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Cytochrome P-450—An Effective Catalyst of the Oxidation of Organic Compounds by Peroxides

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Experimental data on the oxidation of organic compounds by peroxides and other oxidants with participation of cytochrome P-450 from different natural sources are described sytematically and critically. The role of cytochrome P-450 in the oxidative dealkylation of *N*-alkylamines and ethers by hydroperoxides and also its involvement in the peroxide oxidation of aromatic compounds, saturated aliphatic acids, steroids, and other substrates is discussed. Attention is concentrated on the comparison of the mechanism of the oxidation of organic compounds in the complete liver enzyme system, incorporating cytochrome P-450, cytochrome P-450 reductase, and phospholipids as well as NADPH and O_2, and the mechanism of the oxidation of organic compounds by hydroperoxides with participation of cytochrome P-450 alone.
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I. INTRODUCTION

In 1978, 20 years elapsed since the discovery by Garfinkel[1] and Klingenberg[2] of a unique haemoprotein—cytochrome P-450. This haemoprotein is widely distributed in nature and is a component of enzyme systems belonging to the class of oxidoreductases which have been called mono-oxygenases or mixed-function oxidases. Three years after the discovery of cytochrome P-450 Mason et al.[3] and Hayashi et al.[4] independently discovered a new type of oxidoreductases which obligatorily require the presence of reduced pyridine-nucleotides (NADPH or NADH) and which oxidise various substrates, inserting one oxygen atom in the latter and reducing the other to water:

$$AH + NADPH + H^+ + O_2 \rightarrow AOH + NADP + H_2O ,$$

where AH is the substrate and AOH the substrate oxidation product. In 1964 Omura and Sato[5,6] established that cytochrome P-450 is a *b*-type haemoprotein and is characterised by an atypical absorption band of the carbonyl complex in the reduced state at 450 nm, which gave rise to the name of the protein.

During two decades, considerable advances have been made in the study of mono-oxygenases, their composition, properties, function, and structure. These achievements have been reflected in monographs, collections of papers, reviews, and conference proceedings[7-16]. It is now well known that enzyme systems containing cytochrome P-450 enter into the composition of the microsomal fraction of human and mammalian liver cells and are responsible for the metabolism of steroids, prostaglandins, and an enormous number of xenobiotics—foreign substances entering the organism from the environment, among which there are carcinogens, medicinal drugs, and other substances. A steroid hydroxylating system, similar in properties to the microsomal hydroxylating system of the liver, is localised in the mitochondria of the adrenal cortex and is responsible for key stages in the biosynthesis of steroid hormones in man and mammals. Many strains of bacteria contain hydroxylating enzyme systems with cytochrome P-450 as the terminal oxidase and are capable of oxidising hydrocarbons, heterocycles, and compounds of other classes.

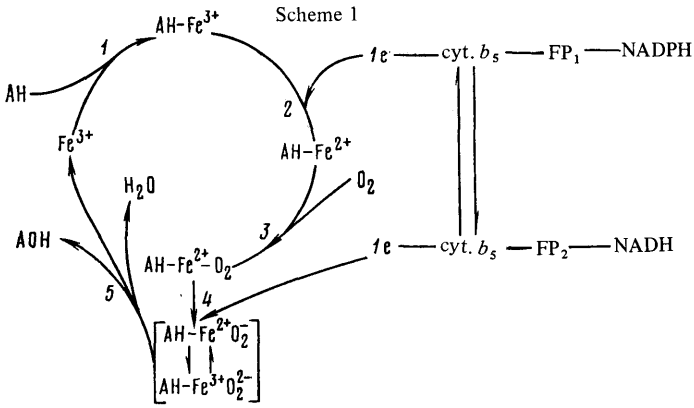
The microsomal hydroxylating system of the liver has particularly attracted the attention of investigators in connection with the significant role of its functions from the point of view of pharmacologists, toxicologists, specialists in chemical carcinogenesis, biochemists, and biophysicists. The microsomal hydroxylating system of the liver consists of three components, each of which has now been isolated and fairly completely characterised[12]: NADPH-cytochrom P-450 reductase (EC 1.6.2.4.), cytochrome P-450 [aryl 4-monoxygenase] (EC 1.14.14.1), and phospholipids. The steroid hydroxylating system from the mitochondria of the adrenal cortex also consists of three main components: adrenodoxin-reductase (EC 1.6.7.1), ferredoxin (adrenodoxin), and cytochrome P-450. The bacterial camphor hydroxylating system also incorporates three enzymes[8]: putidaredoxin reductase, putidaredoxin, and cytochrome P-450_{cam}†.

The mechanism of the action of all three systems is complex and includes many stages. Scheme I presents the sequence of stages in the enzymatic oxidation in the microsomal liver system proposed by Estabrook et al.[17] The majority of these stages have been studied by the methods of stationary and rapid kinetics[18]. The first stage consists in the interaction of the substrate with the oxidised form of cytochrome P-450. The rate constants for this reaction are high and lie in the range 10^2–10^5 litre mol^{-1} s^{-1}.[18] The formation of the enzyme-substrate complex is accompanied by the transition of the

†The following abbreviations have been adopted: cytochrome P-450_{cam} = cytochrome P-450 of camphor hydroxylating bacteria; cytochrome P-450-LM_2 = the form of cytochrome P-450 from the livers of rabbits receiving phenobarbital sodium; PB = phenobarbital sodium; 3-Mech = 3-methylcholanthrene; CHP = cumenyl hydroperoxide; TBHP = t-butyl hydroperoxide; NADPH and NADH can be omitted as they are terms in universal use. AH = substrate in the reduced form.

haem iron from the low-spin to the high-spin state for substrates of type I—hydrocarbons, fully alkylated amines, and unsaturated compounds[19]. The second stage is the reduction of the enzyme–substrate complex by the first electron arriving from the NADPH of the NADPH-specific transport chain via the flavoprotein FP_1 with the possible participation of cytochrome b_5 contained in the membranes of the endoplasmic reticulum of the liver. The second stage consists of two phases: the first phase is characterised by rate constants of 0.2–1 s^{-1} for different substrates and the second (slow) phase is characterised by rate constants in the range 0.09–0.31 s^{-1}.[18]

Scheme 1



The third stage is the formation of the $HA-Fe^{2+}-O_2$ ternary complex. Oxygen adds to the reduced enzyme–substrate complex at high rates of approximately 10^6 mol litre^{-1} s^{-1}.[20] The fourth stage is the reduction of the ternary complex $HA-Fe^{2+}-O_2$ by the second electron, which is believed to be supplied by the NADPH-specific transport chain incorporating the flavoprotein FP_2 and cytochrome b_5. At the present time this reduction stage has not so far been resolved into individual steps for microsomal systems and its rates have not been measured. The fifth stage, which can also be a complex process and can consist of several steps, is characterised by intramolecular reactions of the reduced ternary complex and its decomposition with liberation of a water molecule and a hydroxylated derivative. Cytochrome P-450 is reconverted into the initial form in the fifth stage. Thus the activation of the molecular oxygen in the ternary complex takes place as a result of its reduction by two electrons. The nature of the active form of oxygen responsible for the hydroxylation of chemical compounds of different structure has not so far been established and is controversial.

The mechanism of hydroxylation in the steroid oxidising system and in the camphor hydroxylating bacterial system has, on the one hand, features in common with the oxidation in microsomes and, on the other hand, is characterised by an appreciable difference from the microsomal system[10,11].

The possibility of using organic hydroperoxides for the oxidation of *N*-alkylamines with participation of only cytochrome P-450 in the oxidised form, first discovered by Kadluvar et al.[21], is very important for the study of the mechanism of the activation of molecular oxygen by cytochrome P-450. The use for the hydroxylation of the organic compounds of oxidants containing oxygen already reduced by two electrons (hydroperoxides), $NaIO_4$, $NaClO_2$, and hydrogen peroxide obviates the necessity for the complex electron transport system consisting of macromolecular biocomponents—FP_1, FP_2, and cytochrome b_5.

Thus a possibility arises for simplifying the hydroxylating enzyme system of microsomes by replacing complex components which are difficult to isolate by readily available oxidants. This may be of great importance for the practical application of this unique biocatalyst in the hydroperoxide oxidation of compounds important for pharmacology and medicine. The high capacity of variable-valence metal complexes for the catalytic hydroperoxide oxidation of unsaturated compounds is well known[22,23]: the hydroperoxide oxidation of olefins catalysed by molybdenum complexes constitutes the basis of a familiar method of synthesising epoxypropane developed by the HALCON Company[24].

The aim of the present review is to give a systematic and critical account of reactions involving the oxidation of organic compounds by peroxides with participation of cytochrome P-450 and certain other haemoproteins—haemoglobin, catalase, and cytochrome *c*. We do not consider it necessary to examine here the oxidation of organic compounds by hydrogen peroxide with participation of peroxidases, since peroxide oxidation has been examined in detail in thorough reviews[25,26].

II. OXIDATIVE *N*-DEALKYLATION OF *N*-ALKYLAMINES

The oxidative *N*-dealkylation of alkylamines is one of the commonest reactions catalysed by the microsomal enzyme system. It is believed that this reaction proceeds in two stages, the first of which consists of the insertion of an oxygen atom in a C–H bond of an alkyl substituent with formation of an intermediate hydroxylated compound, which decomposes with liberation of the free amine and formaldehyde:

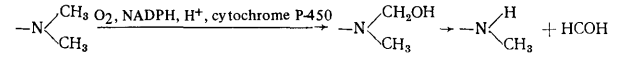


Table 1. The rates of oxidation of amines by pig liver microsomes with participation of cofactors, CHP, and bovine catalase (the rates are given in nanomoles of formaldehyde per minute per miligram of microsomal protein)[21].

Amine	Microsomes, NADPH, O_2	CHP	
		microsomes	catalase
Dimethylaniline	3.4	35.1	451
Aminopyrine	3.5	5.8	312
Benzphetamine	3.3	4.2	10
Propoxyphene	4.7	3.3	0
Ethylmorphine	6.3	2.2	0
Metamphetamine	2.0	0.4	0

The oxidation of aminopyrine, benzphetamine, dimethylaniline, ethylmorphine, and other alkylamines by various peroxides with participation of various animal liver microsomes and highly purified cytochrome P-450 has been investigated[21,27–31]. Table 1 presents data for the oxidation of various amines by cumenyl hydroperoxide (HP) with participation of pig liver microsomes or bovine catalase and compares them with data for the microsomal oxidation of the same substrates in the complete enzyme system containing all the components, NADPH, and O_2.[21] The replacement of the cofactors—NADPH and O_2—by CHP ensures the oxidation of all the amines investigated at rates which in many instances exceed the rate of oxidation in the complete sys-

tem (dimethylaniline and aminopyrine). Catalase is effective only in the oxidation of the first three substrates under the influence of CHP.

The comparison of the efficiencies of various peroxides in the oxidation of the same substrate is of interest (Table 2). The most effective oxidant is CHP. The generation of H_2O_2 in a system containing glucose and glucose oxidase does not ensure high rates of oxidation of aminopyrine. However, comparatively high rates of oxidation of aminopyrine as well as benzphetamine and *p*-nitroanisole by microsomes have been observed in the electrolytic generation of H_2O_2 (or HO_2^-).[28] The participation of cytochrome P-450 in the peroxide oxidation of all amines and many compounds of other classes does not give rise to any doubts. Its involvement has been confirmed by the inhibiting effect of detergents, which convert cytochrome P-450 into the inactive form P-420 (SKF-525A, potassium deoxycholate, etc.)[21] and by the increase of the rate of oxidation of aminopyrine when the microsomes of animals administered phenobarbital are used, since in this case the content of P-450 in the microsomes is significantly increased. The rate of oxidation of aminopyrine by CHP increases in proportion to the cytochrome P-450 concentration. The most convincing demonstration of the direct involvement of cytochrome P-450 in the oxidative dealkylation of amines by peroxides is provided by experiments on the oxidation of benzphetamine involving CHP and highly purified cytochrome P-450 in one of its forms (LM_2) produced in rat liver after induction with phenobarbital[31]. Highly purified cytochrome P-450 is free from reductase, cytochrome b_5, and other components of the microsomal electron transport chains and exhibits a high catalytic activity, which is equal to or exceeds the activity of cytochrome P-450 in microsomes[31].

Table 2. Oxidation of aminopyrine by various peroxides with participation of pig liver microsomes or bovine catalase[21].

Peroxide	Microsomes	Catalase
Cumenyl hydroperoxide	10.8	312
t-Butyl hydroperoxide	3.8	165
Geraniol hydroperoxide	2.0	89
Linoleic acid hydroperoxide	0.7	11
Pregnenolone 17α-hydroperoxide	0.4	8
Di-t-butyl peroxide	0.2	14
H_2O_2-generating system	0.1	0

A detailed kinetic study of the demethylation of many amines by hydroperoxides in the range 20–37 °C has been carried out in our laboratory[27,29,30]. Assuming that the step involving the insertion of activated oxygen in the substrate–cytochrome P-450–ROOH ternary system is rate limiting in the hydroperoxide oxidation of amines and that the subsequent stages involving the liberation of the reaction products are much faster, it is possible to regard the catalytic rate constant as a quantitative characteristic of the decomposition of the ternary complex (Table 3). It follows from Table 3 that the activation energies for the oxidation of amines in hydroperoxide systems (E) are close to the values obtained in the same temperature range and under the same conditions for the complete microsomal system with the cofactors (E^*). The difference between them is within the limits of the error of the determination of the activation energy in a narrow temperature range. The oxidation of amines by hydroperoxides with participation of cytochrome P-450 is characterised by a compensation effect which is described by an equation relating the activation characteristics for the decomposition of the substrate–enzyme–hydroperoxide ternary complex: $\Delta H^*(\text{kcal mol}^{-1}) = 18.7 + 333\Delta S^*$. The compensation effect is characterised by the quantity $\alpha = 333/T_{av} = 1.11$, where T_{av} is the average temperature of the experiments.

Table 3. Parameters of the activation of the enzyme–substrate complexes in the hydroperoxide oxidation of amines with participation of microsomal cytochrome P-450.[29,30]

System	Pre-exponential factor, s^{-1}	E, kcal mol^{-1}	E^*, kcal mol^{-1}	ΔE^a, kcal mol^{-1}
1,2,7-Trimethyldecahydro-4-quinolone + CHP	$6.45 \cdot 10^9$	13.7	14.2	+0.5
Dimethylaniline + TBHP	$7.76 \cdot 10^{10}$	15.8	—	—
Aminopyrine + CHP	$1.12 \cdot 10^9$	12.7	13.4	+0.7
Aminopyrine + CHP[b]	$3.30 \cdot 10^8$	12.0	—	—
Monomethylaniline + CHP	$1.95 \cdot 10^{11}$	16.1	16.6	+0.5
Dimethylaniline + CHP	$5.12 \cdot 10^8$	12.2	13.4	+1.2

Note. [a] $\Delta E = E^* - E$; [b] experiments performed with microsomes of rats receiving phenobarbital.

III. THE HYDROPEROXIDE *O*-DEALKYLATION OF ETHERS

The complete microsomal hydroxylating system in the presence of the cofactors NADPH and O_2 catalyses the oxidative *O*-dealkylation of ethers via the mechanism

$$R{-}OCH_3 \xrightarrow{O_2} R{-}OCH_2OH \rightarrow R{-}OH + HCOH \ .$$

In peroxide systems these reactions are carried out by microsomes in the absence of NADPH and O_2.[32–35]

Table 4. Comparison of the rates of the NADPH-dependent and CHP-dependent *O*-dealkylation in catalysis by rabbit PB-microsomes at 22–25 °C (the rates are expressed in nanomoles of aldehydes per minute per milligramme of microsomal protein)[35].

Substrate	With participation of NADPH and O_2	With participation of CHP
7-Ethoxycoumarin	1.5	3.6
p-Nitroanisole	2.3	12.0
Norcodeine	6.2	6.2
Phenacetine	2.3	2.7

Cumenyl hydroperoxide is effective in the *O*-dealkylation of many substrates (Table 4) and in the oxidation of *p*-nitroanisole the rate of reaction exceeds by a factor of 6 the rate of oxidation in the complete system. *O*-Dealkylation is greatly inhibited by sodium dodecyl sulphate and 1-(2-isopropylphenyl)imidazole[32]. Superoxide dismutase hardly affects the rate of *O*-dealkylation, i.e. the reaction does not involve the active radical-anions O_2^-. It is extremely important that haemin, catalase, horseradish peroxidase, and myoglobin are completely inactive in the reactions involving *O*-dealkylation by hydroperoxides[32].

A detailed kinetic study of the *O*-dealkylation of 7-ethoxycoumarin in the range 0–45 °C and of anisole and *p*-chloroanisole in the range 20–37 °C with participation of CHP and microsomes has been carried out[34,35] (Table 5).

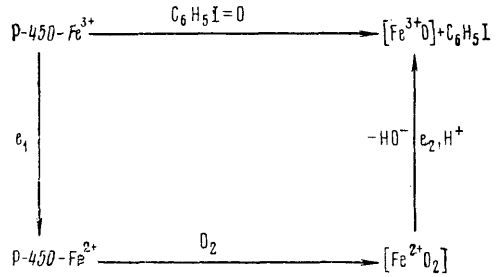
Table 5. Characteristics of the *O*-dealkylation of ethers by cumenyl hydroperoxide[34,35].

Substrate	System	k_0, s^{-1}	E, kcal mol^{-1}	Refs.
Anisole	CHP + intact microsomes	$2.0 \cdot 10^7$	11.4	[34]
p-Chloroanisole	CHP + intact microsomes	$5.5 \cdot 10^7$	11.8	[34]
7-Ethoxycoumarin	CHP + PB-microsomes	—	17.0	[35]

The hydroperoxide oxidation of the same substrate can be achieved via two pathways[34]: TBHP and microsomes demethylate anisole (20%) and at the same time hydroxylate it in the *p*-position (80%) with formation of *p*-methoxyphenol.

For the understanding of the mechanism of oxidation with participation of peroxides and cytochrome P-450, it is of interest that 7-ethoxycoumarin can be *O*-dealkylated by iodosobenzene with participation of microsomes[33], since iodosobenzene contains only one oxygen atom. Presumably the active species attacking the substrates contains only one oxygen atom. Such a species can be the oxenoid complex $[FeO]^{3+}$ (Scheme 2). Scheme 2 illustrates the pathways leading to the formation of the $[FeO]^{3+}$ species by the reaction with iodosobenzene and also in the complete microsomal system.

Scheme 2



IV. HYDROXYLATION OF AROMATIC COMPOUNDS AND EPOXIDATION OF CYCLOHEXENE

A number of studies have been made of the hydroxylation of aromatic compounds by hydroperoxides with participation of liver microsomes and highly purified cytochrome P-450 [36–46] (Table 6).

The hydroxylation of aromatic compounds is accompanied by the decomposition of CHP with formation of cumenyl alcohol. It has been shown[36] that one *p*-aminophenol molecule is formed for each CHP molecule decomposing in the oxidation of aniline. Haemin, catalase, horseradish peroxidase, myoglobin, cytochrome b_5, and cytochrome P-420 are ineffective in the hydroxylation of aniline by cumenyl hydroperoxides[36].

The kinetic characteristics of the hydroxylation of naphthalene by cumenyl hydroperoxide with participation of different enzyme systems have been compared quantitatively[38]. The rate of reaction in the presence of the enzyme system induced by phenobarbital sodium increases in proportion to the increase of the concentration of cytochrome P-450. The catalytic rate constant in induction by 3-methylcholanthrene is reduced by a factor of two compared with the intact systems due to the formation of a new form of haemoprotein (cytochrome P-448) in 3-Mech-induced systems.

Table 6. Comparison of the rates of oxidation of aromatic substrates in the presence of NADPH and O_2 as cofactors and CHP (the rates are expressed in nanomoles of reaction products formed per minute in miligrams of microsomal protein).

System	Substrate	NADPH and O_2 cofactors	CHP
Rat liver microsomes	biphenyl	0.60	1.70
Rat liver microsomes	benzopyrene	0.78	0.89
Rabbit liver PB-microsomes	coumarin	0.062	0.055
Rabbit liver-3-Mech-microsomes	aniline	0.51	50.0

Table 7. The energy characteristics of the oxidation of naphthalene, aniline, and cyclohexene by hydroperoxides.

System	t, °C	E, kcal mol^{-1}	Refs.
Naphthalene			
Male rat PB-microsomes + CHP	5–40	18.8	42
Highly purified rabbit liver cytochrome P-450 (13 nmol/mg of protein) + CHP	5–38	17.2	42
Aniline			
Intact male rate microsomes + CHP	10–40	18.1	42
Male rat PB-microsomes + CHP	5–36	21.3	42
Intact female rat microsomes + CHP	15–37	15.3	40, 41
Intact female rat microsomes + TBHP	15–37	14.5	40, 41
Rat liver cytochrome P-450 (4.4 nmol/mg of protein) + CHP	14–37	15.8	39
Cyclohexane			
Intact male rat liver microsomes + TBHP	20–37	8.15	29

The kinetics of the hydroxylation of naphthalene and aniline by hydroperoxides over a wide temperature range and of the epoxidation of cyclohexene by cumenyl hydroperoxide have been studied in detail[29,37–46]. All the experiments were performed under the conditions involving saturation with respect to the hydroperoxide. The temperature variation of the maximum rates of oxidation of aniline, naphthalene, and cyclohexene obeys the Arrhenius equation, from which the process activation energies were calculated (Table 7). The activation energies for the oxidation of naphthalene by microsomes and highly purified cytochrome in the reaction with the same hydroperoxide are similar. The activation energies for the oxidation of aniline to *p*-aminophenol catalysed by male rat microsomes and purified cytochrome P-450 also differ a little—15.3 and 15.8 kcal mol^{-1}.[39,40,41] Induction by phenobarbital hardly affects the energy characteristics of the oxidation of naphthalene and aniline by cumenyl hydroperoxide[42].

Thus cytochrome P-450 in microsomes and highly purified cytochrome P-450 in solution do not exhibit energy differences in reactions involving the oxidation of the same substrate (naphthalane or aniline) by hydroperoxides. It is therefore of interest to investigate the influence of individual phospholipids on the reactivity of highly purified cytochrome P-450 in the hydroperoxide oxidation of aromatic compounds[43-46], since phospholipids are components of microsomal membranes and are necessary for the reconstruction of the microsomal system for the restoration of its full activity.

The hydroxylation of naphthalene and aniline by cumenyl hydroperoxide with participation of the highly purified LM_2 form of cytochrome P-450 from rabbit liver PB-microsomes (16.8 nmol/mg of protein) has been studied in the presence and absence of various phospholipids[44-46] and it has been shown that the total microsomal phospholipids (TPL), phosphatidylinositol (PI), and lysophosphatidylcholine (LPC) increase the rate of oxidation of naphthalene in the reaction with CHP by a factor of 3–4 when the molar ratio lipids : cytochrome P-450 is 30 and more. Phosphatidylcholine (PC) and sphingomyelin (SM) hardly affect the oxidation of naphthalene up to lipid : cytochrome P-450 ratios of 200–300. The addition of aliphatic acids to the incubation mixture retards the oxidation of naphthalene by cumenyl hydroperoxide.

The addition of TPL, PI, PC, and LPC increases also the rate of oxidation of aniline in the reaction with CHP by a factor of two, PC and SM do not influence the oxidation of aniline, while aliphatic acids retard this process. Thus acid phospholipids (PC, PI, and TPL), containing acid lipid components, activate cytochrome P-450 in the hydroperoxide oxidation of naphthalene and aniline, while lipids existing in the zwitterionic form do not affect the rate of oxidation of a type I substrate (naphthalene) and a type II substrate (aniline)[44-46]. Thus the interaction of cytochrome P-450 with acid lipids affects the rates of hydroperoxide oxidation of aniline. Phosphatidylcholine—the principal component of the microsomal membrane—hardly affects this process. It may be that this factor actually explains the absence of a difference between the activation energies for the hydroperoxide oxidation of naphthalene and aniline with participation of highly purified cytochrome P-450 and membrane-bound cytochrome P-450.[39-42]

The activation of cytochrome P-450 by acid phospholipids in the hydroperoxide oxidation reaction can be accounted for by the stabilisation of the active conformation of the haemoprotein in solution when the lipid molecules are immobilised on the surface of the protein. Coon et al.[47] showed that the interaction of the LM_2 form of cytochrome P-450 with phospholipids is accompanied by an increase of the α-helix content. The spectroscopic changes recorded by differential spectrophotometry in the interaction of LM_2 cytochrome P-450 with phospholipids at different temperatures permit the conclusion that phospholipids can influence the equilibrium involving the spin forms of cytochrome P-450 differing in their ability to interact with substrates of this enzyme[48]. The general considerations concerning the stabilisation of the cytochrome P-450 conformation on interaction with phospholipids can be made specific: cytochrome P-450 assumes the α-helix conformation and can undergo a change in the spin state as a result of conformational rearrangements.

V. OXIDATION OF SATURATED COMPOUNDS AND STEROIDS

Microsomal hydroxylating systems of the liver and kidney, incorporating cytochrome P-450, are known to catalyse the ω-oxidation of aliphatic acids[49,50], the oxidation products being ω1- and ω2-hydroxy acids[50,51]. It has been suggested that liver microsomes contain a highly specific cytochrome P-450 responsible solely for the oxidation to ω1-hydroxy-derivatives, as well as another non-specific cytochrome P-450 which catalyses the ω2-oxidation of aliphatic acids and the hydroxylation of many medicinal drugs and other xenobiotics[51-54]. Kidney microsomes probably contain only one form of cytochrome P-450, which is responsible for both ω1- and ω2-oxidation of aliphatic acids[55,56]. The ratio of the ω1- and ω2-oxidation products is determined by the length of the alkyl substituent in aliphatic acids.

Table 8. Hydroxylation of steroids with participation of rat liver microsomes and different oxidants[60].

Main reaction products	Reaction rates*		
	NADPH, and O_2	$NaIO_4$	CHP
Androstenedione			
6α-OH	0.58	3.97	0.85
7α-OH	0.13	7.13	0.65
15-OH	0.13	0.90	0.12
16α-OH	0.27	0.58	0.43
17β-hydroxy-4-androstene-3,16-dione	0.12	0.70	0.10
Total	1.23	13.28	2.15
Testosterone			
6β-OH	0.74	2.20	1.22
7α-OH	0.34	3.98	0.25
16α-OH	0.16	0.71	0.17
Total	1.24	6.99	1.64
Progesterone			
2α-OH	0.23	0.62	0.12
6β-OH	0.47	1.20	0.48
7α-OH	0	0.44	0
15α-OH	0	0.22	0
15β-OH	0	0.26	0.14
16α-OH	0.41	0.44	0.26
Total	1.11	3.18	1.00
17β-Oestradiol			
Total**	0.38	4.77	1.02

*The rates are given in nanomoles of product formed per minute per miligram of protein.
**The overall data for 6α-OH, 6β-OH, and 16α-OH are indicated.

Cumenyl hydroperoxide and liver microsomes ensure the oxidation of lauric acid to an ω2-hydroxyacid, the ω1-hydroxy-derivative not being formed in detectable amounts[57], while in the complete system containing the cofactors NADPH and O_2 the formation of ω1- and ω2-hydroxy-derivatives in proportions of 1 : 5 is observed. Kidney microsomes and CHP ensure the ω2-oxidation of lauric acid at low rates, but ω1-hydroxy-lauric acid was not detected in this instance[57]. Thus oxidation of lauric acid by CHP with participation of liver and kidney microsomes can yield the same product in both instances—ω2-hydroxylauric acid. This fact confirms the existence in the liver microsomal fraction of two or more forms of cytochrome P-450 responsible for the ω-oxidation of aliphatic acids. Presumably cytochrome P-450 responsible for the ω2-oxidation of aliphatic acids by cumenyl hydroperoxide is identical in liver and kidney microsomes[57].

Rat liver microsomes catalyse the oxidation of aliphatic acids by oxidants such as sodium chlorite, sodium periodate, iodosobenzene, and iodosobenzene diacetate[58]. The oxidants employed hydroxylate aliphatic acids only in the ω2-position

and are much more effective than NADPH and O_2 in the complete microsomal system; iodosobenzene diacetate is the most effective substance. The ω2-oxidation of laurate with participation of liver microsomes is inhibited by a substrate of the microsomal system (aniline), which fully arrests the process[58].

A number of studies have been made on the peroxide oxidation of steroids with participation of cytochrome P-450-containing systems[59–65]. The peroxide oxidation of steroids can be catalysed by liver microsomes[60], cytochrome P-450 from adrenal cortex mitochondria[62], and cytochrome P-450 from liver microsomes[59,63]. Table 8 presents data on the oxidation of steroids in the full microsomal system and also in the presence of $NaIO_3$ and CHP.[60] Evidently the use of $NaIO_3$ and CHP in combination with liver microsomes does not ensure the selective oxidation of steroids, which can be attributed to the presence of many forms of cytochrome P-450 in liver microsomes or to the possible role of radicals in the oxidation, which frequently leads to the absence of selectivity. $NaIO_3$ is the most effective oxidant for steroids[60]; $NaClO_2$, CHP, pregnenolone 17 α-hydroperoxide, t-butyl hydroperoxide, and linoleic acid hydroperoxide give rise to lower rates of oxidation than in the complete system with participation of NADPH and O_2. Oxidants such as H_2O_2, $K_2Cr_2O_7$, CrO_3, $KClO_4$, $KBrO_3$, K_2IrCl_6, and $NaIO_3$ exhibit no activity in the oxidation of steroids with participation of liver microsomes[60].

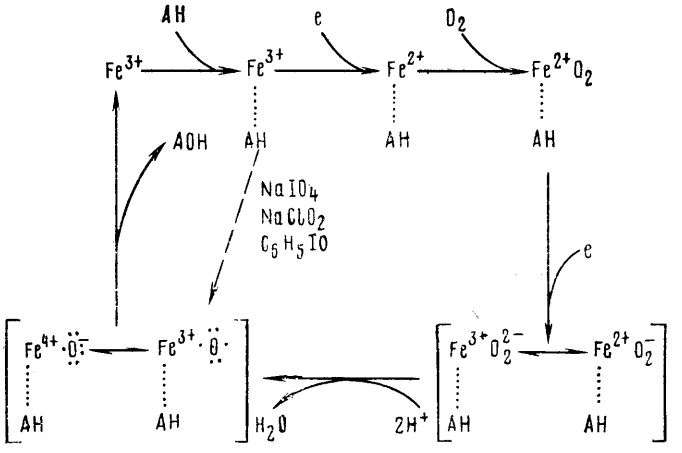
The partly purified cytochrome P-450 from rat liver microsomes oxidises androstenedione when $NaIO_4$, $NaClO_2$, H_2O_2, and organic hydroperoxides are used[59]. Sodium periodate is the most effective oxidant. It is noteworthy that horseradish peroxidase, catalase, and metmyoglobin do not exhibit catalytic properties in the oxidation of steroids by various peroxides[59].

The study of the oxidation of androstenedione by various iodobenzene derivatives with participation of cytochrome P-450 from liver microsomes showed[64] that the effectiveness of the oxidant diminishes in the sequence 2-nitroiodosobenzene diacetate > 2-chloroiodosobenzene diacetate > 2-methoxyiodosobenzene diacetate 4-methyliodosobenzene > iodosobenzene. The effectiveness of the oxidant is determined by several factors: the tendency towards its oligomerisation, which diminishes when the oxidant is coordinated to haem iron, the presence of substituents in the 2-position, and the electron-donating capacity of the substituents. The rates of 7α-, 6β-, and 16α-hydroxylation of androstenedione by 2-nitroiodosobenzene diacetate are higher by factors of 108, 130, and 167 than the corresponding values for the oxidation of this substrate in the same positions in the complete microsomal system. The authors[64] believe that this finding supports the view that there are different rate-limiting stages in the oxidation of androstenedione in the complete microsomal system and in the reaction involving peroxides.

The majority of oxygen donors used in the oxidation of steroids do not ensure process selectivity, in contrast to the observations made in the ω-hydroxylation of saturated aliphatic acids[57,58]. However, in the oxidation of cholesterol by $NaIO_4$ and CHP in the presence of liver microsomes, exceptional selectivity is observed: both oxidants ensure the exclusive 25-hydroxylation of cholesterol[65]. It is of interest to note that, in the oxidation of cholesterol in the complete microsomal system with participation of NADPH and O_2, hydroxylation takes place in the 6β-, 7α-, 12α-, 25-, and 26-positions[65]. In this case the transition to peroxide oxidation is characterised by a greater reaction selectivity. This finding is difficult to account for unambiguously, although one can postulate that competition between the oxidants and cholesterol for binding to cytochrome P-450 renders all the substrate positions except the 25-position inaccessible to attack by the hydroxylating agent. The effectiveness of highly purified cytochrome P-450 in the oxidation of androstenedione by sodium periodate increases following the addition of phosphatidylcholine: the rate of 6β-hydroxylation of this substrate increases by a factor of 2.5 for a cytochrome P-450 : lipid ratio of approximately 10.[63] Detergents such as Triton X-100 also exert an activating effect on cytochrome P-450 in the oxidation of androstenedione by sodium periodate[63].

Hrycay and coworkers[59–62] believe that the active oxidant in the hydroxylation of steroids by various peroxides is the same species, namely the ferryl ion $[Fe^{4+}O^-]$, which is formed when oxygen donors (ROOH, $NaIO_4$, $NaClO_2$, etc.) interact with oxidised cytochrome P-450 (Scheme 3). The postulated species is analogous to the well known complex I (see Scheme 3) formed in the interaction of hydrogen peroxide with peroxidases or catalases[25,26].

Scheme 3



One of the alicyclic compounds oxidised by hydroperoxides with participation of microsomes or purified cytochrome P-450 is cyclohexane[66,31]. In this case the hydroperoxide system (TBHP) is less effective than the complete microsomal system. The affinity of cyclohexane for cytochrome P-450 in the presence of TBHP decreases by a factor greater than 2 and the rate of oxidation falls by a factor of approximately 10.[66]

In conclusion of this section of the review, one should note that both liver microsomes and pure cytochrome P-450 are effective catalysts of the oxidation of saturated compounds such as aliphatic acids and cyclohexanes. It is significant that these compounds are not oxidised at appreciable rates at temperatures close to physiological values by oxidants such as hydroperoxides, hydrogen peroxide, and $NaClO_2$. Cytochrome P-450 accelerates the oxidation of saturated compounds by peroxides. This finding is particularly important from the standpoint that the strength of the C–H bonds at which oxidation occurs is high compared with compounds of other classes (90–95 kcal mol^{-1}). Nevertheless the oxidation of saturated compounds by readily available oxidants with participation of cytochrome P-450 at 20–37 °C is strikingly easy.

VI. PEROXIDASE REACTIONS OF CYTOCHROME P-450

Reactions catalysed by peroxidases are characterised by the following stoichiometry:

$$AH_2 + XOOH \rightarrow A + H_2O + XOH ,$$

where AH_2 is the hydrogen donor and XOOH the peroxy-compound. In 1972 Hrycay and O'Brien[67] showed that cytochrome P-450 exhibits the properties of peroxidases and reduces steroid hydroperoxides to the corresponding alcohols in the presence of a hydrogen donor such as *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPPD). The peroxidase activity of rat liver and borine adrenal cortex microsomes in relation to steroid peroxides is analysed in Table 9.[67] In all cases the rat liver microsomes are more active than the bovine adrenal cortex microsomes.

Table 9. The peroxidase activity of microsomes in relation to steroid hydroperoxides*[67].

Hydroperoxide	Rat liver microsomes	Bovine adrenocortical microsomes
Progesterone 17α-hydroperoxide	92	31
Pregnenolone 17α-hydroperoxide	80	30
Allopregnanolone 17α-hydroperoxide	76	27
Linoleic acid hydroperoxide	75	25
Cumenyl hydroperoxide	34	12
Cholesterol 7β-hydroperoxide	30	17
Cholest-4-en-3-one 6β-hydroperoxide	30	10
Cholesterol 7α-hydroperoxide	22	9
Cholesterol 25-hydroperoxide	17	9
Cholesterol 26-hydroperoxide	15	9
Cholesterol 20α-hydroperoxide	12	5

*The peroxidase activity was measured at 23 °C in 3 ml of phosphate buffer (pH 7.5) containing 0.2×10^{-3} M TMPD, 2 mg of microsomal protein, 0.05×10^{-3} M hydroperoxides, and 1.5×10^{-3} M EDTA.

The peroxidase activity is not influenced by CO, nitrogen, EDTA, and 2-phenyl-2-propanol. Sodium azide has a slight inhibiting effect (20%). The peroxidase activity diminishes by 95% when the microsomes are heated to 80 °C. Substrates of the microsomal enzyme system exert an effective inhibiting influence on the peroxidase reactions of liver microsomes[67]: type I substrates—androstenedione, testosterone, 17β-oestradiol, aminopyrine, hexabarbital, and linoleic acid—inhibit the peroxidase reactions by 65–34%, while type II substrates—aniline, imidazole, pyridine, cyanide, corticosterone, and n-octylamine—inhibit the peroxidase activity by 83–50%. This confirms that cytochrome P-450 is involved in the peroxidase reactions in the oxidised form. The peroxidase activity of cytochrome P-450 from adrenal cortex microsomes is also largely inhibited by substrates of microsomal systems of both types[67].

In the peroxidase reactions of cytochrome P-450, NADH and NADPH can function as hydrogen donors[68]. The combined experimental data[67,68] show that the peroxidase activity is characteristic of the oxidised form of P-450: CO does not inhibit the peroxidase reactions of cytochromes P-450, because it forms a complex solely with the reduced form of this haemoprotein. It has been suggested that, when cytochrome P-450 reacts with hydroperoxides, a complex is formed[25,26] in which the haem iron can exist in a high oxidation state:

$$\text{P-450—Fe}^{3+} + \text{ROOH} \rightarrow \text{P-450—Fe}^{5+} + \text{ROH} .$$

It is known that primary aliphatic alcohols are not substrates of peroxidases[69]. However, rate liver microsomes and highly purified cytochrome P-450 catalyse the oxidation of primary aliphatic alcohols to aldehydes[70]. The susceptibility of alcohols to oxidation by cumenyl hydroperoxide with participation of cytochrome or catalase diminishes with increase of the size of the aliphatic group[70].

Cytochrome P-450 is the most effective catalyst of the oxidation of ethanol by cumenyl hydroperoxide, while cytochrome *c*, haemoglobin, and peroxidase hardly catalyse the oxidation of alcohol. The induction of microsomes by phenobarbital sodium increases by a factor of almost 5 their catalytic activity in the oxidation of ethanol by CHP.[70] The mechanism of the oxidation of aliphatic alcohols with participation of cytochrome P-450 and hydroperoxides is controversial: alcohols can behave as hydrogen donors and can be oxidised via the peroxidase mechanism, which has already been discussed in this section; the second possibility in the oxidation of alcohols is associated with the monooxygenase activity of cytochrome P-450—the insertion of an activated oxygen atom from the P-450–ROOH complex in an alcohol molecule with subsequent decomposition of the unstable intermediate to acetaldehyde and water:

$$CH_3CH_2OH \xrightarrow{\text{cytochrome P-450-ROOH}} \rightarrow \left[CH_3—\underset{\displaystyle OH}{\underset{|}{CH}}—OH \right] + \text{P-450} + \text{ROH}$$
$$\downarrow$$
$$CH_3CHO + H_2O .$$

The mechanism of the oxidation of alcohols by hydroperoxides with participation of cytochrome P-450 has never been studied. The reduction of steroid hydroperoxides by cytochrome P-450 is of great interest for the elucidation of the mechanism of the oxidation of steroids by liver microsomes and in their biosynthesis with participation of mitochondrial cytochrome P-450 from adrenal cortex, since steroid hydroperoxides can behave as intermediates which are then converted into hydroxy-derivatives with participation of cytochrome P-450.

An undesirable side process accompanying the hydroperoxide oxidation of the majority of substrates is the degradation of cytochrome P-450 by hydroperoxides[43–45,71,72]. It has been shown[72] that CHP and TBHP decompose not only cytochrome P-450 but also cytochrome b_5, which plays an important role in electron transport in microsomal chains. The hydroperoxide of linoleic acid decomposes cytochrome P-450 in the microsomal form when this haemoprotein has not been induced, or has been induced by phenobarbital or 3-methylcholanthrene[71]. According to Jeffery et al.[71], two phases—rapid and slow—can be observed in the degradation of microsomal cytochrome P-450. A detailed kinetic study of the degradation of cytochrome P-450 by cumenyl hydroperoxide in microsomes and after purification to different extents in solution has shown that this process is complex and consists of three stages[43–45]. Under the conditions of an excess of CHP, the degradation of cytochrome P-450 can be characterised in terms of bimolecular rate constants for each degradation stage. The extent of the first stage in the degradation of cytochrome P-450 depends on the initial concentration of the hydroperoxide and on the duration of the degradation process regardless of the degree of integration of the cytochrome (microsomes or solubilised cytochrome P-450).[43–45]

Table 10 presents the kinetic parameters characterising the degradation of cytochrome P-450 and its reaction with CHP at different stages of this process. The rate constant k_0 (s^{-1}) characterises the degradation of cytochrome P-450 in the absence of hydroperoxide. This reaction is observed at 25 °C in only one case—for cytochrome P-450 in the LM_2

form when its specific content is 12.0 nmol/mg of protein. The substrates of the microsomal system (aniline and others) greatly inhibit the degradation of cytochrome P-450 by hydroperoxides. The introduction of a radical process inhibitor (1-naphthol) into the incubation mixture entails its consumption and marked inhibition of the degradation process. The inhibiting effect of this antioxidant confirms the radical mechanism of the degradation of cytochrome P-450. The complex of the hydroperoxide and cytochrome P-450, whose structure will be discussed in the next section of the review, decomposes with formation of active radicals:

$$[P-450-Fe^{3+}\ldots OOR] \rightarrow [P-450-Fe^{2+}] + \dot{O}OR + H^+ ;$$
$$\underset{H}{|}$$
$$[P-450-Fe^{2+}] + ROOH \rightarrow [P-450-Fe^{3+}] + HO^- + RO^\cdot .$$

Table 10. Kinetic parameters of the three stages of the degradation of cytochrome P-450 in the reaction with cumenyl hydroperoxide at 25 °C.[43-45]

System	Specific content of cytochrome P-450, nmol g^{-1}	k_0, s^{-1}	K_I	K_{II}, litre mol^{-1} s^{-1}	K_{III}
Intact rat microsomes	0.61	0	18.50	1.80	0.23
Rat PB-microsomes	1.70	0	41.00	1.24	0.22
Rabbit PB-microsomes	2.10	0	8.77	0.47	—
Rat 20-Mech-microsomes	1.00	0	12.67	1.53	0.33
Cytochrome P-450-LM_2**	12.00	$3.0 \cdot 10^{-4}$	0.78	0.43	0.15
" "	13.00	0	0.60—0.97*	0.12—0.39*	0.07
" "	16.84	0	0.69	0.10—0.59*	0.16
Cytochrome P-450-LM_4**	9.40	$0.17 \cdot 10^{-4}$	0.18—0.42*	0.10—0.22*	0.11

*Rate constants for hydroperoxide concentrations in excess of 3.0×10^{-4} M.
**From rabbit PB microsomes.

The radical species RO· and RO_2^- formed attack the porphyrin ring of the cytochrome P-450 haem and the apoenzyme, decomposing the latter.

VII. CATALYSIS OF THE PEROXIDE OXIDATION BY OTHER HAEMOPROTEINS (HAEMOGLOBIN, CATALASE, AND CYTOCHROME *c*)

The elucidation of the catalytic activities of other haemoproteins in the peroxide oxidation is of great interest. Cytochrome P-450 cannot be replaced in all cases by other more readily available haemoproteins: the oxidative dealkylation of *N*-alkylamines is catalysed by horseradish peroxidase in combination with H_2O_2,[73] and by haemoglobin and catalase in combination with CHP or H_2O_2;[74] the oxidation of aniline by cumenyl hydroperoxide is catalysed by haemoglobin[75] and cytochrome *c*;[76] haemoglobin exhibits a peroxidase activity in the presence of H_2O_2 in relation to phenylhydrazine[77].

Data on the catalytic activity of haemoproteins in various reactions are summarised in Table 11. Only cytochrome P-450 is catalytically active in all the reactions, while other haemoproteins can catalyse only one or several peroxide oxidation reactions. None of the haemoproteins, except cytochrome P-450, is capable of catalysing the peroxide *O*-dealkylation of ethers[32] and the oxidation of steroids[59]. Catalase participates in the oxidation of alcohols[70] and in the *N*-dealkylation of amines[21]. Cytochrome P-420 does not exhibit an activity in any of the reactions[32]. It is important to note that the mechanisms of reactions with participation of cytochrome P-450 and other haemoproteins are as a rule different. Hitherto radicals have not been detected directly in any of the peroxide oxidation reactions involving cytochrome P-450. On the other hand, the reactions involving the oxidative *N*-dealkylation of amines in the H_2O_2–peroxidase[73], CHP–catalase, and CHP–haemoglobin[74] systems are accompanied by the formation of radicals. Radical process inhibitors (1-naphthol and others) retard the oxidation of aniline in the haemoglobin–CHP and cytochrome *c*–CHP systems[75,76], which indicates the presence of radical stages in these instances.

Table 11. Catalytic properties of haemoproteins in the oxidation of the organic compounds by peroxides and certain other non-peroxide oxidants.

Reaction	Haemoproteins with catalytic activity*	Refs.	Haemoproteins without catalytic properties	Refs.
N-Dealkylation of amines	cytochrome P-450 (ROOH, H_2O_2)	21, 27-31	cytochrome P-420	21
	catalase (ROOH, H_2O_2)	21		
	peroxidase (H_2O_2)	73		
	haemoglobin (CHP, H_2O_2)	74		
O-Dealkylation of ethers	cytochrome P-450 (CHP, TBHP, C_6H_5IO)	32-35	haemin, catalase, peroxidase, myoglobin, cytochrome P-420	32
Hydroxylation of aromatic compounds	cytochrome P-450 (ROOH, H_2O_2)	28, 36-46	haemin, catalase, peroxidase, myoglobin, cytochrome b_5, cytochrome P-420	36
	haemoglobin (CHP)	75		
	cytochrome *c* (CHP)	76		
Oxidation of steroids	cytochrome P-450 (ROOH, $NaIO_4$, C_6H_5IO, $NaClO_2$)	59-65	peroxidase, catalase metmyoglobin	59
Oxidation of alcohols	cytochrome P-450 (ROOH, H_2O_2)	70	peroxidase, cytochrome *c*, haemoglobin	70
	catalase (ROOH. H_2O_2)			

*The oxidants employed are indicated in brackets.

Table 12. The efficiencies of various systems involving the demethylation of dimethylaniline (0.003 M) at 37 °C in terms of the concentrations of haemoproteins (10^{-6} M).[74]

System	$10^7 w_0^*$, mol litre s^{-1}
Catalase (5.0×10^{-7} M) + CHP (0.001 M)	26.60
Haemoglobin (2.0×10^{-6} M) + CHP (0.003 M)	2.60
Haemoglobin (2.0×10^{-6} M) + H_2O_2 (0.006 M)	4.10
Cytochrome P-450 in microsomes (1.25×10^{-6} M) + CHP (0.001 M)	8.20

*Initial rate of reaction.

In the oxidative dimethylation of aminopyrine in the H_2O_2–peroxidase system radical species have been detected by EPR and are apparently aminopyrine radical-cations[73]. The oxidation of aminopyrine by peroxidase in combination with H_2O_2 is the most effective method for transforming this compound compared with other systems.

The efficiencies of various haemoproteins in the peroxide oxidation of dimethylaniline are compared in Table 12.[74] Catalase is most effective in the peroxide oxidation of dimethylaniline. The inhibiting effect of 1-naphthol has been observed only in reactions involving cytochrome P-450.[74]

At 37 °C in a phosphate buffer (pH 7.4) aniline is hydroxylated to *p*-aminophenol in the haemoglobin–CHP system. Kinetic[75] and spectroscopic[78,79] data show that the hydroxylation of aniline entails the formation of a complex of haemoglobin with aniline and the hydroperoxide: difference spectra of the aniline–haemoglobin system are characterised by an absorption maximum at 425 nm and a minimum at 405 nm.[78] Identical differential spectra have been observed in the interaction of haemoglobin with CHP. The reaction involving the insertion of activated oxygen takes place in the aniline–haemoglobin–CHP ternary complex. A side reaction involving the degradation of haemoglobin by cumenyl hydroperoxide is observed in the course of the process[75].

At 0 °C cytochrome *c* catalyses in combination with CHP the oxidation of aniline to *p*-aminophenol. Aniline forms a complex with cytochrome *c* characterised by an absorption maximum at 420 nm and a minimum at 401 nm at pH 5.8 in a phosphate buffer. Complexes of CHP with cytochrome *c* are formed, which is reflected in the difference absorption spectra, having a maximum at 433 nm and a minimum at 413 nm.[76] 1-Naphthol inhibits the oxidation of aniline by cumenyl hydroperoxide with participation of cytochrome *c*,[76] but does not arrest it completely.

The ability of haemoglobin in combination with H_2O_2 to exhibit a peroxidase activity has been demonstrated[77] in relation to the oxidation of phenylhydrazine in this system. It has been suggested that oxidised haemoglobin (Hb^{3+}) forms a complex with H_2O_2, which decomposes and generates ferrylhaemoglobin, the latter oxidising phenylhydrazine:

$$Hb^{3+} + H_2O_2 \rightarrow [HbO]^{4+} + H^+ + {}^1/_2H_2O_2,$$
$$2[HbO]^{4+} + C_6H_5NH{-}NH_2 \rightarrow 2Hb^{3+} + C_6H_5{-}N{=}NH + H_2O.$$

Table 13. Spectroscopic characteristics of complexes of haemoproteins with hydroperoxides.

System, conditions	λ_{max}, nm	λ_{min}, nm	References
Rabbit PB-microsomes–CHP; pH 7.4	441, 535	422, 579	[81]
Rat liver microsomes–TBHP; pH 7.5; 20°	380–440	418	[82]
Rat liver microsomes–tetralyl hydroperoxide; pH 7.5; 20°	380–440	420	[82]
Cytochrome P-450 from rat liver microsomes–CHP; pH 7.4; 20°	380, 440	418	[39]
Cytochrome P-450–LM_2 from rabbit liver PB-microsomes–CHP	370, 436, 540	421	[83, 84]
Haemoglobin–CHP; pH 8.0; 20°	423	405	*
Cytochrome *c*–CHP; pH 5.8; 20°	433	413	[76]

*Data obtained by E.I.Tishenko in the author's laboratory.

The haemoprotein recently isolated from peas differs from cytochrome P-450 and oxidises a number of substrates via the peroxidase mechanism in the presence of hydroperoxides and H_2O_2: indole is oxidised to indoxyl, phenol is oxidised to hydroquinone, α-naphthol is oxidised to α-naphthohydroquinone, and aniline is oxidised to phenylhydroxylamine[80]. The oxidation of indole in the presence of this haemoprotein is slightly inhibited by KCN and NaN_3, but is very markedly retarded by α,α′-bipyridyl, aniline, and *N*,*N*-dimethyl-*p*-phenyldiamine. The new catalyst, was called the "H_2O_2 reducing haemoprotein[80]. The products formed in the oxidation of the substrates enumerated above with participation of this haemoprotein differ greatly from the products of the microsomal oxidation of the same substrates, which confirms the difference between the new haemoprotein and cytochrome P-450.

In conclusion of this section, it must be emphasised yet again that all the reactions catalysed by cytochrome P-450 are accelerated by other haemoproteins. The most significant difference between the catalytic activities of cytochrome P-450 and other haemoproteins is the absence of radical stages in the case of cytochrome P-450 and their presence in the reactions involving other haemoproteins. Peroxides form complexes not only with cytochrome P-450 but also with other haemoproteins; however, the complexes of cytochrome P-450 and other haemoproteins react via different pathways.

VIII. THE MECHANISM OF THE ACTION OF CYTOCHROME P-450 IN THE PEROXIDE OXIDATION OF ORGANIC COMPOUNDS

Cytochrome P-450, haemoglobin, and cytochrome *c* form complexes on interaction with hydroperoxides[81–84]. The formation of complexes by cytochrome P-450 in a microsomal suspension with cumenyl hydroperoxide was first demonstrated spectrophotometrically[81]. When CHP was mixed with liver microsomes from rabbits receiving phenobarbital, difference absorption spectra were recorded at 5 °C with maxima at 441 and 535 nm and minima at 422 and 579 nm. Analogous spectral changes were observed also in the interaction of microsomes with t-butyl and tetralyl hydroperoxides[82] and also in the interaction of purified cytochrome P-450 with CHP.[39]

The interaction of hydroperoxides and peracids with the highly purified LM_2 form of cytochrome P-450 from rabbit liver microsomes has been studied spectrophotometrically in combination with the stopped flow method[83,84]. Difference absorption spectra were obtained with maxima at 370, 436, and 540 nm and a minimum at 421 nm. The interaction of CHP with P-450-LM_2 is described schematically as follows:

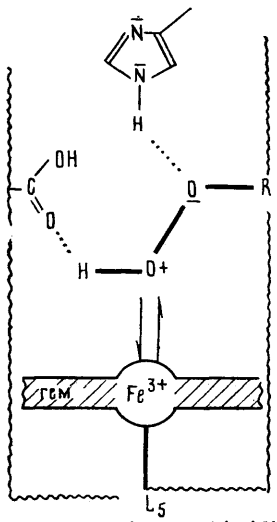
$$P{-}450{-}LM_2 + CHP \underset{k_2}{\overset{k_1}{\rightleftharpoons}} C \underset{k_4}{\overset{k_3}{\rightleftharpoons}} D,$$

where C is the Michaelis complex between CHP and P-450, and D is the spectroscopically detectable species, which can be an oxyferryl derivative of cytochrome P-450. The interaction of CHP and P-450 is very rapid: $k_1 = 5000$ litre $mol^{-1}\,s^{-1}$. The rate constants for the reverse reaction are $k_2 = 50\ s^{-1}$, $k_3 = 1.3\ s^{-1}$, and $k_4 = 0.009\ s^{-1}$.[83] The kinetic parameters determined for this reaction depend little on the pH and on the addition of phospholipids.

Similar spectra characteristics have been obtained in the study of the products of the reaction of P-450-LM_2 with peracetic, *m*-chloroperbenzoic, and *p*-nitroperbenzoic acids as well as other peroxides. The fact that different peroxycompounds form complexes with P-450 having analogous spectroscopic characteristics suggests that the active species arising on complex formation is similar for different oxidants. The spectroscopic parameters of the complexes of haemoproteins with hydroperoxides are presented in Table 13. According to the author, the formation of complexes by hydroperoxides takes place as shown in Scheme 4. The complex is formed by the undissociated form of the hydroperoxide, one oxygen atom of which (from the HO group) is bound to the haem iron, while the other atom forms a hydrogen bond with histidine; the alkyl residue of the peroxide interacts with the apoenzyme via hydrophobic binding. The distal histidine is probably involved in the stabilisation of the haemoglobin complex. Complexes of haemoglobin with

molecular oxygen are known to be stabilised by the distal histidine[85,86].

Scheme 4



L_5 = cysteinyl or histidinyl.

The spectroscopic characteristics (Table 13) probably refer to the product of the conversion of the complex illustrated in Scheme 4 into the oxyferryl derivative, formed as a result of the dissociation of the –O–O– bond and analogous to complex I, known to occur in the reactions of peroxidases:

$$[Fe^{3+}\cdot\ddot{O}\cdot] \rightleftharpoons [Fe^{4+}\ddot{O}:] \xrightarrow{H^+} Fe^{4+}OH\ .$$

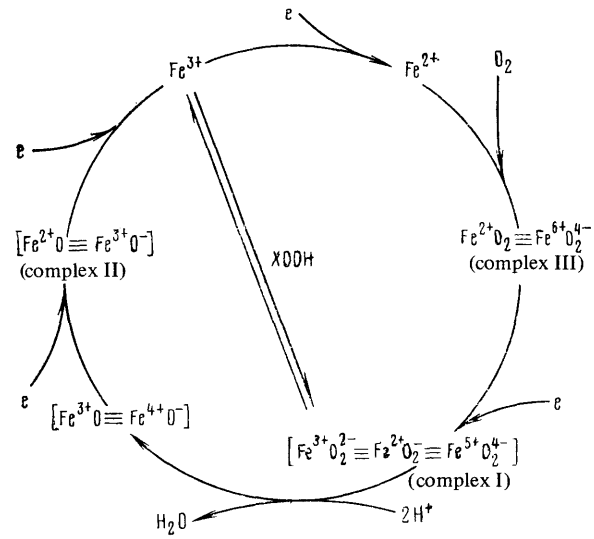
In the interaction of CHP with cytochrome P-450 a signal with a *g*-factor of approximately 2.0, analogous to the EPR signal arising in the interaction of H_2O_2 with metmyoglobin or with cytochrome *c* peroxidase[87,88], was recorded by EPR.[81] It is suggested that this EPR signal is associated with the highest valence states of haem iron, for example, the ferryl 4+ state. When CHP or H_2O_2 is added to metmyoglobin, an EPR spectrum similar to that recorded in the microsomes–CHP system is observed[81]. Yamazaki et al.[89] suggested an overall mechanism which describes the oxidation-reduction reactions of haemoproteins and is distinguished by five valence states of haem iron. This mechanism describes the results obtained in the study of peroxidase and catalase and agrees with the results of the study of the interaction of peroxides with metmyoglobin[90,92].

Scheme 5 illustrates the successive formation of various oxygen-containing species in the functioning of cytochrome P-450 and draws an analogy with the species formed in the functioning of peroxidases. The ternary complex comprising reduced cytochrome P-450, the substrate, and oxygen is equivalent to complex III of peroxidases, as suggested by Ulrich and Staudinger[93]. The valence state of the iron in this complex depends on the distribution of electrons between the oxygen and the iron ion. The one-electron reduction of this complex leads to the formation of complex I or the perferryl ion[89]. The complex I formed can be reduced by a single electron to complex II or simultaneously by two electrons to the initial state (Fe^{3+}–P-450).

Thus the active species formed in the interaction of oxidised cytochrome P-450 with peroxides or in the successive reduction of the substrate–cytochrome P-450–O_2 ternary complex by two electrons can be the same as complex I, which is characterised by a high degree of oxidation of the iron ion.

Complex I can react, receiving in succession one electron from the substrate and being reconverted into the initial form Fe^{3+}–P-450. This peroxidase reaction pathway is characterised by the intermediate formation of complex II. In other cases complex I can obtain immediately two electrons from the substrate and be reconverted into the initial form Fe^{3+}–P-450. This pathway obtains in hydroxylation reactions believed to involve an oxenoid mechanism[93]. The relative contributions of the peroxidase and oxenoid reactions of complex I are determined primarily by the nature of the haemoprotein. In reactions involving hydroperoxides, haemoglobin and cytochrome *c* react via the peroxidase pathway with the intermediate formation of radicals, which has been confirmed by the retarding effect on these reactions by radical process inhibitions. The oxenoid or hydroxylase mechanism is characterised by the absence of radical stages, which is confirmed by the effect of the same inhibitors in hydroxylase reactions[74].

Scheme 5



Complex I, which exists in different forms ($Fe^{2+}O_2^-$, $Fe^{3+}O_2^{2-}$, $Fe^{3+}O$, $Fe^{4+}O^-$) contains oxygen in the triplet or singlet state. The singlet oxenoid inserts an oxygen atom into the substrate via a three-centre synchronous mechanism with formation of a singlet reaction product:

$$\rangle C{-}H + [:\ddot{O}\,Fe^{3+}] \rightarrow \left[\rangle C\overset{OFe^{3+}}{\cdots}H\right] \rightarrow \rangle C{-}OH + Fe^{3+}\ .$$

If the oxenoid is in the triplet state, then the direct insertion of oxygen in the substrate is impossible, because this reaction would be spin-prohibited. In this case oxidation proceeds via the "abstraction of a hydrogen atom–recombination" mechanism or via a latent radical mechanism, which may not be sensitive to the action of radical process inhibitors:

$$\rangle C{-}H + [\cdot\ddot{O}\cdot Fe^{3+}] \rightarrow \rangle C^{\cdot} + [H\ddot{O}\cdot Fe^{3+}] \rightarrow \rangle C{-}OH + Fe^{3+}\ .$$

The different states of the oxygen in complex I can be determined by the nature of the fifth ligand of the haem iron in haemoproteins. The presence of histidine in the fifth position in haemoglobin and cytochrome *c* probably determines significantly the peroxidase reaction pathway with participation of these haemoproteins and hydroperoxides. The cysteinyl group in the fifth position of cytochrome P-450

is responsible for the difference between the spectra of the complexes of this haemoprotein with hydroperoxides and the spectra of the complexes of other haemoproteins (Table 13) and for the hydroxylase reaction pathway.

We shall now consider the possible reaction pathways for the complexes of haemoproteins with hydroperoxides. As a result of the interaction of haemoproteins with hydroperoxides, a Michaelis complex C is formed in which the hydroperoxide is coordinated to the central iron ion via the oxygen atom of the hydroxy-group (see Scheme 4). Evidence for the involvement of the HO groups of cumenyl hydroperoxide in coordination to cobalt and nickel chelates has been obtained by NMR from the chemical shifts of the HO protons in the hydroperoxide[94]. The Michaelis complex C decomposes via reaction (2) with formation of an oxenoid complex or an oxyferryl derivative analogous to complex I. Complex I can react via the hydroxylase mechanism [reaction (3)]. It can receive two electrons in succession from a suitable substrate: the reduction by the first electron leads to the formation of complex II via reaction (4). The Michaelis complex C can decompose in the absence of the substrate with formation of the peroxy-radical $RO_2^{\cdot}$ [reaction (5)]. In this reaction the hydroperoxide behaves as a reductant#:

$$L_5Fe^{3+} + ROOH \rightleftharpoons [L_5Fe^{3+}\ldots\underset{\underset{H}{|}}{OOR}] \text{ (complex C)}; \quad (1)$$

$$C \rightarrow [L_5Fe^{3+}O] + ROH \text{ (complex I)}; \quad (2)$$

$$[L_5Fe^{3+}O] + AH \rightarrow L_5Fe^{3+} + AOH; \quad (3)$$

$$[L_5Fe^{3+}O] + AH \rightarrow [L_5Fe^{2+}O] + A^{\cdot} + H^+ \text{ (complex II)}; \quad (4)$$

$$[L_5Fe^{3+}\ldots\underset{\underset{H}{|}}{OOR}] \rightarrow L_5Fe^{2+} + H^+ + RO_2^{\cdot}; \quad (5)$$

$$L_5Fe^{2+} + ROOH \rightarrow L_5Fe^{3+} + RO^{\cdot} + HO^-, \quad (6)$$

where AH is the substrate and L_5 is the axial ligand in the fifth position.

The reduced form of the haemoprotein is oxidised by another hydroperoxide molecule with formation of the radical $RO^{\cdot}$ [reaction (6)]. Reactions (5) and (6) are well known in the interaction of CHP with metal ions and metal complexes as the Haber–Weiss reactions[95]. The ratios of the rates of reactions (3)–(6) are determined by the nature of the haemoprotein, particularly by the nature of the axial ligand in the fifth position, by the presence or absence of substrates, by the nature of the oxidised substrate, and by other factors. Complex I, formed in the interaction of cytochrome *c* or haemoglobin with cumenyl hydroperoxide, reacts mainly via the peroxidase pathway [reaction (4)], while complex I of cytochrome P-450 reacts mainly via the hydroxylase pathway [reaction (3)]. It is noteworthy that the Michaelis complex C, which is capable of oxidising the substrate in a single stage on interaction with the latter, can serve as the hydroxylating agent. The study of model systems has shown that two hydroxylation mechanisms are possible—consecutive two-state hydroxylation [reactions (2) and (3)] or a single-stage transfer of active oxygen to the substrate[96,97] with the aid of complexes analogous to the Michaelis complex C.

In the presence of substrates or radical process inhibitors, reactions (5) and (6), initiating the degradation of haemoproteins, take place at lower rates. In the absence of substrates or inhibitors, the radicals $RO^{\cdot}$ and $RO_2^{\cdot}$ attack the haem and the apoenzyme and irreversibly decompose them.

The oxygenated form of reduced cytochrome P-450 from liver microsomes was first detected spectrophotometrically by Estabrook et al. in 1971.[17] Gunsalus et al.[98] and Ishimura et al.[99] demonstrated spectroscopically the formation of a complex of molecular oxygen with reduced cytochrome $P\text{-}450_{cam}$, which hydroxylates camphor. The camphor–cytochrome $P\text{-}450_{cam}$–O_2 and reduced cytochrome $P\text{-}450_{cam}$–O_2 complexes have been isolated chromatographically[100,101] below 0 °C in mixed aqueous organic solvents and have been characterised spectroscopically and kinetically. The camphor–cytochrome $P\text{-}450_{cam}$–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[100].

Using the stopped flow method, the formation of a complex of highly purified cytochrome P-450 from liver microsomes with molecular oxygen was investigated spectrophotometrically[102]. In the presence of benzphetamine, this reaction is characterised by two phases: complex A—an oxygenated complex of cytochrome P-450 having maxima at 430 and 450 nm in the difference spectrum—is formed initially. The short-lived complex A is then converted into complex B with a maximum at 440 nm. The formation of complexes of O_2 with reduced cytochrome P-450, can be described schematically as follows:

$$P\text{-}450^{red} + O_2 \overset{k_1}{\rightarrow} \text{complex A} \overset{k_2}{\rightarrow} \text{complex B} \overset{k_3}{\rightarrow} P\text{-}450^{ox}.$$

Complex B has spectroscopic characteristics close to those of complex I and is probably similar to the latter. The reactants are characterised by the following Soret bands: $P\text{-}450^{red}$—424 nm, $P\text{-}450^{ox}$—418 nm, complex A—423 nm, and complex B—421 nm.[102] The rate-limiting stage of the process is the oxidation of cytochrome P-450 in the ternary complex accompanied by the hydroxylation of the substrate. The addition of cytochrome b_5 to the system increases the rate constant k_3, i.e. cytochrome b_5 plays the role of an effector in the reaction.

Thus an oxygenated complex of cytochrome P-450 with properties similar to those of complex I is formed in the complete microsomal system as a result of a series of steps (see Schemes 2, 3, and 5). This complex can transfer an oxygen atom to the substrate with a simultaneous liberation of a water molecule or can initially abstract a water molecule and be converted into the ferryl derivative [$Fe^{3+}O \rightleftharpoons Fe^{4+}O^-$], which transfers an oxygen atom to the substrate.

It is of great interest to elucidate the nature of the rate-limiting stage in the oxidation of substrates in the complete microsomal system. This complex process consists of at least five steps (see Schemes 1 and 5). A kinetic analysis of each of the five steps has been carried out in a review[18], where data obtained in several laboratories were surveyed. Direct measurements of the rate constants for the interaction of substrates with cytochrome P-450, for the reduction of the enzyme–substrate complex by the first electron, and for the formation of the ternary complex comprising reduced cytochrome P-450, the substrate, and oxygen have shown that all three steps are characterised by high rate constants and cannot be rate limiting in the oxidation reaction in the complete system[18].

The rate of this process can be limited either by the reduction of the ternary complex by the second electron or by the step involving the insertion of activated oxygen in the substrate, which is accompanied by the rupture of the attacked bond and subsequent rapid liberation of the reaction product.

#It is noteworthy that the peroxy-radical $RO_2^{\cdot}$ may be bound in a complex, as shown[23] for the decomposition of complexes of hydroperoxides with molybdenum(VI):

$$[Mo^{6+}\ldots\underset{\underset{H}{|}}{OOR}] \rightarrow [Mo^{5+}\ldots{}^{\cdot}OOR] + H^+.$$

Hitherto the conversion of the ternary complex, involving its reduction by the second electron and the subsequent decomposition with formation of the reaction product, has not been resolved into individual stages. However, there are experimental facts showing that the rate-limiting stage of the reaction involves the dissociation of the attacked C–H bond in the substrates. The primary isotope effects in the oxidation of substrates in the complete microsomal system have been measured in a number of studies[103–117]; the results are summarised in Table 14. As a rule, high primary isotope effects are observed except for the oxidation of deuterated cyclohexane, for which an isotope effect has not been observed[105]. Studies in which an intramolecular determination has been made of isotope effects involving substrates which are symmetrical and contain simultaneously non-deuterated and deuterated C–H bonds attacked in the oxidation process, such as, for example, 1,3-diphenylpropane-1,1-d_2, are of special interest[114]. In this case the isotope effect is 11. The same value was obtained in the hydroxylation of norbornane in a reconstructed enzyme system of rabbit liver microsomes[117]. The isotope effects obtained leave no doubt that the rate-limiting stage of the oxidation reaction involves the dissociation of the attacked C–H bond, which supports the "abstraction–recombination" mechanism.

Table 14. The primary isotope effects in the microsomal oxidation of various substrates.

Substrate	Isotope effect	References
CD_3-morphine	1.4	103
[α-D_2]Ethylbenzene	1.8	104
[D_{12}]cyclohexane	1.0	105
[11-D_2]Lauric acid	3.0	106
[9-D_2]Caproic acid	3.0	106
Saturated compounds	1.4	107
[16α-D]Pregnenolone	3.0	108
[7α-^{3}H]Taurodeoxycholic acid	3.8	109
[α-D_2]Isopropylamphetamine	1.6	110
CD_3-*o*-nitroanisole	2.0	111
CD_3-methoxyanisole	10.0	112
Aromatic benzene derivatives	1.3–1.75	113
[1,1-D_2]1,3-Diphenylpropane	11.0	114
Biphenyl	1.4	115
N,N'-Dimethylphentermine	1.4	116
Norbornane	11.5	117

Another piece of evidence for the decisive role of the strength of the bond ruptured in the oxidation process is the existence of the so-called uncoupling compounds—fully fluorinated hydrocarbons which interact with cytochrome P-450 but are not oxidised, since the strong C–F bond cannot be ruptured by the oxidising species[118–121].

Comparison of the energy characteristics of the oxidation of the same substrates in the complete microsomal system and in systems containing only cytochrome P-450 and hydroperoxides showed that in both cases the activation energies for the same substrate are similar or identical (see Table 3 and Akhrem and coworkers[29,30]). This comparison, carried out under rigorously identical conditions for both systems (complete and shunted), supports the occurrence of the same rate-limiting stage in the process in both instances. In the hydroperoxide system there is no need for the transfer of the second electron to the enzyme–substrate–hydroperoxide complex, since the oxygen in this complex is already activated by two electrons, i.e. the ternary complex is not reduced in this case and the rate-limiting stage of the reaction can be only the insertion of an oxygen atom with dissociation of the bond in the substrate oxidised. Consequently the same rate-limiting stage of the process can be postulated in the hydroperoxide system and in the complete microsomal system, namely the insertion of active oxygen in the substrate with subsequent rapid decomposition of the complex and liberation of the reaction product.

Data have been obtained[58–62] showing that the use of oxidants ($NaIO_4$, $NaClO_2$, and iodosobenzenes) in combination with cytochrome P-450 makes it possible to increase the rate of transformation of substrates compared with those attained in the complete system. This led the authors to the view that the rate-limiting stages in the two systems are different[58–62]. Here one should note that the difference between the rates of oxidation can be accounted for not by the difference between the rate-limiting stages in the two cases but by different concentrations of the active oxidising species, which is due to the different affinities of molecular oxygen and the oxidants used for cytochrome P-450.

Naturally the most convincing evidence for the nature of the rate-limiting stage in the oxidation process in the complete microsomal system may be provided by the direct measurement of the rates of all the stages of the process by high-speed kinetic methods. Such a study has been performed for the demethylation of benzphetamine in a reconstructed microsomal system[122]. The rate-limiting stage in the oxidation of benzphetamine may be associated with the reduction of the ternary complex by the second electron or with any process involving the transformation of the ternary complex after its reduction, since all the preceding stages are much faster than the accumulation of the reaction products[122,123].

It is noteworthy that under certain conditions in the microsomal membrane the reduction of cytochrome P-450 by the first electron may be the rate-limiting stage of the oxidation reaction. Below 18–20 °C, the interaction of cytochrome P-450 in microsomes with its reductase (flavoprotein I) is hindered due to a change in the phase state of the phospholipids of the microsomal membranes from the liquid crystal state above 18–20 °C to the crystalline state below these temperatures. The phase transition of the membrane phospholipids is reflected in the activation energy for the oxidation of substrates in the complete system: at 18–20 °C a break is observed in the Arrhenius relation for the maximum rates of oxidation or for the catalytic constants[35,42,124]. The activation energy for the oxidation of substrates of the first type above 18–20 °C is 8–12 kcal mol^{-1} and probably refers to the transformation of the ternary complex; the activation energy below 18–20 °C is 18–24 kcal mol^{-1} and refers to another rate-limiting stage, which may be the reduction of cytochrome P-450 by its reductase under the conditions of hindered contact between them. This contact is facilitated when the state of the membrane changes above 18–20 °C as a result of the possibility of the lateral mobility of its components.

IX. PROSPECTS FOR PRACTICAL UTILISATION

In view of the important functions of hydroxylating enzymes it is desirable to search for ways of using them in practice. At the present time only the first attempts have been made at the practical application of monooxygenases containing cytochrome P-450. The region where cytochrome P-450 and its analogues can be used is extremely wide. The enzyme systems of micro-organisms and yeasts containing

cytochrome P-450 can oxidise virtually all hydrocarbons to alcohols: the American firm Mobil Oil took out a patent in 1967 for the oxidation of petroleum hydrocarbons of all classes by monooxygenases present in bacteria of different strains[125].

"Metabolic reactors" for the investigation of the optimisation of medicinal preparations and also for the extracorporeal detoxidation of blood by oxidising the impurities contained in it can be created on the basis of immobilised cytochrome P-450.[126-128] The cytochrome and its analogues in the immobilised state can be used for the selective oxidation of physiologically active compounds, in the first place steroids and certain amines[129]. It is already possible to quote an example of the successful application of monooxygenases in the sphere of traditional applications of the activity of microbial cells—steroid chemistry; thus Mosbach[130,131] converted Reichstein's substance "S" into prednisolone with the aid of a two-stage system. In the first stage substance "S" is hydroxylated in the 11β-position with formation of cortisol by the mycelium of the fungal culture of *Curvulatia lunata* immobilised in a polyacrylamide gel and the cortisol is converted into prednisolone in the second stage in a column filled with immobilised steroid Δ^{1-2}-dehydrogenase from *Corynebacterium simplex*.

Workers in the German Democratic Republic devised an enzyme electrode based on cytochrome P-450.[126,132] With its aid, it is possible to detect low concentrations of many substrates of cytochrome P-450 by following the consumption of hydrogen peroxide in the reaction with the aid of a sensor electrode.

The practical employment of hydroxylating enzyme systems involves the solution of several complex problems, since such systems consist of multienzyme complexes and require the use of cofactors. Cytochrome P-450 and other components of the system are unstable and expensive. It is therefore necessary to solve the following problems on the way to the practical utilisation of cytochrome P-450 containing monooxygenases:

(1) Simplification of the involved multienzyme complex by decreasing the number of its components and by replacing biomolecules which are difficult to isolate by simpler and more readily available analogues;

(2) stabilisation of cytochrome P-450 by immobilising microsomes or isolated components of the hydroxylating complex[133-139];

(3) practical utilisation of simpler analogues of cytochrome P-450, which retain its function;

(4) immobilisation of micro-organism cells containing hydroxylating complexes[130,141,140].

One of the ways of solving the first problem was proposed by ourselves[29]: the complex electron transport chain of microsomes is replaced by organic hydroperoxides, whose combination with one component of the system (oxidised cytochrome P-450) effects all the reactions characteristic of the complete system. This simplification of the hydroxylating complex greatly decreases the cost of the catalytic system and makes it possible to reduce the involved task of immobilising the entire multienzyme complex to the immobilisation of one of its components—cytochrome P-450.

Further advances in the utilisation of hydroperoxide systems require a successful immobilisation of cytochrome P-450 and its stabilisation with respect to the second component—the organic hydroperoxide. In many laboratories attempts have been made to immobilise cytochrome P-450 from liver microsomes and other natural sources[127,129,133-139] In all cases cytochrome P-450 partly retained its activity, but, after several hours or days, the activity of the immobilised haemoprotein decreases significantly. The task of increasing the stability of the immobilised cytochrome P-450 and of simplifying its immobilisation therefore still remains urgent. It is known that the stability of certain enzymes can be significantly increased by their immobilisation not directly on a matrix but via binding it to an inert protein, which is then in its turn immobilised on a support. In particular such an inert protein may be serum albumin. Berezin and coworkers[142,143] showed that the immobilisation of peroxidase on BrCN-Sepharose, subjected to a preliminary modification with albumin, or the immobilisation of oligomers obtained by the interaction of peroxidase with albumin in the presence of glutaraldehyde leads to a significant stabilisation of peroxidase. The present author and his coworkers[136] immobilised highly purified cytochrome P-450 from rabbit liver microsomes on a modified albumin Biogel P-300 and investigated its activity in the hydroxylation of aniline by cumenyl hydroperoxide. This immobilisation method increases significantly the thermal stability of cytochrome P-450 and ensures the oxidation of aniline to *p*-aminophenol with kinetic characteristics close to those of the same reaction involving non-immobilised cytochrome P-450. After immobilising cytochrome P-450 by sorption on polyamide or nylon-6, specimens of a "dry" catalyst, retaining a high activity for 19 days, were obtained[138].

A detailed study of the degradation of cytochrome P-450 by hydroperoxides suggests that it can be prevented[45,144] by introducing radical reaction inhibitors into the system. The necessity to increase the thermal stability of cytochrome P-450 entails a complex problem. Systematic study of the thermal inactivation of cytochrome P-450 from rabbit liver showed that the stability of the haemoprotein increases significantly when 20% of glycerol is added to the solution and also for certain ways of immobilising cytochrome P-450.[145] According to the present author, further advances in the application of cytochrome P-450 will be achieved using certain new procedures for the stabilisation of enzymes developed by Berezin and Martinek[146,147]: by carrying out the reactions in two-phase "water–water-immiscible organic solvent" systems, by incorporating the enzyme in inverted surfactant micelles in organic solvents, etc.

The studies on the practical utilisation of the unique properties of cytochrome P-450 are only beginning. However, certain advances have already been achieved in this field, but their number is still small. There is no doubt that enzyme reactors and selective oxidation processes will be devised on the basis of cytochrome P-450 and its analogues.

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