium

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$^1O_2\,(^1\Delta)$

HO^+ oxonium ion

$94.1\ kJ\ mol^{-1}$ (ca. 1 eV)   cytochrome P_{450}

cancer phototherapy

H^+

$H_2O + \overset{\cdot\cdot}{\underset{\cdot\cdot}{O}}{:}$ oxene

$h\nu$

| $E^\circ - 0.27\ V$ | $E^\circ + 1.63\ V$ | $E^\circ + 1.46\ V$ | $E^\circ + 2.20\ V$ |
| e^- | e^- | e^- | e^- |

$O_2 \longrightarrow HO_2^\cdot \longrightarrow H_2O_2 \longrightarrow {}^\cdot OH \longrightarrow H_2O$

H^+ H^+ H^+ H_2O H^+

dioxygen   pK 4.5   hydrogen peroxide   hydroxyl radical   water

| $E^\circ - 0.4\ V$ | $E^\circ + 0.90\ V$ | $E^\circ + 1.35\ V$ |
| e^- | e^- | $2e^-$ |

$O_2 \longrightarrow O_2^{-\cdot} \longrightarrow H_2O_2 \longrightarrow H_2O$

$2H^+$ catalase

superoxide ion $2H^+$ pK 15.7

$4e^-$ $4H^+$ pK 11.8

HO_2^- hydroperoxide ion OH^-

hydroxide ion

$E^\circ - 0.80\ V$ pK 20

$2H_2O$ O_2^{2-} peroxide ion

cytochrome oxidase

SCHEME 1

porphyrins exhibit two reversible one-electron oxidations. The first oxidation generates a cation radical and e.p.r. showed that the unpaired electron was delocalized within the aromatic π-system of the porphyrin macrocycle. However, two classes of these π-cation radicals were observed. The highest filled π-orbitals of porphyrins and metalloporphyrins are degenerate with a_{1u} and a_{2u} symmetries (Hanson *et al.* 1976). Removal of a single electron from one or other of these orbitals, determined by the porphyrin peripheral substituents and the metal and its axial coordination, generates either a $^2A_{1u}$ or $^2A_{2u}$ ground state for these π-cation radicals. The $^2A_{1u}$ states are characterized by low unpaired spin density on the *meso*-carbon and nitrogen atoms, while in the $^2A_{2u}$ states, spin density appears on these atoms (Fajer *et al.* 1973).

The two ground states also exhibit quite different, but characteristic optical spectra. Figure 1 shows the optical spectra of the π-cation radicals of the magnesium complexes of octaethylporphyrin (OEP) ($^2A_{1u}$) and *meso*-tetraphenylporphyrin (TPP) ($^2A_{2u}$) ground states. Figure 2 shows the π-cation radicals derived from the one-electron oxidation of $Co^{III}OEP$. In this case, the species $Co^{III}(OEP)^{+\cdot}$ occupies a $^2A_{1u}$ state when coordinated by bromide but a $^2A_{1u}$ state when the bromide is replaced by perchlorate. Of special note is part *b* of figure 2; this shows the optical spectra of catalase and peroxidase after the resting ferric haemproteins have been oxidized, with hydrogen peroxide, by two electrons. Clearly these high oxidation states, known as compounds I, are porphyrin π-cation radicals (Dolphin *et al.* 1971). Mössbauer spectroscopy (Moss *et al.* 1969) had already suggested an oxidation of the iron,
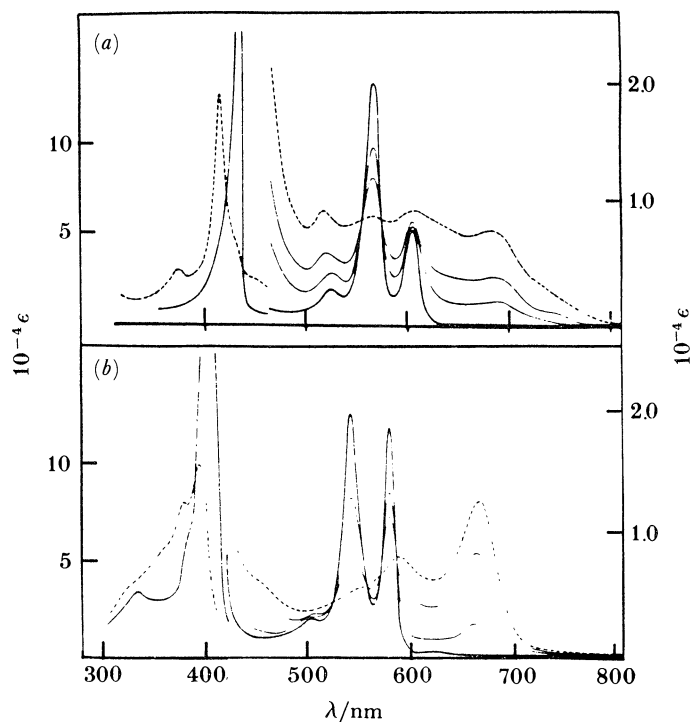
[130]

FIGURE 1. Optical changes during the electrochemical oxidation of magnesium porphyrins. (a) Mg^{II}OEP (solid line); Mg^{II}(OEP)$^{+\cdot}$ (dotted line). (b) Mg^{II}TPP (solid line); Mg^{II}(TPP)$^{+\cdot}$ (dotted line).
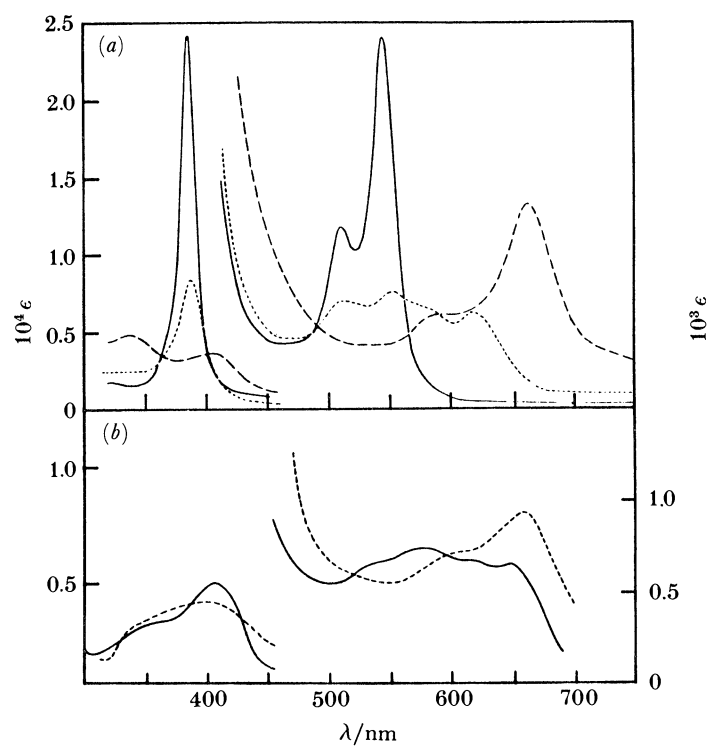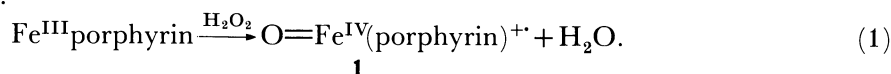

FIGURE 2. (a) Co^{III}(OEP)Br (solid line); Co^{III}(OEP)$^{+\cdot}$ (Br)$_2$ (broken line); Co^{III}(OEP)$^{+\cdot}$ (ClO$_4$)$_2$ (dotted line). (b) Catalase compound I (solid line); horseradish peroxidase compound I (broken line).

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and this coupled with the porphyrin oxidation and the stoichiometry allows the reaction to be represented as in (1).

$$Fe^{III}porphyrin \xrightarrow{H_2O_2} O{=}Fe^{IV}(porphyrin)^{+\cdot} + H_2O. \qquad (1)$$

$$\mathbf{1}$$

The coordination of the oxygen to the iron was established (Hager *et al.* 1972) and elegantly confirmed by ENDOR experiments (Roberts *et al.* 1981).

While the simple porphyrin π-cation radicals exhibit textbook-quality e.p.r. spectra, the same is not true for the compounds I of catalase and horseradish peroxidase. Indeed, only recently has the e.p.r. signal of the Fe^{IV} porphyrin π-cation radical been observed, a difficult task considering it spans thousands of gauss (Schultz *et al.* 1979).

Now that the oxo-Fe^{IV} π-cation radical is well established (excepting for some discussion as to the specific doublet ground state of some haemproteins (Rutter *et al.* 1983) for catalase and the peroxidases we would like to suggest that the cytochromes P_{450} use the 'same' high oxidation state intermediate during their enzymatic cycle.

Scheme 2 shows the enzymatic cycle for the cytochrome P_{450}. Binding of CO to the ferrous intermediate gives the characteristic absorption at *ca.* 450 nm (Hanson *et al.* 1976) for which this family of enzymes are named (Omura & Sato 1962). This atypical haem spectrum was shown to result from the axial coordination of a thiolate (cysteinyl) anion to the ferrous iron (Collman & Sorrell 1975; Chang & Dolphin 1975). The thiolate remains coordinated when the natural ligand, dioxygen, binds (Dolphin *et al.* 1980).

SCHEME 2
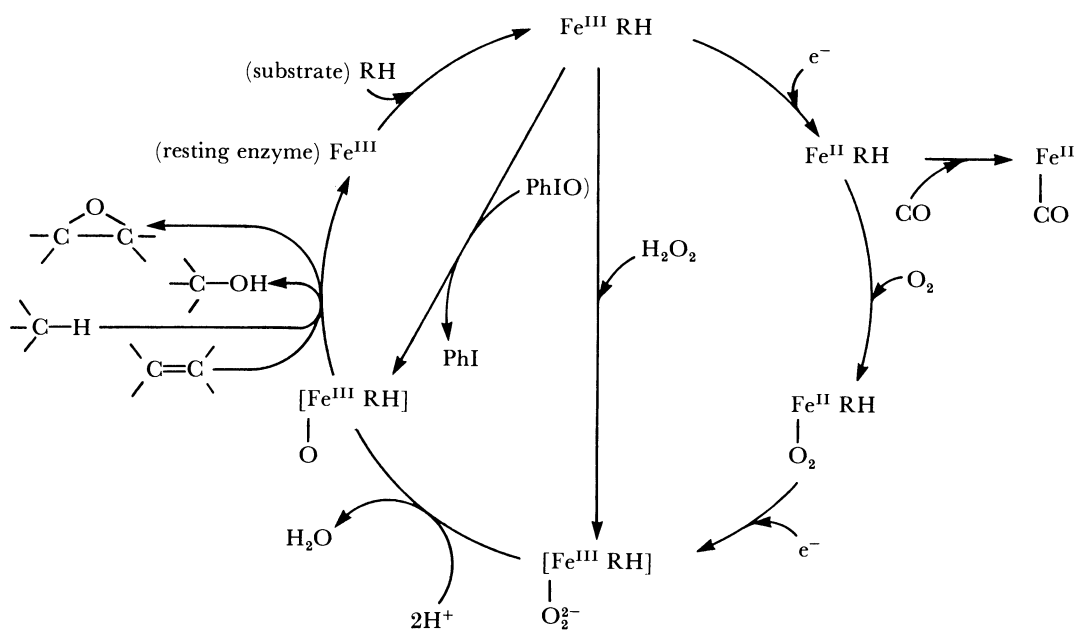
The rate-limiting enzymic step is the second one-electron reduction (Coon & White 1980). No enzymatic intermediates have been observed beyond this step and further discussion relates to studies with model iron and ruthenium porphyrins.

The resting ferric enzyme may be shunted by using hydrogen peroxides or peracids, suggesting that the final one-electron reduction product may be a ferric peroxide complex. A

one-electron electrochemical reduction of oxygenated ferrous OEP (Welborn *et al.* 1981) gives the η^2-ferric peroxide (McCandlish *et al.* 1980) (**2**, scheme 3) suggesting that the same type of intermediate may occur naturally. Heterolytic cleavage of the O—O bond in **2** (and its enzymic equivalent) would, via protonation or peracid formation (Sligar *et al.* 1980) generate a ferric oxene complex, **3** (scheme 4).
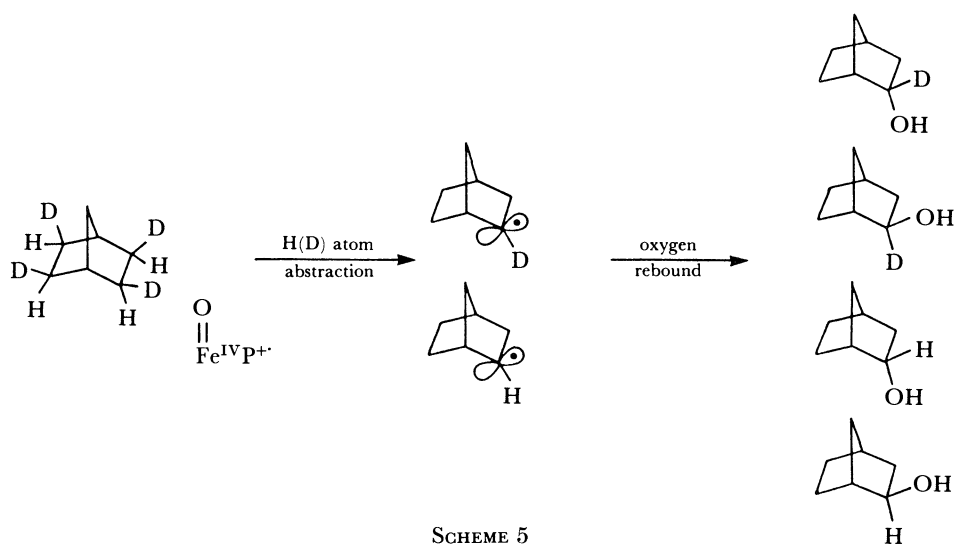

SCHEME 3

SCHEME 4
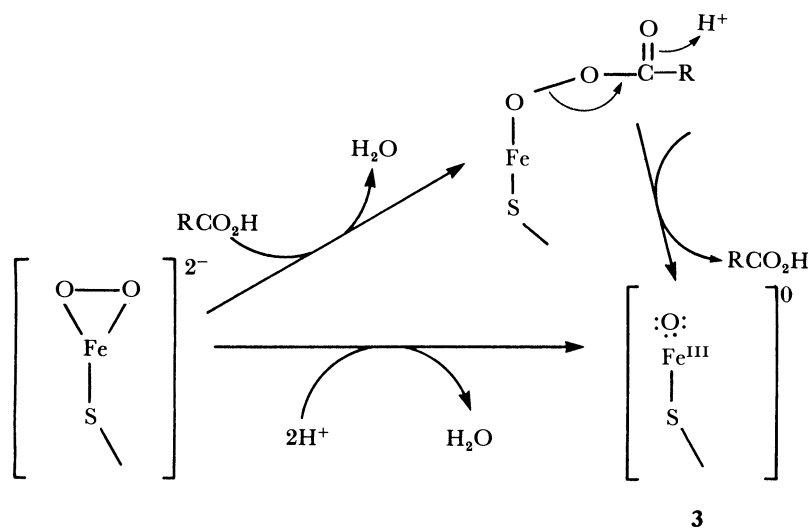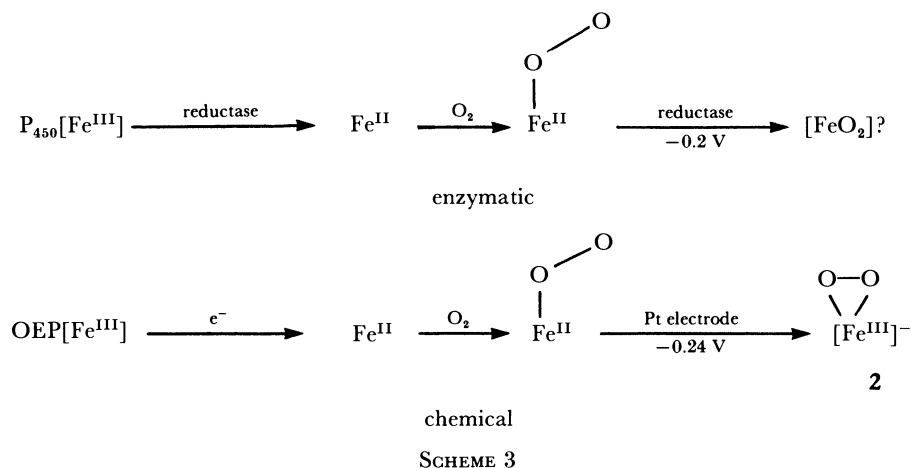
If an oxene were to react like a carbene (Kirmse 1964) or nitrene (Lwowski 1970), then direct insertion into a C—H bond or concerted addition across a double bond might be anticipated. Such chemistry, proceeding as it would without formation of intermediates, might seem appropriate for the known P$_{450}$ chemistry. However, the oxene formulation accounts for none of the observed P$_{450}$ chemistry.

Numerous metal-catalysed oxidations using hydrogen peroxide or dioxygen and a reductant are known. Groves and his colleagues (Groves & McClusky 1976) have shown that Fenton-like reactions of ferrous perchlorate and hydrogen peroxide hydroxylated cyclohexanol (Groves & Van der Puy 1976) possess both regio- and stereoselectively, while at the same time exhibiting a pronounced K_H/K_D isotope effect of 3.2. This reaction was described as occurring via an

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initial hydrogen-atom abstraction by the oxidizing species to generate the organic substrate free radical. Recombination of this radical with a hydroxyl radical, stabilized by coordination to iron, generated the hydroxylated organic product. This stepwise process involving a substrate radical was described as an 'oxygen rebound' mechanism (Groves *et al.* 1978). Deuterium isotope effects and the preference for tertiary hydroxylations (Frommer *et al.* 1970) suggest that direct insertion and carbanionic intermediates were not involved. Similarly, the absence of rearranged products during hydroxylation discounts the intermediacy of any free carbonium ions. Moreover, the generation of charged carbon species in an active site designed to accommodate hydrophobic hosts could be energetically unfavourable.

The elegant studies on the P_{450}-mediated hydroxylation of tetradeuteronorbornane (Groves *et al.* 1978), where retention of deuterium at a hydroxylated carbon (scheme 5) as well as formation of both endo- and exo-alcohols, clearly demonstrate the occurrence of an intermediate in the reaction, and all of the evidence (including an intramolecular K_H/K_D of 11) point to the radical nature of this intermediate.

SCHEME 5

How then can an oxene, generated by the routes described above, account for the observed enzymic chemistry? There are several other resonance structures, perhaps even discrete electronic configurations, that can be written for **3** (scheme 6). One of these, **4**, in which the oxygen contains an octet of electrons, is the $O=Fe^{IV}$ porphyrin π-cation radical, which we
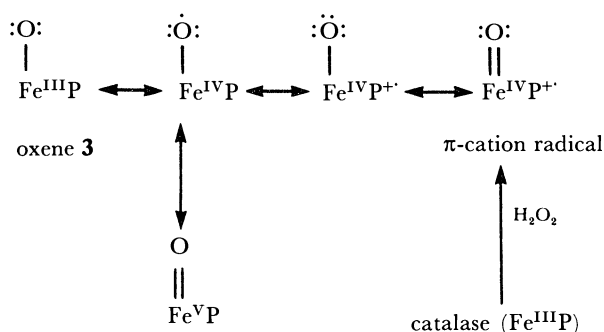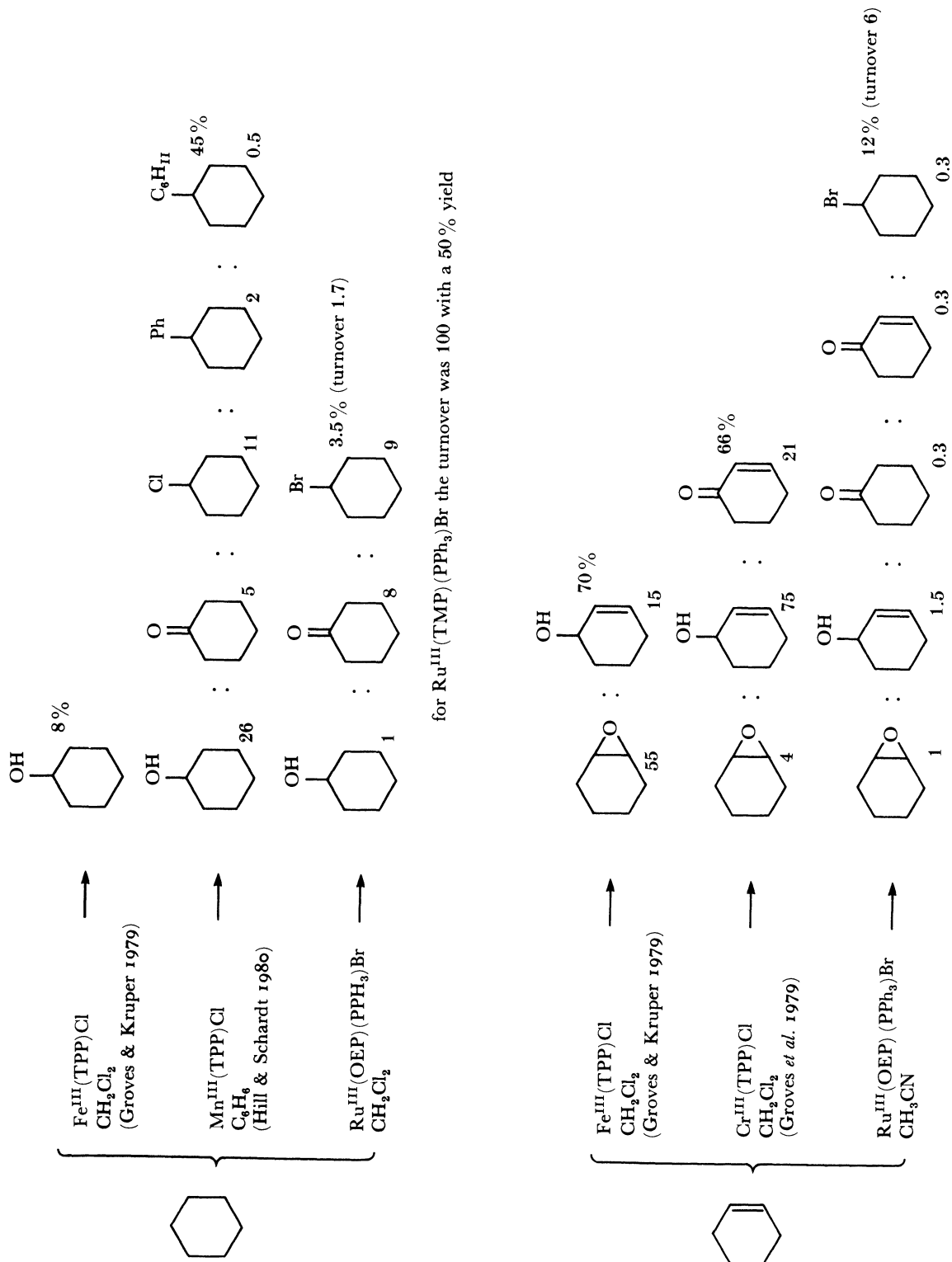

SCHEME 6

[134]

have shown is the 'active intermediate' in catalase and peroxidases (Dolphin *et al.* 1971). Groves and his colleagues have found that model iron porphyrins and the oxygen atom transfer reagent iodosylbenzene will hydroxylate aliphatic hydrocarbons in reactions that closely parallel the enzymic ones, including an isotope effect of *ca.* 13 for cyclohexane hydroxylation (Groves & Nemo 1983). At $-80\,°C$, using the sterically 'protected' *meso*-tetramesityl haemin (TMP, **5**) an intermediate has been observed and characterized as the oxoferryl Fe^{IV} porphyrin π-cation radical (Groves *et al.* 1981).

We have taken a parallel approach to the characterization of such high-oxidation-state intermediates by using ruthenium instead of iron porphyrins. The inorganic chemistry of ruthenium is dominated by much slower rates of ligand exchange compared with iron (Basolo & Pearson 1958), and this experience is observed with ruthenium porphyrins (Dolphin *et al.* 1983). While slow ligand exchange may make the preparation of ruthenium complexes more difficult, it should also stabilize products once they are formed. This is so; scheme 7 outlines the chemistry we have observed with RuOEP (Dolphin *et al.* 1983). The most significant observation, within the current context, is the preparation and characterization of **6** (scheme 7), i.e. $O{=}Ru^{IV}(OEP)^{+\cdot}Br^{-}$, which is 'stable' at room temperature and which hydroxylates
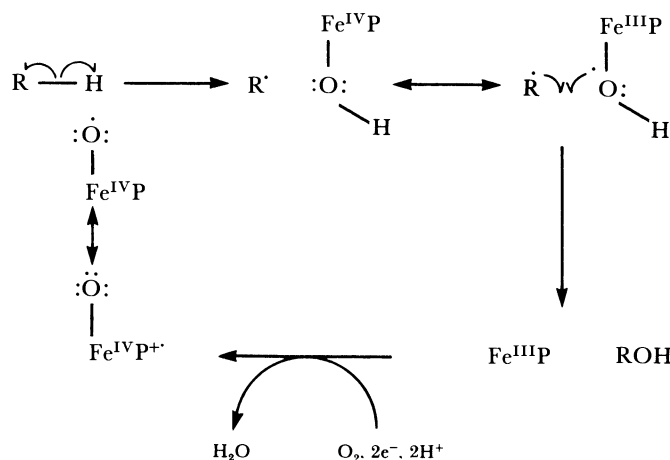

SCHEME 7
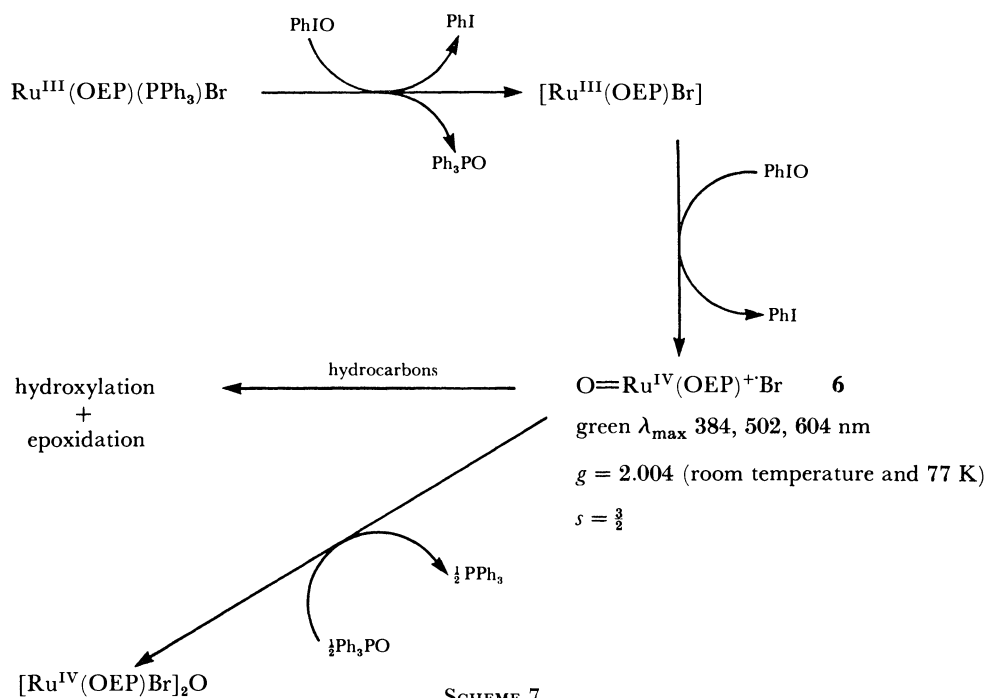
hydrocarbons and epoxidises alkenes in manners analogous to the corresponding iron and manganese systems (table 1). As expected, the catalytic activity of ruthenium porphyrins is lower than that of the corresponding iron complexes. Nevertheless Ru(TMP) does exhibit yields and catalytic turnover numbers comparable with the other metalloporphyrins (T. Leung, B. R. James & D. Dolphin 1985, unpublished results). We find, however, that the benzylic methyl groups of TMP are very reactive (M. Camenzind, D. Dolphin & B. R. James 1985, unpublished results). This accounts for the loss of oxidizing power of the iodosylbenzene and destruction of the catalyst. Replacement of the methyl groups of **5** by chlorines to give **7**

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TABLE 1. HYDROXYLATION OF CYCLOHEXANE AND EPOXIDATION OF CYCLOHEXENE BY USING METALLOPORPHYRINS AND IODOSOBENZENE; YIELDS ARE BASED ON CONSUMED OXIDANT

for $Ru^{III}(TMP)(PPh_3)Br$ the turnover was 100 with a 50 % yield

for $Ru^{III}(TMP)(PPh_3)Br$ the turnover was 120 with a 78 % yield

(scheme 10) dramatically enhances the stability of the catalyst and turnover numbers of more than 10 000 have been observed (Traylor *et al.* 1984).

Scheme 8 shows how the oxoferryl π-cation radical formulation and oxygen rebound mechanism can be incorporated into a P_{450} mechanism. Clearly such a mechanism requires that electrons can readily be transferred from porphyrin and metal to ligand. Our studies on


SCHEME 8

ruthenium porphyrins show that intramolecular transfer between porphyrin and metal is in fact a facile process which can be controlled by axial ligation (Barley *et al.* 1984). Two examples demonstrate the point. Thus oxidation of a ruthenium(II) porphyrin carbonyl complex **8** generates the corresponding $Ru^{II}CO$ porphyrin π-cation radical **9**. Removal of the CO is then accompanied (Barley *et al.* 1984) by an intramolecular electron rearrangement with the electron moving to the periphery to give the Ru^{III} porphyrin (**10**, scheme 9). Conversely the bis-triphenylarsine complex **11** is stable as the oxidized Ru^{III} porphyrin. Replacement of one of the arsine ligands by CO is accompanied by a reversible migration of an electron from the porphyrin to the metal to give the corresponding porphyrin π-cation radical **12**.
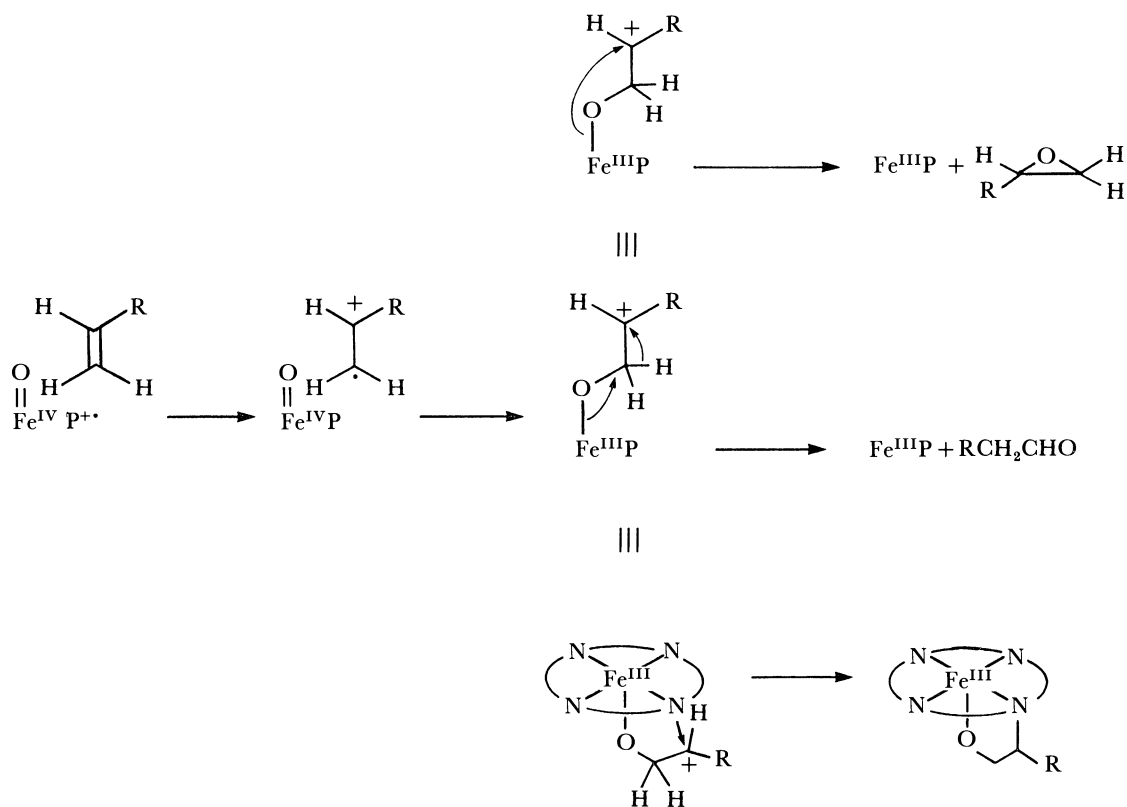

SCHEME 9

[137]
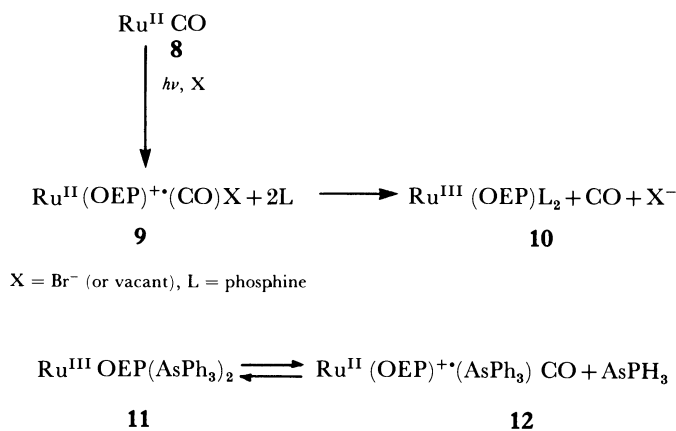
In addition to hydroxylations, cytochromes P_{450} also effect epoxidations (Groves 1980). Although the epoxide often reflects the stereochemistry of the alkene from which it was derived, additional metabolic products require the intervention of an intermediate. While the principle product from *trans*-1-phenyl-1-butene was the *trans* epoxide, both 1-phenyl-1-(and -2-) butanones were produced (Liebler & Guengerich 1983). These ketones were not further metabolites of the epoxide. Moreover, it has been reported (J. T. Groves, 1985, personal communication), for the specific case of propylene, that deuterium can be regio- and stereo-specifically incorporated into the recovered alkene when the enzymic reaction is run in D_2O. Furthermore, deuterium can be lost from deuterated propylene when the reaction is performed in water. Groves has suggested that the reaction proceeds via a metallocycle which generates a metallocarbene upon deprotonation. Be that as it may, the experiment clearly demonstrates the production of an intermediate in these reactions. Of even greater mechanistic and biochemical significance are the observations that both terminal alkenes and alkynes may cause suicide inhibition of cytochromes P_{450} by N-alkylation of the prosthetic haem group. The N-alkyl group contains the oxygen atom that normally generates the epoxide, but N-alkylation does not occur via initial epoxide formation (Ortiz de Montellano *et al.* 1982). We have recently shown that the P_{450} model system using **7** and iodosopentafluorobenzene gives rise to some inactivation and N-alkylation (Mashiko *et al.* 1985). Thus 4,4-dimethyl-1-pentene gives principally the corresponding epoxide; however, the 21-(4,4-dimethyl-2-oxidopentyl)-10,15,20-tetrakis (2,6-dichlorophenyl) haemin (**13**) is also slowly formed, from which the corresponding N-4,4-dimethyl-2-hydroxypentylporphyrin (**14**, scheme 10) can be isolated in greater than

Scheme 10
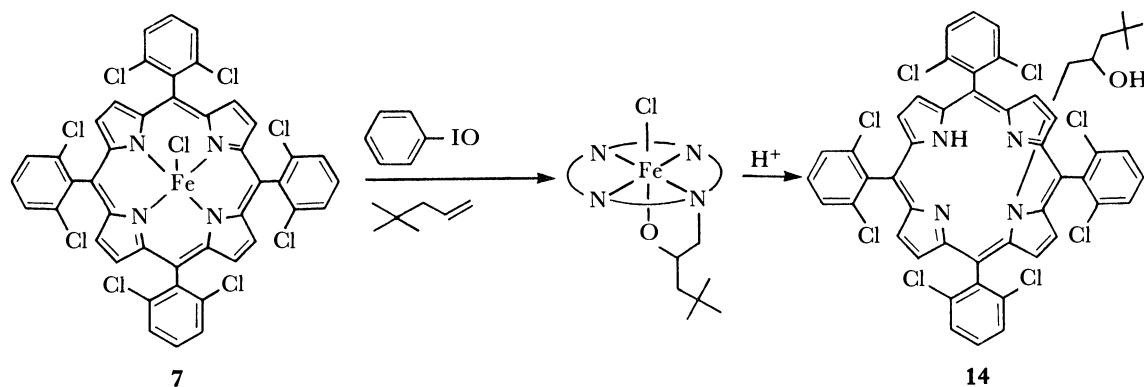
60% yield from the original reaction mixture. This observation establishes even more firmly the use of haemin–iodosobenzene systems as effective models for cytochrome P_{450}, while at the same time confirming the suicide inhibition as a basic reaction of these systems which is not substantially influenced by the apoprotein. Though the exact nature of the intermediates involved . olefin (and alkyne) metabolism are not firmly established (Guengerich & Macdonald 1984), an initial electron transfer to generate a cation radical of the substrate seems the most plausible pathway. Such a radical intermediate can account for all of the observed enzymic reactions (as outlined in scheme 11).

We suggest, in conclusion, that those haemproteins oxidized by hydrogen peroxide including the catalases and peroxidases, and those which generate 'peroxide' by a two-electron reduction of dioxygen (cytochromes P_{450} and the cytochrome oxidases (Blair *et al.* 1983)) all function in a common manner. The key reaction that Nature has learned to achieve and control is the

SCHEME 11

heterolytic cleavage of the O—O bond of the coordinated peroxide to give water and a two-electron oxidation product of the resting enzymes, namely the oxo-iron(IV) porphyrin π-cation radical, **1**. The specific chemistry attainable by these high oxidation states is then controlled by the steric and electronic (including the sixth axial ligand) constraints imposed by the protein. For cytochrome P$_{450}$ (cam) the preliminary X-ray crystallographic results (Poulos 1984) suggest how the subsequent generation of substrate free radicals can be achieved and controlled. It is equally clear that any one specific class of these enzymes can occasionally perform the chemistry associated with the other systems. Thus catalase can cause *N*-demethylation (Kadlubar *et al.* 1974), a reaction typical of cytochromes P$_{450}$, while P$_{450}$ can function as an oxidase and cause the four-electron reduction of dioxygen to water (Gorsky *et al.* 1984).

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