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Activation of Oxygen by Cytochrome P-450 and Other Haemoproteins

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Data on the activation of molecular oxygen by the full microsomal hydroxylating system containing cytochrome P-450 as the terminal oxygenase are examined*. The nature of the hydroxylating agent, which is the oxenoid $Fe^{3+}O$, is analysed. The autoxidation reactions of cytochrome P-450 from various sources, haemoglobin, myoglobin, and peroxidases are compared and the role of the axial ligands of the haem iron and the structure of the active centres of the haemoproteins in this process is demonstrated. The possible mechanisms of the oxidation of organic compounds by peroxides with participation of cytochrome P-450, cytochrome *c*, haemoglobin, and catalase are examined critically. Haemoproteins have been divided into three groups in terms of the type of peroxide oxidation reactions. The relative contributions of the radical and two-electron reactions in the oxidation of compounds by peroxides with participation of different haemoproteins are analysed. The bibliography includes 184 references.

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I. INTRODUCTION

Hydroxylating enzyme systems which employ molecular oxygen as the oxidant and require pyridine nucleotides (NADH or NADPH) for their functioning are widely distributed in the living world. The large number of reactions catalysed by such systems are characterised by the following stoichiometry:

$$AH + NADPH(NADH) + H^+ + O_2 \rightarrow AOH + NADP^+(NAD^+) + H_2O.$$

The enzymes which insert one oxygen atom in the substrate and reduce another oxygen atom to water are called monoxygenases or mixed-function oxidases[1-18]. The most numerous group of monoxygenases consists of involved multienzyme complexes containing unique haemoproteins as the terminal oxidase—cytochromes P-450. Hydroxylating systems of this type are present in human and animal liver microsomes and adrenal cortex mitochondria and form part of the composition of the cells of many bacteria, yeasts, and fungi, are constituents of the liver cells of cold-blooded animals, and are present in certain higher plants.

The liver microsomal hydroxylating system has attracted particular attention in connection with the great importance of its functions. This system oxidises endogenic substrates (steroids and cholesterol) and numerous xenobiotics entering the organism, including drugs, poisons, carcinogens, industrial chemicals from the environment, etc. The liver microsomal hydroxylating system consists of three components, each of which has been fully characterised[7,11,17,18]: NADPH–cytochrome P-450 reductase (EC 1.6.2.4), cytochromes P-450 (EC 1.14.14.1), the existence of many forms of which has been convincingly demonstrated[11], and phospholipids[9]. The steroid hydroxylating system from adrenal cortex mitochondria also consists of three components: adrenodoxin reductase (EC 1.6.7.1), ferredoxin (adrenodoxin), and two forms of cytochrome P-450, one of which is responsible for the reactions involving the scission of the cholesterol side chain ($P\text{-}450_{scc}$) and the other is responsible for the 11-β-hydroxylation reaction of steroids ($P\text{-}450_{11\beta}$).[18] The camphor-hydroxylation bacterial system also includes three components: putidaredoxin reductase, ferredoxin (putidaredoxin), and cytochrome $P\text{-}450_{cam}$.[18,19] As a result of the efforts of investigators from many countries, the most significant aspects of the structure, functions, and mechanism of the action of enzymatic hydroxylating complexes have been extensively investigated and surveyed in a series of monographs, reviews, and collections[1-19]. However, two most important problems have not been finally solved, namely the mechanism of the activation of molecular oxygen by cytochrome P-450 and the organisation of multienzyme complexes in the membranes of the endoplasmic reticulum, the adrenal cortex mitochondria, and bacterial cells.

The aim of the present review is a critical consideration and survey of the experimental data on the activation of molecular oxygen by cytochrome P-450 obtained very recently for the microsomal hydroxylating system of the liver. The ability of cytochrome P-450 to oxidise many substrates of different chemical types in combination with organic peroxides and other oxidants is extremely significant for the understanding of the mechanism of the activation of oxygen by cytochrome P-450. In this connection much attention has been devoted to reactions involving the oxidation of a substrate by hydroperoxides and other oxidants with participation of cytochrome P-450. The mechanism of the action of cytochrome P-450 differs from that of the other haemoproteins capable of catalysing many oxidative processes. One of the aims of this review is the consideration and analysis of the causes of this difference between cytochrome P-450 and other haemoproteins in oxidation-reduction processes. In order to solve the latter problem, the author has confined himself to the range of haemoproteins listed in Table 1, in view of the availability of data permitting a comparison of the reactions involving these haemoproteins and also the possibility of analysing the role of axial ligands and the structure of apoproteins in the activation of oxygen. Much attention has been devoted to the nature of the hydroxylating agents and

† The following abbreviations are used in the review: $P\text{-}450\text{-}LM_2$ = cytochrome from liver microsomes of rabbits administered phenobarbital-sodium; $P\text{-}450\text{-}LM_4$ = cytochrome from liver microsomes of rabbits administered 3-methylcholanthrene or β-naphthoflavone; $P\text{-}450_{cam}$ = cytochrome P-450 from camphor-hydroxylating bacteria; $P\text{-}450_{scc}$ = cytochrome from adrenal cortex mitochondria catalysing the scission of the cholesterol side chain; CHP = cumenyl hydroperoxide: TBHP = t-butyl hydroperoxide; DMA = *N*,*N*-dimethylaniline; ROOH = organic hydroperoxides and peracids; AH = substrate; AOH = reaction product.

Table 1. Certain characteristics of haemoproteins.

Haemoprotein	Molecular mass	Axual ligand		Absorption bands, nm			Natural source	References
		L_5	L_6	oxidised	reduced	complex with CO		
Cytochrome P-450: LM_2	48 700	S (Cys)	H_2O or N(His)	418, 535, 568	413, 544	450, 552	rabbit liver	[11, 13]
LM_4	55 300	S (Cys)		396, 645	411, 545	448, 550	rabbit liver	[11, 13]
P-450$_{cam}$	45 000	S (Cys)	N(His)	417, 535, 571	411, 540	447, 550		[19]
P-450$_{scc}$	48 000	S (Cys)	N(His)	394, 540, 565	416, 548	448, 550	mitochondria	[25]
P-450$_{11\beta}$	45 000	S (Cys)	N(His)	394, 510, 645	416, 548	448, 550	bovine adrenal cortex	[25]
Cytochrome b_5	11 000	N(His)	N(His)	413	424, 526, 556	none	calf liver	[5]
Cytochrome c	13 000	N(His-18)	S(Met-80)	407	415, 521, 550	none	horse heart	[26]
Peroxidase (EC 1.11.1.7)	40 000	N(His)	H_2O	420, 541	432, 535, 565	—	horseradish	[27, 28]
Myoglobin	17 000	N(His)	H_2O or HO^-		435, 555	423, 541, 578	sperm whale heart	[29]
Haemoglobin	68 000	N(His-82α, 92β)	H_2O or HO^-	407, 504, 538, 630	~430, ~555, ~590	419, 539, 569	human and horse blood	[29, 30]
Catalase (EC 1.11.1.6)	232 400	N(His)	HO^- or H_2O	—	—	—	ox liver, horse liver	[28, 31]

the relative contributions of the homolytic and heterolytic stages in oxidation processes with participation of the haemoproteins enumerated.

II. ACTIVATION OF MOLECULAR OXYGEN BY THE FULL MICROSOMAL HYDROXYLATING SYSTEM

We shall employ the concept of the full microsomal hydroxlating system to designate the reconstructed systems containing NADPH-cytochrome P-450 reductase, cytochrome P-450, NADPH, oxygen, and the substrate, and also in the description of the oxidation by liver microsomes with participation of NADPH and oxygen in contrast to the oxidation of substrates by hydroperoxides with participation of microsomes or individual forms of cytochrome P-450.

The process involving oxidation by microsomes and reconstructed systems is complex and consists of at least eight stages (Fig.1). In the first stage the substrate forms a complex with the oxidised form of cytochrome P-450, which is accompanied by a change in the oxidation-reduction potential and the spin state of the haemoprotein: the majority of substrates convert the low-spin form of cytochrome P-450 into the high-spin form, while certain amines induce the reverse transition. It has been suggested that the high-spin form of cytochrome P-450 loses the axial ligand in the sixth position but retains the cysteinyl group as the fifth ligand[20]. The rates of formation of the enzyme–substrate complexes are high and are characterised by constants in the range 10^2–10^6 litre mol^{-1} s^{-1} depending on the nature of the substrate[21]. The binding of substrates to cytochrome P-450 facilitates the reduction of the enzyme–substrate complex by the first electron arriving from the NADPH-specific electron transport chain[18,22]. The chain incorporates NADPH-cytochrome P-450 reductase (FP_1) and possibly cytochrome b_5. NADPH-cytochrome P-450 reductase is a flavoprotein (FP_1) with a molecular weight of approximately 79 000 and containing one FAD group and one FMN group in the molecule[23,24]. FMN reductases receive two electrons from NADPH, while FAD is involved in the transfer of these electrons to cytochrome P-450.[24] The mechanism of the action of the reductase has not been ultimately elucidated, since the relative positions of the two flavin fragments in the enzyme molecule are unknown. The second stage of the process in microsomes and in reconstructed systems consists of two phases[12]. A possible cause of the two phases in the reduction of the enzyme–substrate complex by the first electron in microsomes may be the heterogeneity of cytochrome P-450 [12,17] and, what is more likely, the different rates of reduction of different spin forms of cytochrome P-450,[32] since high-spin haemoproteins are reduced faster than the low-spin haemoproteins[20].

In the third stage the enzyme substrate complex reduced by the first electron combines rapidly with molecular oxygen: the rate constants for the addition of O_2 to different complexes vary in the range 10^4–10^6 litre mol^{-1} s^{-1}.[21] The oxygenated form of reduced cytochrome P-450 from liver microsomes was first detected spectrophotometrically in 1971 by Estabrook et al.[22] At the same time Gunsalus et al.[33] and Ishimura et al.[34] demonstrated spectroscopically the formation of a complex of oxygen with the reduced camphor-hydroxylating cytochrome P-450$_{cam}$. The camphor–P-450$_{cam}$–O_2 and P-450$_{cam}$–O_2 complexes were isolated for the first time in 1976 [35,36] by chromatography at temperatures below 0 °C in mixed aqueous organic solvents. These complexes have been characterised spectroscopically and kinetically. The camphor–P-450$_{cam}$–O_2 ternary complex undergoes autoxidation: the activation energy for this process is 18 kcal mol^{-1} in water and 19 kcal mol^{-1} in an aqueous organic medium[35].

The oxygenated complex of cytochrome P-450 was isolated for the first time in 1979 from rabbit liver microsomes at −30 °C in aqueous glycerol solution[37]. The complex is characterised by absorption maxima at 420 and 557 nm and the activation energy for [its] autoxidation is ~10 kcal mol^{-1}.[37] The ternary complex of cytochrome P-450$_{scc}$ from bovine adrenal cortex mitochrondria with cholesterol and oxygen was purified quite recently in the same medium: the complex has an absorption maximum at 414 nm and an extinction coefficient of 84 ± 2 litre mmol^{-1} cm^{-1},[38]

The complex formation reaction of highly purified cytochrome P-450 from rabbit liver microsomes (P-450-LM) has been investigated spectrophotometrically using stopped flow methods[39]. The reaction was carried out in two stages in the presence of the substrate (benzphetamine) and was found to consist of two stages: the short-lived oxygenated Complex A, having absorption maxima in the difference spectrum at 430 and 450 nm, is formed initially and is then converted into

Complex B with an absorption maximum at 440 nm. The complex formation reaction between oxygen and the reduced cytochrome is described by the scheme

$$P\text{-}450^{r}_{LM} + O_2 \xrightarrow{k_1} A \xrightarrow{k_2} B \xrightarrow{k_3} P\text{-}450^{o}_{LM}\ .$$

The rate constants are $k_1 > 6.0 \times 10^4$ min^{-1}, $k_2 = 270$ min^{-1}, and $k_3 = 3.0$ min^{-1}.[39] Complex B has spectroscopic characteristics close to those of Complex I in the reactions of peroxidases with hydrogen peroxide[28] and is probably analogous to it. The reaction components have the following Soret bands: P-450^r—424 nm, P-450^o—418 nm, A—432 nm, and B—421 nm. The oxidation of cytochrome P-450 in the ternary complex is the rate-limiting stage of the process and leads to the hydroxylation of the substrate.

The structure of the oxygenated haemoprotein is of primary importance for the mechanism of the activation of oxygen by cytochrome P-450. The state of oxygen in the substrate—P-450—O_2 complex and its configuration are still unknown. By analogy with oxyhaemoglobin, it has been suggested that oxygen receives an electron from the haem iron and its state is best reproduced by the structure proposed for oxyhaemoglobin by Weiss[40]: $Fe^{3+}O_2^-$ (Fig.1). By analogy with oxyhaemoglobin, one can postulate that the oxygen in the oxygenated cytochrome P-450 has the configuration Fe—O=O, in which the Fe—O=O angle is close to 120°. Attempts have been made to calculate the most favourable configuration for the oxygenated cytochrome P-450 by quantum-mechanical methods[41,42]. The calculations showed that the unusual spectroscopic properties of the oxygen and carbonyl complexes of cytochrome P-450 are fully associated with the thiol ligand in the fifth axial position and depend very strongly on the distance between the haem iron atom and the sulphur atom of the thiol ligand. An increase of this distance by 0.2 Å and more is quite sufficient to convert cytochrome P-450 into the inactive form P-420, which differs from the former by the maximum of the Soret band of the carbonyl complex of the cytochrome in the reduced state[42]. It would be premature and risky to reach definite conclusions about the configuration of oxygen in the oxygenated cytochrome P-450 on the basis of quantum-mechanical calculations, since theoretical calculations for the Fe—O_2 bond in oxyhaemoglobin proved unconvincing. Such calculations, carried out by the molecular orbital method[43], give preference to Pauling's structure[44], whereas it follows from calculations by the MO-LCAO method carried out by other workers[45] that only the closed Griffith structure[46] ensures the existence of the stable low-spin complex of oxyhaemoglobin. An X-ray diffraction study of oxygenated myoglobin confirmed Pauling's model, i.e. the axis of the O_2 molecule forms an angle close to 120° with the plane of the porphyrin ring[47].

The substrate—P-450—O_2 ternary complex in microsomal hydroxylating system is unstable and undergoes autoxidation. The reduction of this complex by yet another electron, which occurs in the fourth stage of the process (Fig.1), is necessary for the hydroxylation of the substrate. It has been suggested that the second electron comes from the NADH-specific microsomal chain containing NADH-cytochrome b_5 reductase and cytochrome b_5. Hitherto this stage of the process has not been resolved into individual steps and its rates have not been measured for microsomal systems. The important role of cytochrome b_5 in the transport of the second electron has been confirmed in the study of oxidation by microsomes[48—53] and in the oxidation of a series of substrates in reconstructed systems[54—65]. It is believed that cytochrome b_5, reduced in the NADH-specific electron transport chain, can transmit the second electron directly to cytochrome P-450.[48] The participation of cytochrome b_5 in the transport of the second electron to the ternary complex is indicated by a whole series of experimental data, which have been discussed[66].

Firstly, in the microsomal oxidation of substrates of the first type (hydrocarbons, tertiary amines, and alicyclic compounds) with participation of two pyridine-nucleotides (NADH and NADPH), a synergistic effect is observed, reflecting the important role of the two electron transport chains in the oxidation process[48,50]. The synergistic effect is not observed for substrates of the second type (aniline and other amines)[17]. It is important to note that only endogenic cytochrome b_5 participates in the synergistic effect, while the exogenic cytochrome b_5 (incorporated in the microsomal membranes) does not always stimulate microsomal oxidation and actually retards the reactions of aminopyrine and DMA.[49,66] Evidently the cytochrome b_5 incorporated in the membrane differs from the native cytochrome b_5 by the fact that it cannot enter a cluster containing cytochrome P-450 and reductase and cannot participate in the reduction of cytochrome P-450. The inhibiting effect of the cytochrome b_5 incorporated in microsomes can be explained by its ability to receive electrons from NADPH-cytochrome P-450 reductase and to consume the latter non-productively from the standpoint of the hydroxylation process. The reduction of cytochrome b_5 by NADPH-cytochrome P-450 reductase was initially merely postulated[48,50] but now it has been confirmed experimentally[17,56,59]. The different effects of the endogenic and exogenic cytochrome b_5 on the microsomal oxidation can be explained by the different oxidation-reduction potentials of the native haemoprotein and the haemoprotein incorporated in the membrane.

Secondly, in the oxidation by microsomes or reconstructed systems cytochrome b_5 frequently accelerates the oxidation of some substrates, but is indifferent in the reactions of others; the oxidation of benzo[*a*]pyrene,[53,57] acetanilide[57], 4-ipomeanol[52], benzphetamine[54], 7-ethoxycoumarin[53,55,61], and *p*-nitroanisole[59—61] is accelerated by cytochrome b_5, while the oxidation of aminopyrine[49] and aniline[66] is inhibited by this haemoprotein. It may be that the substrates of the first group form complexes with cytochrome P-450 whose oxidation-reduction potentials favour the reduction of the substrate—P-450—O_2 complexes by cytochrome b_5; on the other hand, aniline and aminopyrine form complexes with oxidation-reduction characteristics which prevent their reduction by cytochrome b_5.[49,66]

Thirdly, the influence of cytochrome b_5 on oxidation in the reconstructed systems is determined to a large extent by the nature of the cytochrome P-450 itself. Cytochrome P-450 with an increased affinity for cytochrome b_5 has been isolated recently[60] and it has been shown that the oxidation demethylation of *p*-nitroanisoles is in this case effectively accelerated by cytochrome b_5.[56,59] Cytochrome b_5 accelerates the oxidation of the same substrate, namely benzo[*a*]pyrene with different efficiencies under identical conditions in the presence of two different forms of cytochrome from rabbit liver (P-450-LM and P-450-LM$_4$), i.e. the nature of cytochrome P-450 determines to a significant extent the stimulating possibilities of cytochrome b_5.[62] Direct evidence has been obtained recently for the reduction of oxygenated cytochrome P-450 from liver microsomes by cytochrome b_5:[65] it was shown that the autoxidation of the oxygenated cytochrome P-450 is accelerated in the presence of reduced cytochrome b_5.

Thus the influence of cytochrome b_5 on the oxidation reactions of various substrates in microsomes and in reconstructed systems is determined by several factors: the nature of cytochrome b_5 (exogenic or endogenic) and its oxidation-reduction potential, the nature of the substrate oxidised and its influence on the oxidation-reduction characteristics

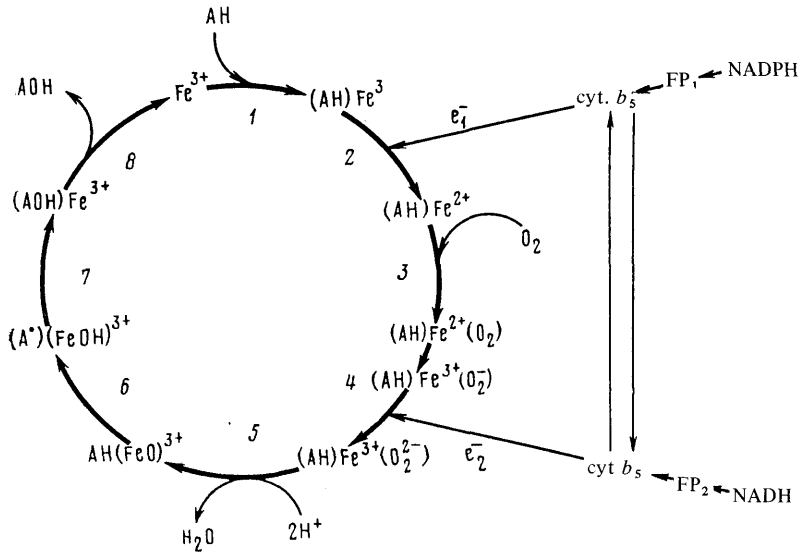


Figure 1.

of the ternary substrate–P-450–O_2 complex, and finally the nature of cytochrome P-450 itself and its affinity for cytochrome b_5. We may also note the influence of the methods used to reconstruct the enzyme systems, the content of admixtures of detergents which have not been removed, and the reaction conditions. For a favourable ratio of the oxidation-reduction parameters of cytochrome b_5 and the ternary complex, the haemoprotein stimulates the oxidation of substrates, while under the conditions of an unfavourable ratio of the above parameters, cytochrome b_5 can receive electrons from NADPH-cytochrome P-450 reductase but is incapable of transmitting them to cytochrome P-450, which retards the oxidation process.

The fourth stage of the microsomal oxidation is a complex process in which several macromolecular biocomponents of the microsomal system can participate. The rates of this stage have not so far been measured. We may note that the stage in which the ternary camphor–P-450_{cam}–O_2 complex is reduced by the second electron in the camphor-hydroxylating system has been studied in detail and characterised by its rate constants. This process proceeds with participation of an effector—putidaredoxin, and is the rate-limiting stage in the oxidation of camphor in a bacterial system[19,67].

After the reduction of the ternary complex by the second electron, the fourth stage of the process involves the hydroxylation of the substrate, the liberation of the molecules of water and the reaction product, and the regeneration of the initial catalyst in the oxidised form (Fig.1). It must be emphasised that at the present time none of these processes has been investigated separately and furthermore it is not known in what sequence the processes occur. One cannot rule out the possibility that the number of stages after the fourth may be greater or less than the four stages indicated in Fig.1. It is possible that after the fourth stage the hydroxylation step is followed by the simultaneous liberation of the molecules of the reaction product and water from the ternary complex, i.e. the complex hydroxylation step constitutes a cooperative process, which is frequently encountered in enzymatic reactions. Another possibility has been considered by White and Coon[12]: the fourth stage is followed by the cleavage of the O–O bond in the ternary complex with formation of water, the hydroxylating agent ruptures the C–H bond in the substrate, and this is followed by the formation of the reaction product, which is released in the last stage of the process. The oxidising agent has not been identified directly in any of the hydroxylating enzyme systems. Its nature and properties must be inferred from indirect data concerning the stereoselectivity and specificity of the microsomal oxidation, the presence or absence of isotope effects in the oxidation of labelled substrates and, what is very important, the similarities and differences in the oxidation of the same substrates in the complete system and with participation of hydroperoxides in combination with cytochrome P-450. The inhibition of the reactions by antioxidants, which retard the radical oxidation processes, plays an important role in the consideration of the relative contributions of the homolytic and heterolytic stages in the microsomal oxidation process[68,69]. Stages *5–8* of the process (Fig.1) will be henceforth discussed taking into account all the data concerning different aspects of the microsomal oxidation of substrates.

It is believed[12] that the fourth stage of the process is followed by the cleavage of the O–O bond in the ternary complex with formation of a hydroxylating species containing only one oxygen atom. This claim is supported by the high oxidising capacity of iodosobenzene $C_6H_5I{=}O$ and its derivatives in combination with the oxidised cytochrome P-450, first observed by Ullrich and coworkers[70]. Indeed iodosobenzene can transmit to the oxidising agent only one oxygen atom, since it has no other. Two groups of investigators were able to obtain with the aid of iodosobenzene relatively stable compounds simulating the hydroxylating agent of cytochrome P-450, which contains only one oxygen atom[71,72]: the oxidation of hydrocarbons by iodosobenzene is catalysed by chlorodimethylferriprotoporphyrin IX,[71] while in the presence of octaethylporphyrinatoiron(III) chloride in $CHCl_3$ or CH_2Cl_2 2-iodoso-*m*-xylene gives rise to a species whose magnetic susceptibility corresponds to the high-spin state of iron(IV).[72] It is believed[72] that the structure of the compound obtained is analogous to that of Complex I of

peroxidases, catalases, and chloroperoxidase and is characterised by the following formulae:

$$P^{+}Fe^{IV}O^{2-} \longleftrightarrow PFe^{IV}O^{-} \longleftrightarrow PFe^{III}O \ ,$$

where P is the porphyrin. The high degree of oxidation of iron in these model complexes is stabilised, according to the authors, by the porphyrin ring, which gives up one electron to the iron or oxygen atom. In many instances evidence has been obtained for the existence of porphyrin cations[73,74]. The species whose structure is illustrated above is called oxene[18,75], since its oxygen atom contains six electrons in the outer shell and is distinguished by a high reactivity, by analogy with carbenes and nitrenes[76,77]. As a result of the cleavage of the O–O bond in the ternary complex, such a species is in fact formed in the scheme proposed by White and Coon[12]. The O–O bond dissociation process takes place with participation of the carboxy-group of one of the aminoacids of the active centre of cytochrome P-450 (Fig.2). The authors believe that the structure of the peracid formed on interaction of the carboxy-group of the oxygen complex of cytochrome P-450 is favourable for the subsequent cleavage of the O–O bond and the formation of the hydroxylating species. This consideration is confirmed by the high hydroxylating capacity of peracids in relation to aromatic and aliphatic hydrocarbons, which increases when the peracids are coordinated to metal complexes. It is not fortuitous that peracids are convenient models of monoxygenases simulating the step in which oxygen is inserted in the substrates of the microsomol system[4,75,78]. It is believed[12] that the oxenoid formed in stage 5 (Fig.1) is stabilised by the thiol ligand of cytochrome P-450, which constitutes an unusual reservoir of electrons in the formation of the hydroxylating species and its reactions:

$$S^{-}Fe^{V}O^{2-} \longleftrightarrow S^{-}Fe^{IV}O^{-} \longleftrightarrow S^{-}Fe^{III}O \leftrightarrow S^{\cdot}Fe^{III}O^{-} \ .$$

The possibility of the involvement of the carboxy-group of one of the aminoacids of the active centre of cytochrome P-450 in the formation of the hydroxylating agent has been confirmed experimentally[79] in relation to cytochrome P-450$_{cam}$.

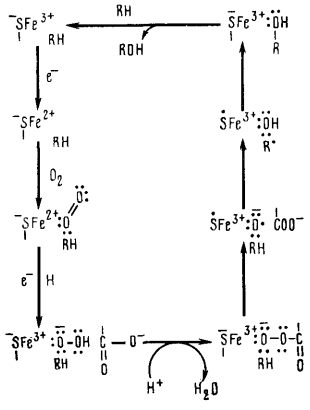
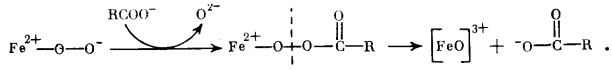


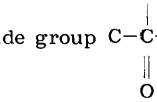
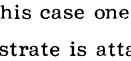
Figure 2.

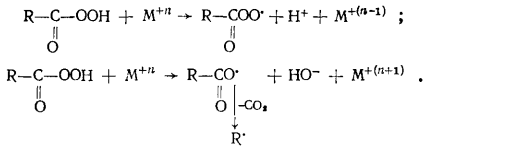
Using labelled oxygen ($^{18}O_2$), it has been shown[79] that dihydrolipoic acid acylates the distal oxygen atom in the oxygenated complex P-450$_{cam}$ $^{18}O_2$, forming an intermediate peroxy-compound, the dissociation of the O–O bond of which is greatly facilitated, which results in the insertion of one ^{18}O atom in camphor and of the other in the carboxylate group of dihydrolipoic acid:



The heterolytic decomposition of an acyl peroxide results in the formation of the oxenoid $[Fe^{18}O]^{3+}$ and lipoic acid labelled in the carboxy-group:



As an alternative to the heterolytic decomposition of the acyl peroxide formed as a result of the covalent binding of the carboxy-group of the aminoacid to the oxygenated cytochrome P-450, the possibility of the homolytic decomposition of the intemediate peroxy-compound, formed with participation of the amide group , has been considered[12]. In this case one must postulate that the C–H bond in the substrate is attacked by the amidyl radical . We believe that this reaction pathway is unlikely, because the amidyl radical is relatively stable and at temperatures close to physiological temperatures cannot rupture the stable C–H bond in the majority of substrates of cytochrome P-450. The homolytic dissociation of the O–O bond in the intermediate acyl peroxide must also be ruled out, because the homolysis of this bond with participation of metal complexes and ions is accompanied by the decarboxylation of the acylperoxy-radical formed[80]:

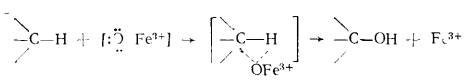


As a result of the last reaction, the acid of the apoenzyme participating in the covalent fixation of oxygen in the oxygenated cytochrome P-450 would be irreversibly consumed and the catalyst would rapidly break down.

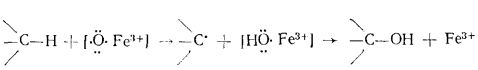
Thus the most probable pathway leading to the formation of the oxenoid in the complete microsomal system is heterolytic decomposition of the acyl peroxide formed on interaction of the carboxy-group of one of the aminoacids of the active centre with the activated oxygen of the ternary substrate–P-450–O_2 complex reduced by the second electron. It is important to note that the oxygen coordinated to cytochrome P-450 is not only fixed to the matrix of the enzyme, as suggested for oxyhaemoglobin[44,81], but is covalently bound to one of the acids of the apoenzyme with formation of an intermediate peroxy-compound.

In the sixth stage of the process (Fig.1) a C–H bond in the substrate molecule is ruptured. It is believed[12] that this stage proceeds via a radical mechanism. This claim is supported by experimental data, which we shall consider in detail below. The electronic structure and reactivity of the oxenoid $[Fe^{3+}O \leftrightarrow Fe^{4+}O^{-}]$ are completely unknown. Presumably one of two mechanisms of attack on the substrate

obtains[16,18]: the first (oxenoid) or two-electron mechanism:



and the second (cryptoradical) or one-electron mechanism:



It would be incorrect to claim that one of the reaction pathways is universal. If the oxygen in the oxenoid is in the singlet state, the first mechanism operates, in which the oxygen atom is inserted in the C–H bond without its dissociation, as happens in the reactions of carbenes, nitrenes, and atomic oxygen with aromatic and aliphatic hydrocarbons[76,77,78]. This reaction pathway is most likely in oxidation reactions of aromatic compounds and is the only one possible in the microsomal oxidation of cyclohexene and other unsaturated compounds and it is difficult to imagine the formation of epoxy-derivatives of cyclohexene and olefins via a radical mechanism. If the oxenoid is in the triplet state, then the direct insertion of the oyxgen atom in the substrate is impossible—such a reaction is spin-prohibited. In this case the one-electron, cryptoradical mechanism operates, which is reflected in the scheme (Figs.1 and 2). The radical mechanism of the oxidation reaction in the complete microsomal system has been supported by a number of pieces of important evidence.

Table 2. The primary isotope effects (k_H/k_D) in the microsomal oxidation of various substrates.

Substrate	k_H/k_D	References
[CD_3]Morphine	1.4	83
[α-D_2]Ethylbenzene	1.8	84
[D_{12}]Cyclohexane	1.0	85
[11-D_2]Lauric acid	3.0	86
[9-D_2]Caproic acid	3.0	86
Saturated compounds	1.4	87
[16α-D]Pregnenolone	3.0	88
[7α-^{3}H]Taurodeoxycholic acid	3.8	89
[α-D_2]Isopropylamphetamine	1.6	90
[CD_3]-*o*-Nitroanisole	2.0	91
[Cd_3]Methoxyanisole	10.0	92
Aromatic compounds	1.3-1.75	93
[1,1-D_2]-1,3-Diphenylpropane	11.0	94
Biphenyl	1.4	95
N,N'-Dimethylphentermine	1.4	96
Norbornane	11.5	97
7-Ethoxycoumarin	13.5	98

Firstly, a mixture of methylcyclohexanols with the isomer ratios 1° : 2° : 3° = 0.072 : 1 : 1.25 has been obtained in the hydroxylation of methylcyclohexene with participation of cytochrome P-450-LM_2.[82] Such proportions of the reaction products are consistent with a radical process mechanism.

Secondly, the dissociation of the C–H bond in the microsomal oxidation process is supported by the primary isotope effects, which have been observed in a number of instances (Table 2). It is remarkable that, in the oxidation of aromatic compounds of the benzene series and biphenyl, the isotope effects are small: k_H/k_D = 1.3–1.8. Small isotope effects have also been obtained for the oxidative dealkylation reactions of [CD_3] morphine[83], *N,N'*-dimethylphentermine[96], [CD_3]-*o*-anisole, and [α-D_2]isopropylamphetamine[90,91]. This means that the oxidation of aromatic compounds and oxidative dealkylation of certain substances either do not involve the dissociation of the C–H bonds or this process is not rate-limiting. In a number of instances measurements have been made of the intramolecular isotope effect which occurs when the molecule contains hydrogen and deuterium in the same position. The microsomal oxidation of *exo, exo, exo, exo*-tetradeuteronorborane yielded a mixture of *endo*- and *exo*-2-norborneols[97]. The ratio of the *exo*- and *endo*-alcohols was 3.4 : 1 for norbornane, decreasing to 0.76 : 1 for the deuterated substrate. The isotope effect was 11.5 in this instance[97]. It was shown in the same study that the oxidation of norbornane is not accompanied by the complete retention of configuration on formation of the alcohol, which confirms the intermediate formation of the carbon-containing radical. Measurements have been made recently of the isotope effect in the *O*-de-ethylation of 7-ethoxycoumarin[98] in the oxidation by two cytochromes from rat liver microsomes—P-450 and P-448. The ratios k_H/k_D were 11.8 and 13.5 for P-450 and P-448 respectively. The large isotope effects in a number of instances confirm that the rate-limiting stage of the microsomal oxidation process involves the dissociation of the C–H bonds in the substrates.

Thirdly, systematic kinetic studies of the influence of 1-naphthol and mannitol on the oxidation of aniline and DMA by microsomes with participation of NADPH and O_2 and by reconstructed systems with participation of the same cofactors demonstrated an effective retarding effect of 1-naphthol[69]. The 1-naphthol concentrations were significantly lower than those of both substrates and the mixed type of inhibition in the oxidation of the two substrates cannot therefore be accounted for solely by their competition with the inhibitor for binding to cytochrome P-450. Under the experimental conditions, 1-naphthol serves as an effective trap for active radicals participating in the oxidation reaction, whereas mannitol does not retard the oxidation process. The results led to the conclusion that radicals may participate in the oxidation of aniline and DMA in the complete microsomal system[69].

Thus the proportions of the reaction products in the oxidation of hydrocarbons, the large primary isotope effects in the oxidation of a series of substrates, the inhibiting effect of antioxidants on the oxidation of aniline and DMA, and the partial retention of configuration in the oxidation of norbornane confirm the possibility of radical stages in oxidation by the complete microsomal system.

The last stage of the microsomal oxidation reaction (Fig.1) is associated with the liberation of the reaction product from the ternary complex and the regeneration of cytochrome P-450 in the initial stage. This stage is rapid, since the more polar reaction products are less strongly bound to cytochrome P-450 than the less polar substrates and are easily displaced by the substrate molecules from the active centre of the enzyme.

The problem of the determination of the rate-limiting stage of the microsomal oxidation reaction is fairly complex. We may note immediately that the rate-limiting stages in the oxidation of the same substrate in microsomes and reconstructed systems may differ.

Extensive kinetic data concerning the oxidation of various substances by microsomes and the rates of invdividual stages of the process were considered previously[16,18]. Here the reader should merely be reminded that some of the stages of the microsomal oxidation reactions are known beforehand to

be incapable of being rate-limiting. The interaction of many substrates with oxidised cytochrome P-450 takes place at high rates[18,21] and the reduction of the enzyme–substrate complex by NADPH-cytochrome P-450 reductase is also rapid and it has been shown[21,99] that it cannot limit the rate of the entire oxidation process. The third reaction stage, namely the binding of molecular oxygen to the reduced enzyme–substrate complex, has high rate constants in the range 10^3–10^6 litre $mol^{-1}s^{-1}$.[21] It has so far been impossible to estimate the rate of reduction of the ternary complex by the second electron in the fourth stage of the microsomal oxidation process. It is sometimes suggested that this stage can in fact limit the rate of the process as a whole[12,17]. An analogy is frequently drawn between the microsomal system and the bacterial camphor-oxidising hydroxylating system in which the stage involving the reduction of the ternary camphor–P-450_{cam}–O_2 complex by the second electron is the rate-limiting stage for the process as a whole[19,67]. However, this analogy may be erroneous, because the reduction of the ternary complex by the second electron in the bacterial system involves yet another protein—putidaredoxin. On the other hand, the turnover number in the microsomal system is less than one-tenth of that in the hydroxylation of camphor with participation of P-450_{cam}.[12]

The most correct solution of the problem of the nature of the rate-limiting stage in the microsomal oxidation process involves the measurement of the rates of all the stages of the complex process and their comparison. Such a study has been carried out in Sato's laboratory in connection with the oxidation of benzphetamine in a reconstructed system[99]. Table 3 presents the kinetic characteristics of the individual stages of the process.

Table 3. The kinetic parameters k and ν of the N-demethylation of benzphetamine in the reconstructed systems of liver microsomes[99].

Stage of the process	Kinetic characteristics
Binding of benzphetamine to cytochrome P-450	$k_1 = 50\ s^{-1}$
Reduction of enzyme-substrate complex by first electron	$k_2 = 4\text{–}12\ s^{-1}$
Binding of oxygen to enzyme-substrate complex	$k_3 = (1.0\text{–}1.3) \times 10^3\ s^{-1}$
Decomposition of ternary complex with formation of reaction products	$\nu \geqslant 2.3$
Oxidation of NADPH	$\nu = 0.8\text{–}1.2$
Accumulation of formaldehyde	$\nu = 0.5\text{–}0.7$

Note. The rates of the processes are given in nmol s^{-1} per nmol of cytochrome P-450.

Analysis of the rates of the individual stages of the oxidation of benzphetamine led the authors[99] to the conclusion that the rate-limiting stage may be either the reduction of the ternary complex by the second electron or any process after this step, since the reaction product accumulates at rates less than the rate of decomposition of the ternary complex.

Some years ago we suggested that the rate-limiting stage of the microsomal oxidation process is the attack on the substrate in the ternary complex by the hydroxylating agent accompanied by the dissociation of the attacked C–H bond[100,101]. This hypothesis is supported by several experimental facts. Firstly, as already mentioned, large primary isotope effects are observed in the microsomal oxidation in most cases (Table 2), which implies the dissociation of the C–H bonds in the substrate molecule in the rate-limiting stage of the reaction. If such dissociation occurred before or after the rate-limiting stage, it would be impossible to obtain isotope effects of 11–13.

Secondly, there exist the so-called uncoupling compounds which are bound effectively to cytochrome P-450 but are not oxidised by microsomes. They include fully fluorinated hydrocarbons[102–105]; in this case the C–F bonds are very stable and cannot be ruptured by the oxidising agent in the rate-limiting stage.

Thirdly, we have shown that, in the complete microsomal system and with participation of hydroperoxides, the kinetic and energetic characteristics of the oxidation of the same substrates are in both cases very close or do not differ at all. This may reflect the attack on the substrate by the same oxidising agent in both systems. However, in the hydroperoxide oxidation there is no necessity for the transport of two electrons to the oxidising agent, since the oxygen of the hydroperoxide is already activated by two electrons. Consequently both systems may have the same rate-limiting stage, involving the transformation of the ternary complex containing the substrate, cytochrome P-450, and oxygen in the first case and the substrate, cytochrome P-450 and the hydroperoxide in the second. This is apparently entirely valid for the majority of substrates, although one cannot ignore certain differences between the oxidation by microsomes in the complete system and the oxidation with participation of hydroperoxides. The differences between the two systems will be discussed below.

In conclusion of this section, it must be emphasised that the rate-limiting stage of the microsomal oxidation reaction can vary as a function of the reaction conditions. A change in the process temperature is extremely significant from this point of view. The microsomal phospholipids undergo phase transformations at temperatures in the range 17–20 °C, which affect the energetic characteristics of the oxidation of the same substrates: the activation energies for the oxidation of substrates of the first type above the phase transition temperatures vary in the range 9–14 kcal mol^{-1}; at temperatures below the phase transition temperature, the activation energies increase to 17–25 kcal mol^{-1}.[106] The break in the relations between the oxidation rate constants and temperature and the points corresponding to the phase transitions of the microsomal phospholipids can denote a change in the rate-limiting stage of the process: above the break point under the conditions of enhanced mobility of cytochrome P-450 and the reductase, their interaction is facilitated and does not limit the rate of oxidation; below the phase transition point, the liquid-crystal state of the membrane lipids hinders contact between the two enzymes and the stage involving the reduction of cytochrome P-450 by the reductase may limit the rate of the entire process. The activation energies below the break point may therefore refer to the stage in which cytochrome P-450 is reduced by the reductase, while the activation energies above the break point may refer to another stage, for example, the decomposition of the ternary complex with formation of the reaction product or to the dissociation of a C–H bond by the hydroxylating agent in the ternary complex.

III. AUTOXIDATION OF HAEMOPROTEINS

Comparative analysis of the autoxidation of haemoproteins is useful from two points of view: firstly, the autoxidation of myoglobin and haemoglobin constitutes the only method of

activation of molecular oxygen by these proteins and, secondly, comparison of the rates of autoxidation of haemoproteins makes it possible to estimate the role of the axial ligands and the structure of the apoenzyme in the activation of oxygen by haemoproteins. Table 4 presents certain characteristics of the oxygenated complexes of haemoproteins. It shows that the oxygenated complexes of five haemoproteins are characterised by similar absorption spectra. All the proteins enumerated undergo autoxidation. It has been shown that the oxidation of cytochromes P-450$_{cam}$ and P-450-LM$_2$ results in the formation of the superoxide radical-anions $O_2^{\cdot-}$.[108-111] Somewhat unexpectedly, the $O_2^{\cdot-}$ radical-anions could not be detected by direct methods in the autoxidation of cytochrome P-450-LM$_4$ from the liver microsomes of rabbits given β-naphthoflavone[112]. Some authors erroneously attributed to the $O_2^{\cdot-}$ radical-anion the role of the hydroxylating species in the microsomal oxidation on the grounds that the oxidation processes are inhibited by superoxide dismutase and copper complexes which can model this enzyme[113]. A detailed study of the inhibition of the oxidation of DMA by copper(II) ions and the copper(II) complexes of tyrosine and lysine showed that the role of the copper compounds reduces to the interaction with the $O_2^{\cdot-}$ radical-anion and to displacing the equilibrium to the right[114]:

$$[Fe^{2+}O_2 \equiv Fe^{3+}O_2^{\cdot-}] \rightleftharpoons Fe^{3+} + O_2^{\cdot-} .$$

This process leads to the non-productive consumption of the oxygenated cytochrome P-450 and competes with the reduction of the latter by the second electron, i.e. lowers the concentration of the hydroxylating species in the system and thereby retards the oxidation. Copper compounds also enter into other reactions which, together with that discussed above, are responsible for the complex mixed type of inhibition in the oxidation of DMA by rabbit liver microsomes[114].

Table 4. Characteristics of oxygenated complexes of haemoproteins.

System	λ_{max}, nm	E^*, kcal mol^{-1}	References
S-P-450$_{cam}$-O_2	418, 552	17.0; 18.0	19, 35, 36
P-450$_{cam}$-O_2	—	24.0; 14.5	19, 35, 36
S-P-450$_{scc}$-O_2	414	—	38
P-450-LM$_2$-O_2	420, 557	9.6	37
Haemoglobin-O_2	412, 542, 577	17.7	29, 107
Myoglobin-O_2	417, 544, 582	—	29

*E = activation energy for the oxidation reaction.

Myoglobin and haemoglobin are autoxidised very slowly, which is not surprising, since their main function is to transfer molecular oxygen and not to activate it. Nevertheless it has been shown repeatedly that the oxidation of myoglobin and haemoglobin results in the formation of the radical-anions $O_2^{\cdot-}$.[81] The autoxidation of oxygen-transferring proteins has attracted the attention of investigators for a long time[115]. At the present time it is entirely possible to consider the main pathways leading to the autoxidation of myglobin and haemoglobin. There are three mechanisms of the oxidation of these proteins:

$$Fe^{2+}O_2 + H_2O \rightleftharpoons Fe^{3+}OH_2 + O_2^{\cdot-} , \quad (1)$$

$$Fe^{3+}O_2 + Fe^{2+} \rightleftharpoons Fe^{2+}O_2Fe^{2+} \rightarrow 2Fe^{3+} + O_2^{2-} , \quad (2)$$

$$Fe^{3+}O_2 + H \text{ (or } e^-) \rightarrow Fe^{3+}O_2H \rightarrow \text{products.} \quad (3)$$

Reaction (1) is an important stage of the autoxidation of iron-porphyrins, but the formation of $O_2^{\cdot-}$ or $HO_2^{\cdot}$ is hindered by the hydrophobic environment within the protein. Comparison of the corresponding oxidation-reduction potentials shows that the reactions of simple ions are endothermic and for this reason can hardly constitute the main pathway in the autoxidation of myoglobin and haemoglobin at pH 7:

$$Fe^{2+} + O_2 \rightarrow Fe^{3+}OH_2 + O_2^{\cdot-} ,$$
$$Fe^{2+}O_2 \rightarrow Fe^{3+}OH_2 + O_2^{\cdot-} ,$$

Reaction (1) may become the principal stage in the autoxidation of haemoglobin if the oxidation-reduction potential of the Fe^{2+}/Fe^{3+} pair is significantly reduced. Such reduction of the potential is possible when histidine in the fifth axial position is replaced by another ligand, for example, tyrosine or cysteine, which is observed in certain mutant haemoglobins. However, the axial ligand is not the only species which can decrease the oxidation-reduction potential of the haemoprotein; this can be done by the protein itself. Indeed, myoglobin, haemoglobin, and peroxidase have the same fifth axial ligand (histidine), but they have different oxidation-reduction potentials and as a consequence their oxygenated complexes have different capacities for autoxidation (Table 5). Table 5 shows that the capacity for autoxidation of oxygenated haemoproteins increases with decrease of the oxidation-reduction potential, which decreases markedly on replacement of the fifth ligand (histidine in myoglobin, haemoglobin, and peroxidases) by cysteine (in cytochromes P-450).

Table 5. The oxidation-reduction potential (E_0') of haemoproteins and qualitative comparison of the stabilities of their oxygenated complexes $Fe^{2+} + O_2$ (K).

Haemoprotein	E_0' (pH 7), V	K	References
Haemoglobin	+0.2	very stable	115
Myoglobin	+0.1	very stable	115
Turnip peroxidase, P$_7$	-0.12	stable for 20 min	115
Horseradish peroxidase	-0.25	oxidises rapidly, not detected	115
S-P-450$_{cam}$	-0.17	oxidises very rapidly	19, 35, 36
P-450$_{cam}$	-0.27	stable only at -30 °C	19, 35, 36

Reaction (2) is unlikely for protein iron-porphyrins, since the protein hinders the dimerisation of the oxygenated form by reaction with another porphyrin molecule, but such dimerisation has been observed for non-protein iron-porphyrins. It is noteworthy that the dismutation of two Fe^{2+}–P-450$_{cam}$–O_2 complex species has been demonstrated for the bacterial hydroxylating system[19]:

$$2[Fe^{3+}\text{—P-450}_{cam}\text{—}O_2^{\cdot-}] \rightarrow Fe^{3+}\text{—P-450}_{cam} + O_2^{2-} + Fe^{3+}\text{—P-450}_{cam} + O_2 .$$

This is possible only in the case of the association of two molecules of cytochrome P-450$_{cam}$, which cannot be ruled out.

The oxidation of the oxygenated myoglobin and haemoglobin usually takes place with participation of a donor of a hydrogen atom or an electron [reaction (3)]. Such donors can be nitrite[116], phenol[116], 1-naphthol[107], aniline[30], phenylhydrazine[117], and other organic and inorganic reductants. It has been shown that oxyhaemoglobin oxidises these reductants via a radical mechanism[107,118]. The activation energy for the oxidation of 1-naphthol by oxyhaemoglobin is 17.7 kcal mol^{-1}.[107]

In the absence of electron donors, oxyhaemoglobin is fairly stable and different techniques, for example, flash photolysis (λ = 300 nm), must be used to generate the O_2^- radical-anion[119].

Thus oxyhaemoglobin and oxymyoglobin are distinguished under normal conditions by a greater stability compared with other oxygenated haemoproteins. It has been suggested that this stability is ensured by the structure of the oxygen-transferring proteins and, in particular, by the important role of the distal histidine moiety, which stabilises oxygen by forming hydrogen bonds with it.[44] However, slow oxidation of myoglobin and haemoglobin nevertheless occurs and in oxygenated blood there is usually a steady-state concentration of methaemoglobin $Fe_3^{3+}OH_2$, amounting to 1–2%. In the presence of H^+ ions or electron donors, the oxidation of haemoglobin is accelerated: for example, the aniline hydroxylase activity of erythrocytes has been convincingly demonstrated. Haemoglobin, which is not usually regarded as an enzyme, catalyses the oxidation of aniline to *p*-aminophenol[120]:

$$\text{aniline} + Fe^{2+}O_2 \rightarrow \text{aniline}\cdot Fe^{2+}O_2 \xrightarrow{e^-,2H^+} p\text{-aminophenol} + Fe^{3+} + H_2O .$$

It is important to note that the oxygenated cytochromes P-450$_{cam}$ and P-450-LM$_4$ are incapable of oxidising their substrates[18,19]. In the former case the oxidation requires reduced putidaredoxin—the effector of the oxidation of camphor and in the latter case oxidation takes place in the presence of reduced cytochrome b_5.[121] This parallelism suggests that in the microsomal system cytochrome b_5 can play the role of an effector analogous to that of the Fe- and S-containing putidaredoxin in the bacterial camphor-oxidising system. In the literature there is only one indication of the oxidising capacity of oxygenated cytochrome P-450-LM$_2$ in the absence of electron donors[122]. However, we believe that these data have been obtained using a doubtful analysis of the reaction product and require additional confirmation.

In conclusion of this section, we emphasise yet again that the autoxidation of haemoproteins takes place with formation of the O_2^- radical-anion, which is not itself a hydroxylating species but on recombination can give rise to hydrogen peroxide, which is a source of the active oxidant radical-anions $HO^{\cdot}$. The rates of oxidation of haemoproteins are determined by the oxidation-reduction potentials of the Fe^{2+}/Fe^{3+} pair and increase sharply when histidine in the fifth axial position (myoglobin, haemoglobin, and peroxidases) is replaced by cysteine (cytochromes P-450). The oxidation-reduction potential of iron-porphyrins is determined not only by the nature of the fifth axial ligand but also by the structure of the protein itself. The distal histidine in the myoglobin and haemoglobin molecules probably significantly stabilises the oxy-complexes of these oxygen-transferring haemoproteins, protecting them from autoxidation with liberation of the toxic radical-anion O_2^-. The activation of oxygen in the coordination sphere of cytochrome P-450 is ensured by its anomalously low oxidation-reduction potential.

IV. THE MECHANISMS OF THE CATALYSIS OF PEROXIDE OXIDATION BY HAEMOPROTEINS

It has already been mentioned that oxidised cytochrome P-450 combined with organic hydroperoxide catalyses the oxidation of many substrates of the microsomal system. After the first study by Kadlubar et al.[123], in which the ability of liver microsomes to catalyse reactions involving the oxidative dealkylation of amines by hydroperoxides was demonstrated, numerous investigations, surveyed and discussed in a series of reviews[12,16,124], were carried out. It is known that cytochromes P-450-LM$_2$, P-450$_{cam}$, and certain others are catalytically active in peroxide oxidation[16,125]. On the other hand, it has not been possible to detect a catalytic activity of cytochrome P-450-LM$_4$ in peroxide oxidation[126,127]. The range of oxidants employed is extremely wide and includes, apart from organic hydroperoxides (CHP and TBHP), also peracids, hydrogen peroxide[128], sodium periodate[129], sodium chlorite[130], iodosobenzene[70], iodosobenzene diacetate[131], and pyridine *N*-oxide[132]. The number of reactions catalysed by cytochrome P-450 when peroxy- and non-peroxy-oxidants are used is also large: oxidation of aromatic compounds[106,133–140], oxidative *O*- and *N*-dealkylation of aromatic compounds[106,133–140], oxidative *O*- and *N*-dealkylation of ethers and tertiary amines[123,130,141–148], epoxidation of cyclohexene[100], oxidation of saturated and alicyclic compounds[149–151], and oxidation of aliphatic alcohols[152]. Thus all reactions catalysed by microsomal cytochrome P-450 in the complete system can take place in the presence of peroxides[16,18]. The peroxide oxidation of the lipids in the microsomal membranes, in which radical initiation reactions may play a significant role on interaction of lipid peroxides with membrane haemoproteins, occupies an important place. The formation of lipid peroxides has been investigated in detail[5]. Two systems for the peroxide oxidation of unsaturated aliphatic acids exist in the microsomal fraction: a non-enzymatic system in which iron is reduced by ascorbic acid and an enzymatic system in which the reductant is NADPH. The endogenic haem iron atoms serve as the inducing centres. The consideration of the important class of lipid peroxide oxidation reactions is outside the framework of this review, since it is of independent interest. We shall merely mention that the reactions of lipid peroxides with cytochrome P-450 may become an important source for the generation of the active radicals $RO^{\cdot}$ and $RO_2^{\cdot}$ via mechanisms which will be discussed in detail below. In the first stages of their investigations, several groups of workers postulated that the oxidising agent in peroxide oxidation is Complex I (Fig.3), analogous to the well known intermediate complex in the reactions of peroxidases, catalases, and chloroperoxidase[100,133–143]. The formation of Complex I in the hydroperoxide system and its comparison with the formation of the same agent in the complete microsomal system are presented in Fig.3. This analysis is supported by several important experimental data. Firstly, different spectra, identical with the spectra of Complex I for certain peroxidases (for example, cytochrome *c* peroxidases), have been recorded in the interaction of hydroperoxides with microsomes or with the highly purified cytochrome P-450-LM$_2$.[153] Table 6 presents the characteristics of the difference spectra of the complexes of hydroperoxides with cytochrome P-450 and other haemoproteins. Secondly, when microsomes were mixed with CHP, the EPR spectrum of a product having a *g*-factor of 2.0 was recorded, i.e. a radical species analogous to Complex I is formed[154]. Thirdly, the oxidation of [4-^{3}H]acetanilidine in the hydroperoxide system yielded the same NIH shifts as in the complete microsomal system, i.e. the mechanism of the insertion of an oxygen atom in acetanilide is the same in both cases[155]. Fourthly, as already mentioned, the oxidation of many substrates in the complete and hydroperoxide systems yielded similar or identical kinetic and energetic parameters for the oxidation of the same substrate in the two systems[18,100,101]. All the facts enumerated above confirm the hypothesis put forward by several workers that the mechanisms of the oxidation reactions in the complete and hydroperoxide systems are similar[16,100,146] and make it possible to postulate the existence of the same hydroxylating agent in both systems—the oxenoid [$Fe^{3+}O \leftrightarrow Fe^{4+}O^-$], analogous to Complex I of per-

oxidases and catalases. However, a series of results have been obtained recently indicating a more complex mechanism of the hydroperoxide oxidation and possible differences between the hydroxylating agent and Complex I.

Table 6. Spectroscopic characteristics of the complexes of haemoproteins with hydroperoxides.

Haemoprotein	Hydroperoxide	λ_{max}, nm	λ_{min}, nm	K_S, mmol litre^{-1}	References
Microsomes	CHP	441	422	—	154
" "	tetralinyl hydroperoxide	380, 440	420	—	160
" "	TBHP	380, 440	418	—	160
" "	CHP	380, 440	418	—	136
" "	CHP	440	418	0.043	161
Cytochrome P-450-LM_2	CHP	440	413—415	0.120	162
Cytochrome P-450-LM_2	ROOH*	436—440	418	—	128
Cytochrome *c*	CHP	433	413	0.083	163
Haemoglobin	CHP	425	405	0.100	164

*Substituted perbenzoic acids, substituted CHP, and substituted benzyl hydroperoxides were used here; K_S = dissociation constant of complex.

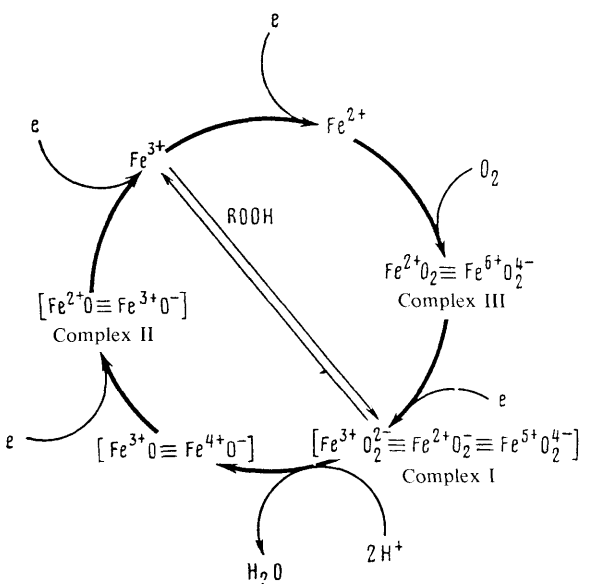
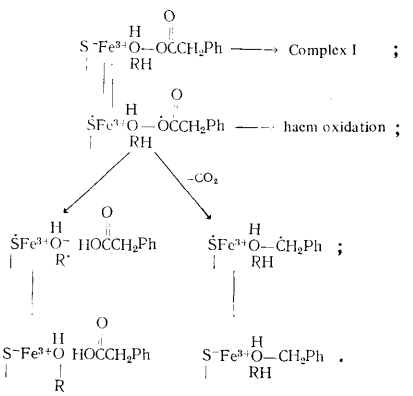


Figure 3.

The application of spectrophotometry and stopped flow techniques permitted a detailed investigation of the interaction of a large number of substituted perbenzoic acids, CHP derivatives, and benzyl hydroperoxide derivatives with cytochrome P-450-LM_2 and to reconstruct the absolute absorption spectra of the intermediate compounds formed[128]. Spectra differing appreciably for different peroxides were obtained as a result. The absolute absorption spectrum of a mixture of *m*-chloroperbenzoic acid with cytochrome P-450-LM_2 is characterised, in particular, by maxima at 375, 425, and 540 nm. This spectrum differs significantly from the known spectra of Complexes I: Complexes I of peroxidase and catalase have an absorption maximum at 400 nm, while Complex I of chloroperoxidase has a maximum at 395 nm.[74,156,157] The complex detected for cytochrome P-450 is analogous to Complex I of cytochrome *c* peroxidase (λ_{max} = 419 nm) [153] and Complex II of horseradish peroxidase (λ_{max} = 420 nm).[156] The second significant difference between the complexes of cytochrome P-450 with hydroperoxides and Complex I of peroxidases and catalyses consists in the kinetics and stoichiometry of their formation[128]. Peroxidases and catalases react with H_2O_2 or hydroperoxides (ROOH) in accordance with 1 : 1 stoichiometry and their reaction is believed to be irreversible. Admittedly very recently it has been shown that the formation of Complex I and Complex II by peroxidases can be a reversible process; Complex I can be reduced to ferri-peroxidase in two stages with the intermediate formation of Complex II when K_2IrCl_6 is used as the reductant[158]. Blake and Coon[128] showed that the interaction of hydroperoxides with cytochrome P-450 is complex and consists of at least two reversible stages:

$$\text{P-450} + \text{ROOH} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{C} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \text{D},$$

where C is the enzyme–substrate complex and D an unstable compound which can be detected spectroscopically according to the authors. The rate constant ratio k_1/k_2 depends greatly on the type of hydroperoxides and is correlated with their hydrophobic properties[128]. It has also been shown that the rate constants k_3 and k_4 are determined by the structure of the peroxides and the spectra of compound D differ for different peroxides, i.e. the nature of the peroxide has a marked influence on the interaction with cytochrome P-450 and on the characteristics of the intermediate compounds. This led White and Coon[12] to the conclusion that the intermediate complex of cytochrome P-450 with ROOH differs from Complex I of peroxidases and catalases. They therefore proposed[12] a new mechanism of the peroxide oxidation differing by the fact that the O–OR bond in the ternary substrate–P-450–ROOH complex undergoes homolytic dissociation and the oxidising agent is the alkoxy-radical RO$^\cdot$ derived from the hydroperoxide (Fig.4). The homolytic pathway in the dissociation of the O–OR bond was confirmed in a study of the interaction of cytochrome P-450-LM_2 and R-450_{cam} with phenylperacetic acid[125]. The homolysis of the O–OR bond is supported by the decarboxylation of phenylperacetic acid catalysed by cytochrome P-450:



In the peroxide oxidation mechamism[128] (Fig.4) an important role is attributed to the thiol ligand of cytochrome P-450. The possible involvement of this ligand in the homolytic

decomposition of hydroperoxides was suggested previously by the present authors[139]:

$$ROOH + Fe^{3+}—SH \rightarrow RO^{\cdot} + H_2O + Fe^{3+}—S^{\cdot} .$$

Thermochemical calculation shows that, under favourable solvation conditions, this reaction is entirely feasible close to physiological temperatures[159]. The thiol ligand is in fact an electron donor facilitating the homolytic dissociation of the O–OR bond. The complex formed after the homolysis of the O–OR bond may be equivalent to Complex II of peroxidases and catalases:

$$S^{\cdot}Fe^{2+}OH^{\cdot} \leftrightarrow S^{\cdot}Fe^{3+}OH^{-} \leftrightarrow S^{-}Fe^{3+}OH^{\cdot} \leftrightarrow S^{-}Fe^{4+}OH^{-} .$$

It may be that the intermediate compound detected spectroscopically after mixing ROOH and cytochrome P-450 is in fact Complex II: it is not fortuitous that the absorption maxima of Complex II of peroxidases and of the mixture of cytochrome P-450 with ROOH coincide (at 420 nm).[156]

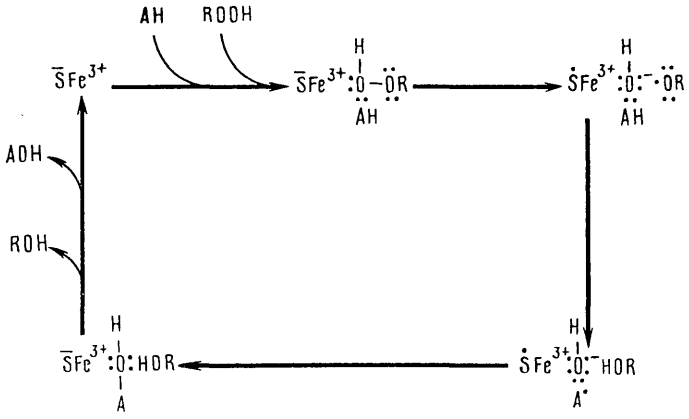


Figure 4.

Without denying the possibility of homolytic decomposition reactions of organic hydroperoxides in the presence of cytochrome P-450, which has been demonstrated in a number of studies[125,139,159], we shall nevertheless make a number of significant comments concerning the interpretation of data obtained by Coon and coworkers[12,128]. Firstly the absorption spectra of Complexes I for peroxidases and cytochrome P-450 need not necessarily be identical and, furthermore, they should in fact differ, since the hydroperoxides become ligands of the haemoprotein, occupying the sixth coordination site, while the fifth axial position contains histidine in peroxidases and cysteine in cytochrome P-450. The differences between the ligands are quite sufficient to account for the difference between the spectra of Complexes I for different haemoproteins. It follows from Table 6 that the nature of the fifth ligand is responsible for the difference between differential absorption spectra of the complexes of ROOH with cytochrome *c* and haemoglobin, on the one hand, and cytochrome P-450, on the other. Secondly, the formation of Complexes I of peroxidases as well as the interaction of ROOH with cytochrome P-450 can be reversible[158]. Thirdly, the oxidation of cyclohexene in the microsomes–TBHP system leads to the formation of epoxycyclohexane, which is inconsistent with the radical process mechanism[100]. Fourthly, the oxidation of labelled acetanilide yielded identical NIH shifts in the complete and hydroperoxide systems, which confirms the similarity of the mechanisms in the two cases. Bearing in mind all the data available at present, one may claim that the hydroperoxide systems involve, apart from the reactions via the oxenoid pathway similar to the reactions in the complete system, also radical processes which are responsible in many instances for the oxidation of certain substrates and the degradation of cytochrome P-450. The processes where the radical stages may predominate include the *N*-dealkylation of tertiary amines, which is effectively retarded by radical process inhibitors (1-naphthol, etc.[69]) and the oxidation of alcohols[14,155]. On the other hand, the oxidation of aromatic compounds and the epoxidation of olefins proceed via the oxenoid pathway, considered in the second section of this review. It is noteworthy that some of the most reactive aromatic compounds can be oxidised simultaneously via two pathways—oxenoid and radical. Benzo[*a*]pyrene is probably oxidised in this way in hydroperoxide systems[165]; the oxenoid reaction pathway leads to the formation of epoxides, while radical processes result in the accumulation of phenols and quinones. The claim[165] that there is a fundamental difference between the mechanisms of the oxidation of benzo[*a*]pyrene in the complete system and with participation of CHP, based on the difference between the reaction products in the two systems, is unduly categorical: we simply have the situation that in the hydroperoxide system yet another reaction pathway (the radical pathway) occurs in addition to the oxenoid pathway.

Other haemoproteins can also catalyse the peroxide oxidation reactions of certain substrates (Table 7), although not all the reactions characteristic of cytochrome P-450 are accelerated by other iron-porphyrins: peroxidase cannot oxidise saturated compounds and alcohols and cytochrome P-450 cannot participate in any of the reactions quoted in Table 7. The question arises how to explain the different reactivities and the differences between the mechanisms of the peroxide oxidation reactions involving different haemoproteins. The first stage of the process is the same for all haemoproteins—the formation of complexes with the peroxides (Table 6). Table 6 does not include data for the known reactions of peroxidases and catalases with hydroperoxides. Complexes of ROOH with different haemoproteins differ in the parameters of the difference absorption spectra—the difference between the axial ligands in the fifth position has an effect. Apart from the axial ligands, the aminoacid residues in the active centres can play an important role in complex formation between ROOH and haemoproteins. It has been suggested that the hydrogen bonds formed between the carboxy-groups of the aminoacids and the distal histidine play a very significant role in the complexes of ROOH with haemoproteins[16] (Fig.5). An important role in the heterolytic cleavage of the O–OR bond in cytochrome *c* by peroxidase has been attributed to the distal histidine-52, arginine-48, and tryptophan-51 [166] (Fig.6). Fig.6 shows that the distal histidine-52 is a kind of acid–base catalyst, the arginine-48 residue stabilises the charge, while the proximal histidine-174 stabilises the haem iron in a high oxidation state, i.e. the active centre of peroxidase is directly involved in the formation of Complex I. It is also known that aspartate-43 of horseradish peroxidase is incorporated via its carboxy-group in the formation of a complex with H_2O_2.[174] In the myoglobin molecule the glycine E8 occupies the same site as aspartate-43 in the horseradish peroxidase molecule and for this reason myoglobin does not form Complex I, in contrast to peroxidase[174]. Thus, despite

the fact that all haemoproteins form complexes with ROOH, not all of them are capable of generating Complex I owing to the differences between the structures of the active centre.

Table 7. Catalytic properties of haemoproteins in the oxidation of organic compounds by peroxides and certain non-peroxide oxidants[16].

Reaction	Haemoprotein exhibiting catalytic activity	References	Haemoprotein without catalytic properties	Ref.
N-Dealkylation of amines	cytochrome P-450 (ROOH, H_2O_2)	123, 141-145	cytochrome P-420	123
Ditto	catalase (ROOH. H_2O_2)	123	ditto	123
"	peroxidase (H_2O_2)	167	"	123
"	haemoglobin (CHP, H_2O_2)	168	"	123
O-Dealkylation of ethers	cytochrome P-450 (CHP, TBHP, C_6H_5IO)	146-148	haemin, catalase	146
Ditto	ditto	146-148	peroxidase, myoglobin, cytochrome P-420	146
Hydroxylation of aromatic compounds	cytochrome P-450 (ROOH, H_2O_2)	100 133-140	haemin, catalase	133
	haemoglobin (CHP)	169	peroxidase, myoglobin, cytochrome b_5, cytochrome P-420	133
	cytochrome *c* (CHP)	163	ditto	133
Oxidation of steroids	cytochrome P-450 (ROOH, NaClO, C_6H_5IO, $NaClO_2$)	129 170-173	peroxidase, catalase, metmyoglobin	129
Oxidation of alcohols	cytochrome P-450 (ROOH, H_2O_2)	152	peroxidase	152
Ditto	catalase (ROOH, H_2O_2)	152	cytochrome *c*	152

*The oxidants employed are indicated in brackets.

The coordination of hydroperoxides at the sixth site in haemoproteins is well known[16,175,176]. The fate of the complexes of hydroperoxides with haemoproteins can be various; peroxidases and catalases form Complex I, while myoglobin does not give rise to such a complex. The formation of radicals in the interaction of certain haemoproteins with hydroperoxides has been demonstrated. The detection of a low-intensity chemiluminescence as a result of the recombination of the peroxy-radicals ROO· showed that such radicals are formed on interaction of hydroperoxides with cytochrome *c*, myoglobin, methaemoglobin, and horseradish peroxidase[177], i.e. in the cases enumerated there is a possibility of radical stages in the oxidation of the substrate introduced into the system. It is at present possible to compare the efficiencies of various haemoproteins in the oxidative dealkylation reactions of *N*-alkylamines and to discuss the differences between the mechanisms of their action (Table 8). Horseradish peroxidase is most effective in the oxidation of amines by hydrogen peroxide[167], while catalase is most effective in the oxidation of DMA by cumenyl hydroperoxide[168]. The radical-cations of the substrate have been detected by EPR in the H_2O_2–peroxidase system on oxidation of aminopyrene[167]. The oxidation of aminopyrene in the CHP–cytochrome P-450 and CHP–myoglobin systems takes place with the same efficiencies and is accompanied by the formation of substrate radicals[178]. Griffin and Ting[179] believe that all the oxidative dealkylation reactions of amines listed in Table 8 proceed via a radical mechanism. Griffin proposed the following mechanism for the oxidative dealkylation of *N*-alkylamines[179]:

$$R_2N{-}CH_3 \xrightarrow[-e^-]{} R_2\dot{N}{-}CH_3 \xrightarrow[-H^+]{} R_2\dot{N}{=}CH_2 \rightarrow \xrightarrow{H_2O} R_2\dot{N}H_2 + H_2C{=}O\,.$$

A distinctive feature of this mechanism is the radical nature of the process and the incorporation in formaldehyde of an oxygen atom from water and not from an O_2 molecule. We confirmed the radical stages of the reactions in the oxidation of DMA by cumenyl hydroperoxide with participation of microsomes, cytochrome P-450-LM_2,[69] and haemoglobin[168] using 1-naphthol as the radical process inhibitor. The complex nature of the inhibition of the oxidation of DMA by 1-naphthol is consistent with the possible role of radical reactions in the peroxide oxidation with participation of CHP and haemoglobin, catalase, or cytochrome P-450.

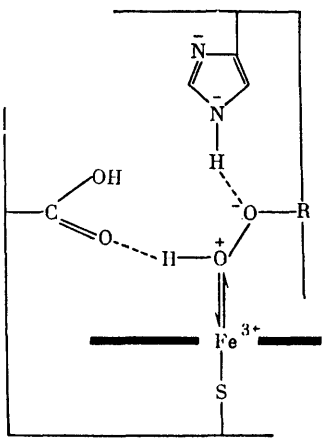


Figure 5.

Thus radical stages are observed in reactions involving the oxidative dealkylation of *N*-alkylamines by hydroperoxides with participation of horseradish peroxidase, myoglobin, haemoglobin, catalase, protohaemin, and cytochrome P-450. In the oxidation of aminopyrine radicals were detected by EPR,[182] while in the oxidation of DMA effective process inhibition by an antioxidant (1-naphthol) was noted[69,168].

We carried out a systematic comparative kinetic study of the oxidation of aniline by cumenyl hydroperoxide with participation of cytochrome P-450,[69,106,136] haemoglobin[169], and cytochrome *c*.[163] Aniline forms complexes with the three haemoproteins (Table 9). The most stable complexes are formed between cytochrome P-450 and aniline. The difference spectra of the complexes of cytochrome P-450 and cytochrome *c* differ owing to the different nature of the axial ligands in the fifth position (Table 1). An allosteric effect was observed in the interaction of aniline with haemoglobin and the complexes were characterised in this case by three

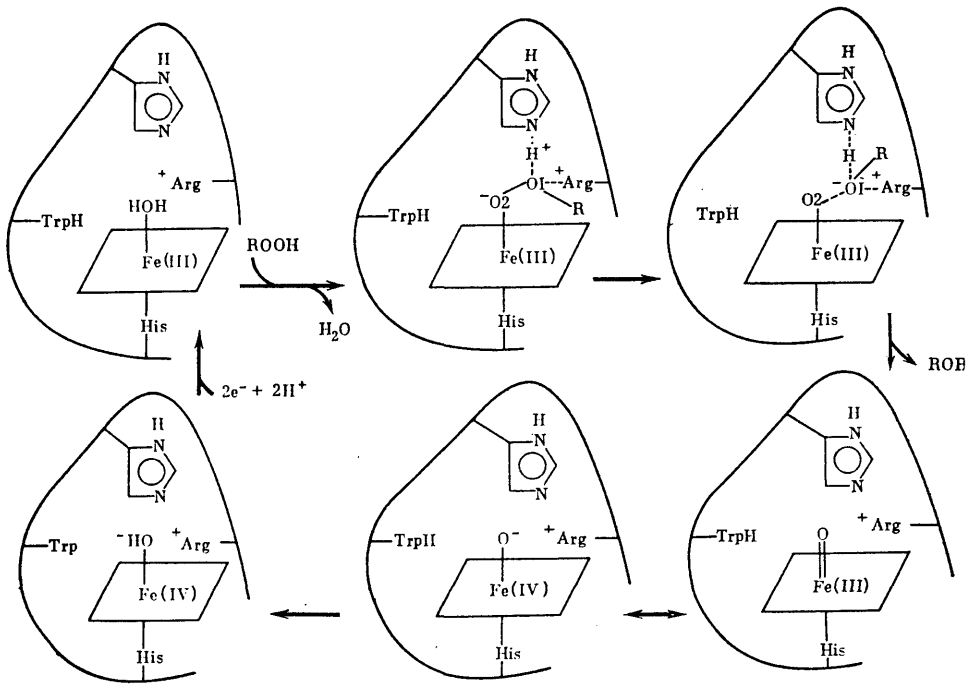


Figure 6.

dissociation constants—K_R, K_T, and K_S. All three haemoproteins catalyse effectively the oxidation of aniline to p-aminophenol. 1-Naphthol inhibits all three reactions, but does not arrest them completely. The reactions involving cytochrome *c* and haemoglobin have a radical mechanism, whereas in our view the reactions catalysed by cytochrome P-450 may involve not only radical stages but also the oxenoid pathway[69].

Table 8. Systems for the oxidative dealkylation of *N*-alkylamines exhibiting features of radical reactions.

Haemoprotein	Oxidant	Substrate	References
Horseradish peroxidase	H_2O_2	aminopyrine	167, 179
Ditto	"	DMA	180
"	"	DPT	180, 181
Metmyoglobin	H_2O	aminopyrine	179
Metmyoglobin	CHP	aminopyrine	182
Methaemoglobin	H_2O_2	DMA	168, 180
Methaemoglobin	H_2O_2	DPT	180
Methaemoglobin	CHP	DMA	168
Catalase	CHP	DMA	168
Protohaemin	H_2O_2	aminopyrine	179
Cytochrome P-450 from rabbit liver PB-microsomes	CHP	aminopyrine	178
Rat liver microsomes	CHP	DMA	168
Rabbit liver PB-microsomes	CHP	DMA	69
Cytochrome P-450-LM_2 from rabbit liver PB-microsomes	CHP	DMA	69

Note. DPT = *N*,*N*-dimethyl-*p*-toluidine; PB denotes microsomes induced by phenobarbital-sodium.

Table 9. Spectroscopic parameters of the complexes of aniline with haemoproteins.

Haemoprotein	λ_{max}, nm	λ_{min}, nm	K_S, mmol litre^{-1}	References
Cytochrome P-450:				
in PB-microsomes	431	398	0.60	160
in MC-microsomes†	431	399	0.027	160
in microsomes	429, 440	385, 397	0.160	160
P-450-LM_2 from rabbit microsomes	433	412	3.230	162
P-450-LM_4 from rabbit microsomes	427	391	1.430	162
Cytochrome *c*	421—417	401—399	24—13	183
Ditto	420	405	24*	163
Human haemoglobin	425	405	K_R 75 K_T 260 K_S 105	30
Horse haemoglobin	425	405	K_R 33 K_T 125 K_S 53	164

*Value obtained at pH 5.8; the remaining values of K_S for the aniline–cytochrome *c* complex were obtained at different pH.

†MC = 3-methylcholanthrene (Editor of Translation).

The hydroperoxide oxidation reaction of DMA is inhibited by a $CuCl_2$ and the copper complexes of lysine and tyrosine[144]. The inhibition of the oxidation of DMA by $CuCl_2$ in the reaction involving CHP and cytochrome P-450-LM^2 is non-competitive, while the copper complexes of lysine and tyrosine inhibit this process via a mechanism of the mixed type. The inhibiting effect of copper compounds on the oxidation of

DMA by cumenyl hydroperoxide can be explained by the decomposition of the hydroperoxide via the Haber–Weiss reaction with formation of the radicals $RO^{\cdot}$ and $RO_2^{\cdot}$, which causes the degradation of cytochrome P-450. Copper chloride at concentrations of ~0.001 M and above denatures cytochrome P-450 in the absence of CHP.[144]

The similarities and differences between the hydroperoxide oxidation reactions with participation of cytochrome P-450 and other haemoproteins can be discussed on the basis of the data presented in this section[16,18]. These processes are characterised by the following sequence of reactions:

$$Fe^{3+} + ROOH \rightleftarrows Fe^{3+}(H)-O-OR \quad ; \qquad (4)$$

$$Fe^{3+}(H)-O-OR \rightarrow [Fe^{3+}O] + ROH \quad ; \qquad (5)$$

Complex I

$$Fe^{3+}(H)-O-OR \rightarrow Fe^{2+} + H^+ + RO_2^{\cdot} \quad ; \qquad (6)$$

$$Fe^{3+}(H)-O-OR \rightarrow [FeOH]^{3+} + RO^{\cdot} \quad ; \qquad (7)$$

$$[Fe^{3+}O] + AH \rightarrow Fe^{3+} + AOH \qquad (8)$$

$$[Fe^{3+}O] + AH \rightarrow [Fe^{2+}O] + A^{\cdot} + H^+ \quad ;$$

Complex II

$$[Fe^{2+}O] + AH \xrightarrow{H^+} Fe^{3+} + A^{\cdot} + H_2O \quad . \qquad (10)$$

As already mentioned, all haemoproteins (with the exception of cytochrome P-450-LM_4) form complexes with ROOH [reaction (4)]. Peroxidases, catalases, and cytochrome P-450 subsequently form Complex I [reaction (5)]. Myoglobin, haemoglobin, and cytochrome *c* apparently do not form Complex I owing to the differences between the structure of the active centre and the structures of peroxidases, catalases, and cytochrome P-450. The formation of Complex I (where this occurs) is accompanied by the simultaneous reactions involving the decomposition of the complexes of haemoproteins with ROOH into radicals [reactions (6) and (7)]. All haemoproteins without exception can generate the radicals $RO^{\cdot}$ and $RO_2^{\cdot}$, which are capable of oxidising the majority of substrates of monoxygenases and can decompose the porphyrin ring in haemoproteins[16,159]. Reaction (5), on the one hand, and reactions (6) and (7), on the other, depend on the type of haemoprotein. Reactions (6) and (7) predominate for myoglobin, haemoglobin, and cytochrome *c*, while reaction (5) is the main one for cytochrome P-450, peroxidases, and catalyases, i.e. one group of haemoproteins (myoglobin, haemoglobin, and cytochrome *c*) decompose hydroperoxides via the homolytic mechanism, while the other group (cytochrome P-450, peroxidases, and catalases) cleave the O–OR bond mainly via the heterolytic mechanism with formation of Complex I. The presence of the substrates of cytochrome P-450 (aniline, DMA, and naphthalene) and radical reaction inhibitors (1-naphthol and substituted phenols) suppresses reactions (6) and (7) and promote the specific enhancement of reaction (5).[16,139]

The mechanism of the oxidation of Complexes I is determined, in the first place, by the nature of the haemoprotein and, in the second place, by the nature of the substrate. Complex I of peroxidases and catalases is characterised by the presence of an iron ion in a high oxidation state (Fe^{4+}) and is distinguished by its ability to undergo one-electron oxidation processes, since it is a radical [reaction (9)]. Reaction (9) results in the formation of Complex II, which oxidises in its turn yet another molecule of the substrate AH. The activity of Complexes I of peroxidases and catalases is fairly high and these complexes readily oxidise a multiplicity of substrates—phenols, amines, etc.[27,28], but cannot rupture the strong C–H bonds of saturated hydrocarbons, which are not substrates of peroxidases and catalases.

Complex I of cytochromes P-450 is capable of undergoing simultaneously two-electron oxidation reactions (with the oxenoid or hydroxylase mechanism) and one-electron oxidation reactions (with the radical or peroxidase mechanism). Presumably the reactivity of Complex I of cytochromes P-450 is higher than the reactivity of the same complex of peroxidases and catalases, i.e. the iron ion in Complexes I of cytochrome P-450 can exist in a higher oxidation state than the iron ion in Complexes I of peroxidases and catalases. The highest oxidation state of iron in Complex I of cytochrome P-450 is stabilised not only by the porphyrin ring, as in peroxidases[73,74], but also by the thiol ligand in the fifth coordination site. The main difference between cytochrome P-450 and other haemoproteins, responsible for its unique properties, consists in the possibility of the stabilisation of Complex I via two pathways—with participation of the porphyrin ring and the thiol ligand. The relative contributions of the radical and heterolytic stages in oxidation by Complex I of cytochrome P-450 are determined by the nature of the substrate: *N*-alkylamines, saturated hydrocarbons, ethers, and alcohols are oxidised by the radical mechanism, while aromatic compounds and olefins are oxidised mainly via the oxenoid heterolytic pathway, i.e. with the transfer of two electrons from the substrate to Complex I simultaneously. Naturally, in many instances there is a possibility of the simultaneous occurrence of the one- and two-electron oxidation reactions. This probably happens in the oxidation of benzo[*a*]pyrene and cyclohexene by hydroperoxides: the epoxyarene derivatives of benzo[*a*]pyrene and epoxycyclohexane are formed via the oxenoid mechanism, while the phenols derived from benzo[*a*]pyrene and 2-cyclohexanol are formed via the radical mechanism. Tertiary amines readily give up an electron to many oxidants and are therefore oxidised mainly via the radical mechanism, which has been proposed and proved by Griffin[179], with the intermediate formation of the relatively stable iminium cation $R_2\overset{+}{N}=CH_2$, which is converted into a secondary amine and formaldehyde in an aqueous medium.

Table 10 summarises the available information about the nature of the oxidation of different substrates by peroxides with participation of various haemoproteins. We have divided haemoproteins into three groups: the reactions of myoglobin, methaemoglobin, and cytochrome *c* *proceed only via the radical pathway* with participation of the radicals $RO^{\cdot}$ and $RO_2^{\cdot}$, the second group of haemoproteins—peroxidases and catalases—catalyse reactions via the radical mechanism with formation of Complexes I of these proteins, and finally the third group consists of cytochromes P-450, which, depending on the nature of the substrates, can convert them via the heterolytic pathway with participation of Complexes I {cyclohexene, naphthalene, aniline, and benzo[*a*]pyrene} or via the radical pathway with participation of the same Complex I and the radicals $RO^{\cdot}$ and $RO_2^{\cdot}$ (DMA, aminopyrine, cyclohexane, cumene, ethylbenzene, alcohols, and camphor).

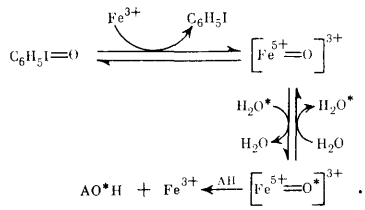
Table 10 shows that there are substrates with "amphoteric" properties, capable of being oxidised simultaneously via two pathways—the homolytic and heterolytic pathways {benzo[*a*]pyrene and possibly other aromatic hydocarbons}. It follows from the data presented here that there is no universal mechanism of the catalysis of the peroxide oxidation by haemoproteins. The mechanism of the peroxide reactions of haemoproteins is determined by several factors:

1. The nature of the haemoprotein, the structure of its active centre, and the fifth axial ligand.
2. The nature of the substrate oxidised, its ability to undergo one- or two-electron redox reactions, and the strength of the C–H bonds dissociated in hydrocarbons or the oxidation-reduction potential of *N*-alkylamines.
3. The nature of the oxidant employed.

Table 10. Reactions in ROOH–haemoprotein systems.

System	Possible oxidising agents	Substrate	
		one-electron reactions	two-electron (oxenoid) reactions
Myoglobin + CHP	$RO^{\cdot}$, $RO_2^{\cdot}$	aminopyrine [182]	none
Haemoglobin + CHP	ditto	aminopyrine [182]	none
Ditto	»	aniline [169]	none
»	»	DMA [168]	none
Cytochrome *c* + CHP	»	aniline [163]	none
Peroxidase + H_2O_2	$[Fe^{3+}O]$	aminopyrine [167]	none
Ditto	ditto	DMA [180]	none
Catalase + CHP	»	DMA [168]	none
Cytochrome P-450 from liver microsomes + ROOH	$[Fe^{3+}O]$, $RO^{\cdot}$, $RO_2^{\cdot}$	DMA [69]	cyclohexene [100]
Ditto	ditto	aminopyrine [178]	naphthalene [100]
»	»	cyclohexane [125]	aniline [69]
»	»	cumene [125]	—
»	»	ethylbenzene [125]	—
»	»	benzo[*a*]pyrene [165]	benzo[*a*]pyrene [165]
»	»		—
Cytochrome $P\text{-}450_{cam}$ + phenylperacetic acid	$[Fe^{3+}O]$, $RO^{\cdot}$, $RO^{2\cdot}$	alcohols [152] camphor [125]	—

The last postulate has been confirmed by the different behaviour in oxidation reactions with participation of cytochrome P-450 of oxidants such as hydrogen peroxide, organic hydroperoxides[12,128], and iodosobenzene[184]. It has been shown recently that, in the oxidation of camphor by iodosobenzene with participation of cytochrome $P\text{-}450_{cam}$ using $H_2{}^{18}O$, the oxygen incorporated in the oxidation product originates from water and not from the oxidant[184]. The authors[184] suggest that in the presence of $P\text{-}450_{cam}$ iodosobenzene forms a unique oxenoid capable of exchanging its oxygen rapidly with that of water:



In the absence of cytochrome $P\text{-}450_{cam}$, there is no isotope exchange between iodosobenzene and water. In our view, there is a possibility of another mechanism which explains the results of the above study: cytochrome P-450 catalyses the decomposition of iodosobenzene with formation of an oxygen atom, whose high reactivity explains its rapid isotope exchange with $H_2{}^{18}O$. The hydroxylating agent may be the ^{18}O atom, which exhibits a high hydroxylating capacity[18].

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In conclusion one should note that the final determination of the mechanisms of the catalytic action of haemoproteins in oxidation reactions requires further studies of several different kinds: comparative investigation of the oxidation of a wide range of substrates with participation of different haemoproteins under comparable conditions and the search for new approaches to the study of the intermediate stages in processes with participation of cytochrome P-450 in order to elucidate the nature and structure of the hydroxylating agent. The determination of the nature of the active species in microsomal oxidation will lead to new possibilities for a specific search for simple catalytic chemical systems capable of replacing a unique haemoprotein, namely cytochrome P-450, and for devising oxidation processes which will not be inferior in their efficiency and selectivity to enzymatic processes.

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