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# Mono-oxygenase Enzymes and Their Simulation

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Information on reactions catalysed by mono-oxygenases is summarised and classified. The composition and properties of these enzymes are considered, together with the kinetics and the mechanism of their action in the hydroxylation of aliphatic and aromatic compounds and in the epoxidation of alkenes. Methods for imitating the action of mono-oxygenases are discussed and systematised, and the reaction mechanisms of the model systems are examined critically. A list of 193 references is included.

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## I. INTRODUCTION

It is well known that molecular oxygen is relatively inert, and is unable directly to oxidise amino-acids, steroids, hydrocarbons, and other compounds in living organisms. Indeed, the reaction between an oxygen molecule and a hydrocarbon RH + $O_2$ → R· + $HO_2$· is strongly endothermic ($Q_{H-O_2} - Q_{R-H} = 47 - 93 = -46$ kcal mole$^{-1}$ [1]) and can occur at a measurable rate only at very high temperatures. Under conditions approximating to biological the oxygen molecule cannot be converted into two active atoms, since the strength of the O=O bond is 117.2 kcal mole$^{-1}$ [1]. Oxygen will become an active oxidant when it undergoes stepwise reduction to the radicals $HO_2$· and HO· and to hydrogen peroxide. Stepwise reduction of molecular oxygen may take place thermally, radiolytically, or in the concerted oxidation of organic compounds[2]. The active oxidants—$H_2O_2$, HO·, $\cdot O_2H$—operate very non-selectively on substrates, so that a large number of reaction products are formed. Yet micro-organisms are able to assimilate various hydrocarbons and other compounds, and to oxidise them under mild conditions with extraordinary selectivity[3-7]. Such complicated organic molecules as steroids can be transformed selectively into hydroxy-derivatives (hydroxylated) by micro-organisms[8,9]. The hydroxylation of various substrates—amino-acids, steroids, hydrocarbons, heterocyclic compounds, etc. — is one of the most important among physiological processes. It can now be accepted as proved that hydroxylation is accomplished by means of enzyme systems—termed mono-oxygenases--belonging to the class of oxidoreductases. Enzymes of this type, discovered in 1954 by Mason[10-13], have the distinguishing feature that, during reactions which they catalyse, one atom of an oxygen molecule is consumed in oxidising the substrate (S), while the other atom is reduced to water, so that the overall hydroxylation is represented by the equation S + $O_2$ + $DH_2$ → SO + $H_2O$ + D, where $DH_2$ is the cosubstrate, which is dehydrogenated by the reaction. If the substrate itself acts as cosubstrate ($SH_2$ + $O_2$ → SO + $H_2O$), the mono-oxygenase is said to be "internal" [14].

Mason himself names the type of enzymes he had discovered "mixed-function oxidases", thereby stressing that the enzymes belonged to the class of oxidases and registering the unusualness of the insertion of oxygen into a substrate. Hayaishi [12-14] suggested the term "mono-oxygenases", which is now widely used, since it enables mono-oxygenases to be distinguished from dioxygenases, which insert two oxygen atoms together into a substrate.

Mono-oxygenase enzymes have recently been intensively studied, and the research results are reflected in several reviews, devoted mainly to biological aspects of the action of mono-oxygenases[4,11,15]. The aim of the present Review is to describe the mono-oxygenases known at the present time, to classify them according to types of reactions and structure, to assess their role in biological oxidation, and to discuss their composition and properties. We would like to lay special emphasis on the role of models in the study of the mechanism of reactions catalysed by mono-oxygenases. We have considered mainly recent papers, since they provide the newest information and cover that obtained earlier. Generally accepted biochemical abbreviations are used in the Review.

## II. OCCURRENCE, SITE AND ROLE OF MONO-OXYGENASES IN BIOLOGICAL OXIDATION

All intracellular respiratory processes can be divided into two classes—(i) oxidation associated with the accumulation of energy and (ii) free oxidation, unconnected with the formation of high-energy (macroergic) compounds. Concerted phosphorylating oxidation provides an energy supply for the cell and is affected by means of a poly-enzymic complex whose structure is completely obscure. This complex consists of several electron carriers, whose reduction potentials at pH 7.0 lie between those of the initial donor (NAD–NADH = −0.320 V) and the final acceptor ($O_2$–$H_2O$ = 0.815 V). Thus a large potential difference arises, and the initial energy of oxidation of the substrate is involved in the process. This energy is partly (~40%) consumed in forming adenosine triphosphate.

The energy of hydrolysis of ATP is used by the cell in biosynthetic reactions, which otherwise would be thermodynamically disadvantageous. The role of oxygen in concerted oxidation is to accept electrons liberated by dehydrogenation. Degradative metabolism in the cell and this function of oxygen are now quite well known, and are described in detail in handbooks of biological chemistry[16].

Free oxidation, unconnected with the formation of high-energy compounds, is effected by means of enzymes, which can be divided into two classes—oxidases and oxygenases—but proteins—the oxygen carriers haemoglobin, myoglobin, and haemocyanin—are excluded from consideration. Oxidases are dehydrogenases, using oxygen as electron acceptor and reducing it to hydrogen peroxide[17]. Depending on the nature of the prosthetic groups oxidases are flavoproteins, metal flavoproteins, or copper proteins.

**Table 1.** The most studied mono-oxygenases.

| Enzyme | Electron donor | Occurrence | Ref. |
|---|---|---|---|
| steroid mono-oxygenases | | | |
| Squalene 2,3-mono-oxygenase EC 1.12.1.3 | NADPH | liver | 28, 29 |
| 11β-Mono-oxygenase EC 1.14.1.6 | NADPH | adrenal mitochondria | 30—32 |
| $C_{(21)}$-Mono-oxygenase EC 1.14.1.8 | NADPH | adrenal-gland endoplasmic retirulum | 33, 34 |
| Cholesterol 20-mono-oxygenase EC 1.14.1.9 | NADPH | adrenal mitochondria | 35, 36 |
| 1α, 1β, 2β, 6α, 6β, 7α, 7β, 15α, 16α, 18α-Mono-oxygenase | NADPH | liver endoplasmic reticulum | 37—40 |
| Mono-oxygenases of amino-acids and their derivatives | | | |
| *L*-Kynurenine 3-mono-oxygenase EC 1.14.1.2 | NADPH NADH | liver mitochondria | 41, 42 |
| Dopamine β-mono-oxygenase EC 1.14.2.1 | ascorbate | adrenal glands | 43, 44 |
| *p*-Hydroxyphenylpyruvate 1-mono-oxygenase EC 1.14.2.2 | aldehyde formed | liver cytoplasm | 45, 46 |
| Phenylalanine 4-mono-oxygenase EC 1.14.3.1 | tetrahydrobiopterin | liver cytoplasm | 47, 48 |
| Proline 4-mono-oxygenase | ascorbate | | 49, 50 |
| other mono-oxygenases | | | |
| *gem*-α-Mono-oxygenase | NADPH | liver endoplasmic reticulum | 51 |
| Aliphatic-acid ω-oxygenase | NADPH | liver endoplasmic reticulum | 52—54 |
| Arene *p*-mono-oxygenase | NADPH | liver endoplasmic reticulum | 55, 56 |
| Alkane mono-oxygenase | NADPH | liver endoplasmic reticulum | 53, 57—59 |
| *p*-Hydroxybenzoate hydroxylase EC 1.14.1.12 | NADPH | *Pseudomonas desmolytica* | 60 |
| Camphor 5-mono-oxygenase | NADPH | *Pseudomonas putida* | 61, 62 |

In contrast to oxidases, oxygenases catalyse reactions during which oxygen combines with the substrate. If one oxygen atom is attached, the enzyme is a mono-oxygenase; if two oxygen atoms are introduced into the substrate, the enzyme is termed a dioxygenase. As already noted, one oxygen atom is consumed in oxidising the substrate in the actions catalysed by mono-oxygenases, while the other oxygen atom is reduced to water. It is the latter atom which serves as electron acceptor, as with oxidases. This explains the term "mixed-function oxidase". This function of oxidases requires the presence of a cosubstrate, which undergoes dehydrogenation during the reaction. If the substrate itself acts as cosubstrate, the enzyme is termed an internal mono-oxygenase. Thus mono-oxygenase enzymes catalyse reactions in which the role of oxygen is not restricted to serving as electron acceptor but involves incorporation into the substrate, i.e. oxidation of the latter. Examples of internal mono-oxygenases are—lysine oxygenase[14,18], lactate decarboxylase[19], tryptophan oxygenase[20], and arginine oxygenase[21].

Reactions catalysed by mono-oxygenases are widely represented in biological systems. A special respiratory chain comprising free-oxidation enzymes is localised in microsomes in the liver, where most foreign compounds and several natural metabolites undergo transformations leading to non-toxic polar products, which are then excreted by the kidneys. The mono-oxygenase-catalysed hydroxylation of medicinal, carcinogenic, and toxic substances is of great interest for pharmacology, oncology, and toxicology[4,22]. A hydroxylation chain similar in composition to the microsomal chain functions in adrenal mitochondria, where it is involved in the synthesis of steroid hormones. Malfunctioning of such processes underlies several pathological conditions.

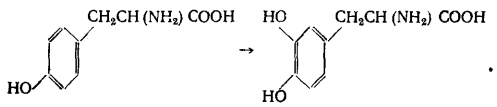
Several micro-organisms hydroxylate hydrocarbons, using them as nutrients[3,5-7,23,24]. This ability has been examined during recent years with the aim of producing foodstuffs and fodder from petroleum hydrocarbons and natural gas[23,24]. The American Mobil Oil Company in 1967 patented the enzymic oxidation of all classes of hydrocarbons[6]. The universal character of the oxidation is explained by the use of various strains of four species of micro-organisms—*Pseudomonas*, *Nocardia*, *Bacillus*, and *Mycobacterium*.

Hydroxylation of foreign compounds can be accomplished not only in the endoplasmic reticulum of liver cells but also in the lungs, gastrointestinal tract, skin, and kidneys[25-27]. The endoplasmic reticulum is a system of membranes ~80 Å thick located in the cytoplasm. On differential centrifugation, fragments of these membranes form the so called microsomal fraction remaining in the supernatant liquid after the separation of heavy particles—nuclei and mitochondria.

Complicated hydroxylating systems, wherever they are found—in mammalian liver microsomes, in adrenal mitochondria, or in bacteria— are in all cases multicomponent in character. Table 1 lists the most important mono-oxygenases studied during recent years.

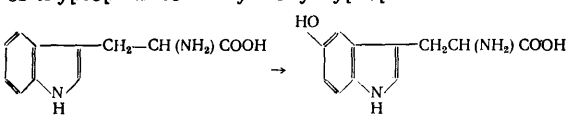
The review by Maslova et al.[4] has discussed in detail aspects of the physiological role of the mono-oxygenases of liver microsomes, induction of the synthesis of the microsomal hydroxylating system, and its hormonal regulation. We would like to consider only the part played by mono-oxygenases in the physiological state of the brain. During recent years great advances have been made in investigating enzymes of the central nervous system, especially those affecting metabolism of the biogenetic amines involved in the specific functions of nerve cells. Such amines include tyramine, dopamine, adrenaline, noradrenaline, serotonine, etc. Their biosynthesis and catabolism are regulated by the activity of enzyme systems, in particular mono-oxygenases, and govern the normal physiological state of the brain[63]. In the nerve endings and in certain compartments of the cerebrum tyrosine

hydroxylase catalyses the hydroxylation of tyrosine to 3',4'-dihydroxyphenylalanine[64]:



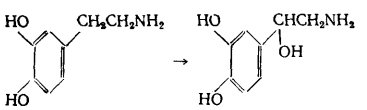
The purified enzyme was obtained from the medullary layer of bovine adrenal glands[64]. The search for multiple forms of tyrosine hydroxylase is continuing. This enzyme is active under aerobic conditions in the presence of tetrahydrofolic acid or 2-amino-5,6,7,8-tetrahydro--4-hydroxy-6,7-dimethylpteridine and of $Fe^{2+}$ and $K^+$ ions[65]. It is inhibited *in vitro* by amino-acids and their derivatives, in particular by noradrenaline, catechols, and indoles. Normally it hydroxylates only tyrosine. In Parkinson's disease the hydroxylation is assumed to be possible of dihydroxyphenylethylamine, whose injection into animals causes "chemical sympathectomy", i.e. cessation of the functions of the nerve cells mediated by pulses of noradrenaline, and also complete inhibition of the activity of tyrosine hydroxylase[66].

Tryptophan hydroxylase, which catalyses the hydroxylation of tryptophan to 5'-hydroxytryptophan



has been detected in brain[67-70], especially in the stem[71]. The purified enzyme has been obtained from bovine brain[71]. The presence of 2-amino-5,6,7,8-tetrahydro-4-hydroxy--6,7-dimethylpteridine is necessary for functioning of the enzyme. In contrast to the liver enzyme, brain tryptophan hydroxylase hydroxylates only *L*-tryptophan at position 5. The hydroxylation of tryptophan limits the rate of biosynthesis of serotonine in the brain[72].

Dopamine $\beta$-hydroxylase, which catalyses the hydroxylation of dopamine to noradrenaline



was identified as early as 1964. It catalyses the concluding stage in the biosynthesis of noradrenaline, which mediates the nervous impulses in the synapses of the sympathetic nervous system. The development of certain forms of psychic depression is attributed to an insufficiency of adrenaline. Many psychotropic agents change the noradrenaline content of the brain. Accumulation of excess of noradrenaline in a cell inhibits further hydroxylation of dopamine.

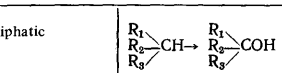
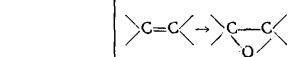
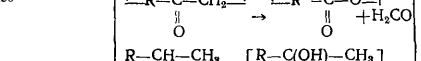
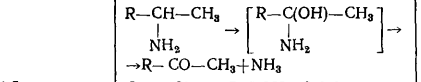
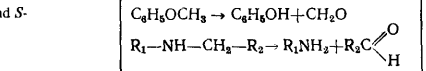
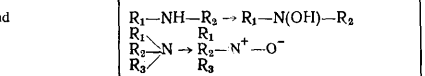
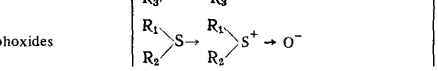
## III. TYPES OF REACTIONS CATALYSED BY MONO-OXYGENASES

The reactions catalysed by mono-oxygenases are extremely diverse (Table 2). Liver microsomes are able to metabolise substances of various structures, i.e. to effect various reactions, among which the most common is the hydroxylation of aliphatic compounds. In practice this is the most important reaction in the microbiological transformation of steroidal compounds[8,9]. Steroid hydroxylases of micro-organisms are known for almost all positions in the steroid system—ring system, side-chain, angular methyl groups—but all the hydroxylations studied in the steroid series occurred at a saturated carbon atom. Steroid hydroxylases are met not only in micro-organisms but also, as already noted, in animal tissues[78-80]. Fermentation by micro-organisms usually introduces one oxygen atom—more rarely two atoms—into the steroid molecule. Liver microsomes oxidise the terminal groups of aliphatic hydrocarbons to primary alcohols[53] and effect the $\omega$-hydroxylation of aliphatic acids to $\omega$-hydroxy-acids[53,81-84]:

$$CH_3(CH_2)_nCOOH \rightarrow HOCH_2(CH_2)_nCOOH.$$

The second most common reaction catalysed by mono-oxygenases is the hydroxylation of aromatic systems[47,48,55,56,60,85] with the formation of phenols. This reaction plays an important part in the metabolism of aromatic amino-acids. The position of the entering hydroxy-group is often determined by the presence of functional substituents in the aromatic ring.

**Table 2.** Reactions catalysed by mono-oxygenase enzymes.

| Type of reaction | Reaction | Ref. |
|---|---|---|
| 1. Hydroxylation of aliphatic compounds |  | 12, 14, 30–40, 73, 74 |
| 2. Hydroxylation of aromatic compounds | ArH→ArOH | 47, 48, 55, 56, 60, 64, 67–70 |
| 3. Epoxidation |  | 8, 9, 75 |
| 4. Formation of lactones |  | 76 |
| 5. Deamination |  | 4 |
| 6. Oxidative *N*-, *O*-, and *S*-dealkylation |  | 14 |
| 7. *N*-Hydroxylation and *N*-oxidation |  | 77 |
| 8. Formation of sulphoxides |  | 12 |

Epoxidation occurs in microbiological transformations of unsaturated steroids[8,9]. Enzymic formation of $\alpha$-oxides has now been described from $\Delta^5$-, $\Delta^{9(11)}$-, $\Delta^{11}$-, and $\Delta^{14}$-steroids. The epoxidation and the hydroxylation of steroids are very often accomplished by the same enzyme system[8]. Fat-soluble amines are deaminated in the presence of mono-oxygenases, being converted into ketones and ammonia. Amphetamine and its analogues undergo this reaction in the animal liver. Mono-oxygenases catalyse also *N*-, *O*-, and *S*-dealkylation, hydroxylate at a nitrogen atom, and oxidise substrates at nitrogen and sulphur atoms.

With all their variety the reactions of mono-oxygenases have an important common feature: only one oxygen atom is transferred to the substrate by the reaction.

## IV. COMPOSITION AND PROPERTIES OF MONO-OXYGENASE ENZYME SYSTEMS

Hydroxylating enzyme systems are complicated multicomponent protein structures. All mono-oxygenases can be divided into two large groups: the first group includes *enzymes containing no metal ion or complex*; the second group comprises *enzymes containing one or more metal atoms*, which are therefore metal proteins.

Examples of this first group of enzymes are imidazolyl acetate mono-oxygenase [14] (the electron donor for which is NADH, while the prosthetic group contains flavin adenine dinucleotide (FAD) and is a flavoprotein), kynurenine 3-mono-oxygenase [41,42,86,87], salicylate hydroxylase [73], *p*-hydroxybenzoate hydroxylase [74], and *L*-lysine mono-oxygenase [88]. The prosthetic groups of these enzymes are usually FAD-containing proteins [89-93]. The kinetics and the mechanism of the action of enzymes of this group will be discussed in detail in Section V.

Enzymes of the second group, which contain a metal ion, are more numerous and have been more thoroughly studied. Among them is tyrosinase, a copper protein, the cosubstrate being an *O*-dihydroxyphenol reaction product [94]. Dopamine β-mono-oxygenase contains two copper atoms per enzyme molecule [95]. Phenylalanine hydroxylase, containing 1–2 iron atoms per enzyme molecule, has now been well studied [96-100]; the enzyme isolated from rat liver exists in two forms separable by electrophoresis, each of which may be either a monomer, a dimer, or a tetramer (with respective molecular weights 51 000–55 000, 110 000, and 210 000). The dimers dissociate into the monomeric form when the enzyme is diluted and the temperature is raised from 0 to 30°C.[96] The stoichiometry of the reaction of phenylalanine hydroxylase can be expressed

Tetrahydropterin + Phenylalanine + $O_2$ → Quinonoid dehydropterin + Tyrosine + $H_2O$.

Tetrahydropterin is the cosubstrate undergoing dehydrogenation. The iron atom is present in the enzyme in the high-spin form of $Fe^{3+}$. The hydroxylase is inhibited by loss of the iron atom or on addition of a compound which chelates the metal. The electron spin resonance spectrum of purified phenylalanine hydroxylase is characterised by a signal with $g = 4.23$, which is due to the high-spin form of the iron ion. Addition of a substrate to the enzyme leads to disappearance of this signal, which indicates that the iron has been reduced to $Fe^{2+}$. [98] The enzyme inactivated by removal of iron is reactivated by the addition of iron(II) chloride. The study of phenylalanine hydroxylase isolated from human liver by autopsy has begun quite recently [99,100]. Two forms of the enzyme—dissolved at 6000 *g* and precipitated at the same acceleration—are obtained [100]. Much in common is found between the properties of the enzymes isolated from human and rat livers. The Michaelis constant $K_m$ varies with pH and the 2-amino-5,6,7,8-tetrahydro-4-hydroxy-6,7-dimethylpteridine concentration; at the optimum pH it is $5 \times 10^{-4}$ M. [100]

Enzymes containing as prosthetic group the so called cytochrome P-450 form the largest group studied intensely during recent years. These enzymes can in turn be divided into three subgroups depending on their occurrence in Nature—mono-oxygenases in microsomes of the mammalian liver [102-112], in mitochondria of the adrenal glands, and of bacterial origin [60-62, 89-93, 118, 119]—between whose enzyme systems exist substantial differences, although they all usually contain cytochrome P-450. Scheme 1 shows for comparison the electron-transfer chains established in the three subgroups of enzymes [4].

Scheme 1

*liver microsomes*

NADPH → flavoprotein II → non-haemin Fe protein → cytochrome P-450 → $O_2$
↓
cytochrome c

*adrenal mitochondria*

NADPH → flavoprotein III → non-haemin Fe protein → cytochrome P-450 → $O_2$
(adrenodoxin)

*bacterial mono-oxygenases*

Methylene hydroxylase
NADPH → flavoprotein → putidaredoxin → cytochrome P-450 → $O_2$
ω-Hydroxylase
NADPH → flavoprotein → rubredoxin → unidentified cytochrome → $O_2$

Scheme 1 shows that the electron (hydrogen) donor is NADPH, or NADH in the case of bacterial mono-oxygenases. Hydrogen is transferred from the donor to the flavin group of the protein reductase together with an electron pair by a mechanism of hydride migration. The protein reductase is equally capable of two- and one-electron transfers. The next link in the chain of electron transfer is a protein generally containing iron and sulphur atoms, termed a non-haemin iron protein adrenodoxin with mitochondrial, putidaredoxin with methylene hydroxylase, and rubredoxin in alkane carboxylic acid systems. The role of the iron protein may be different in different cases: although its presence is not necessary in the last system, it is highly specific with methylene hydroxylase and cannot be replaced by any other protein. Putidaredoxin and adrenodoxin play a very direct part in the transfer of electrons along the chain, and we shall discuss them in greater detail later. There is as yet no direct indication of the participation of non-haemin iron in electron transfer in microsomes, although it is supposed to act as a component between flavin and cytochrome P-450.

The terminal electron carrier in microsomal, mitochondrial, and bacterial chains is cytochrome P-450, which has now been isolated from liver microsomes [101-105], adrenal mitochondria [113-116], and bacteria [118,119]. It is a haem protein, which in the reduced state forms with a molecule of carbon monoxide a stable complex having an absorption maximum at 450 nm. The cytochrome is oxidised fairly readily by molecular oxygen, and can be reduced again with NADPH or dithionite. It contains not only iron but also copper. Treatment with proteases, amides, ketones, phenols, and anilines converts the cytochrome into a form possessing no enzymic activity, with the Soret band of the carbonyl complex at 420 nm, which is characteristic of other cytochromes. The spectrum of the oxidised form of cytochrome P-450 contains absorption maxima at 360, 416, 535, 570, and 650 nm. The enzyme from liver microsomes contains a mixture of low-spin and high-spin forms of iron. The spectrum of the reduced enzyme is distinguished by a low absorption coefficient and displacement of the Soret band to shorter wavelengths with maxima at 412 and 557 nm. In the oxidised state, cytochrome P-450 from liver microsomes gives an e.s.r. signal with components $g_x$, $g_y$, and $g_z$ of the *g*-factor of 1.91, 2.25, and 2.41 respectively. This signal characterises a low-spin state of tervalent iron in an asymmetric environment. The low-spin form of the cytochrome passes into a high-spin form with an anisotropic e.s.r. signal either at low pH or on treatment with thiol inhibitors. The cytochrome from liver microsomes contains a lipid component, which confers on it the ability to combine with hydrophobic compounds. It has recently been shown [120] that reduced cytochrome P-450 from microsomes forms with molecular oxygen a complex having an absorption maximum at 440 nm, twenty times as rapidly

as the carbonyl derivative: the rate constants for the interaction of cytochrome P-450 with molecular oxygen and with carbon monoxide at 25°C are respectively $4.4 \times 10^6$ and $2.4 \times 10^5$ $M^{-1}$ $s^{-1}$. Cytochrome P-450 isolated from the mitochondria of adrenal glands differs little from liver samples. Specimens from the two sources have analogous optical and e.s.r. spectra [114].

The cytochrome isolated from camphor-oxidising microorganisms is now the most thoroughly studied [118,119,121]. Its optical and e.s.r. spectra have been well investigated, as well as its Mössbauer spectra, for which the natural cytochrome was enriched with the iron-57 isotope. In the presence of the substrate (camphor) the oxidised cytochrome consists of a mixture of high-spin ($S = 5/2$) and low-spin ($\frac{1}{2}$) haem iron $Fe^{3+}$. Removal of camphor converts the former into the latter form of iron. Anaerobic reduction of cytochrome P-450 gives the high-spin form of $Fe^{2+}$ ($S = 2$), no paramagnetic effects being observed even in a strong field. The Mössbauer spectra of cytochrome $P\text{-}450_{camph}$ are analogous to those of oxygenated haemoglobin. Cytochrome $P\text{-}450_{camph}$ is a protein of molecular weight 45 000 consisting of a polypeptide chain and iron protoporphyrin IX. Axial coordination of haem P-450 still remains an open question: it is assumed merely that cysteine sulphur and histidine nitrogen are axial ligands. The reduced enzyme gives a complex with molecular oxygen, which has been observed by optical methods [118,121]: a new spectral band appears with maxima at 555, 418, and 355 nm. [118] This is accompanied by disappearance of the e.s.r. signal having $g = 2.26$, characteristic of the low-spin $Fe^{3+}$ form. The complex cytochrome $P\text{-}450_{camph} \rightarrow O_2$ is fairly stable at low temperatures (4–8°C), but is slowly converted into the high-spin form of $Fe^{3+}$ on standing. Thus the haem nature of P-450 cytochromes has now been firmly established, as has their direct participation in the hydroxylation of various substrates. Formation of an oxygenated derivative of the cytochromes on enzymic hydroxylation has been proved.

Cytochrome P-450 is preceded in the electron-transfer chain by so called iron- and sulphur-containing proteins, which are distinguished from other iron-containing proteins by the fact that the metal is not attached to complexing organic ligands [122]. In rubredoxin a single iron atom is coordinated with four cysteine sulphur atoms. The biological role of rubredoxin is not always clear: it consists mostly in electron transfer. In steroid-hydroxylating systems this protein is termed adrenodoxin. It has been isolated in a free form [117] and has been well characterised. Adrenodoxin contains 2 g-atoms of iron and 2 moles of labile sulphur per mole of protein; its molecular weight is 12 500 and its simple polypeptide chain contains 114 amino-acids. The structure of the active centre is dinuclear–tetrahedral. The most recent value [114] for the redox potential of adrenodoxin is −0.27 V at pH 7.0. Adrenodoxin can be completely reduced by dithionite, NADPH, or adrenodoxin reductase.

A protein named putidaredoxin, which contains both iron and sulphur, plays an especially important part in the hydroxylation of camphor: it ensures transfer of a second electron to the ternary complex of cytochrome with the substrate and oxygen [119]. Putidaredoxin is very specific, and cannot be replaced by another sulphur-containing protein, e.g. adrenodoxin, a component of steroid 11 β-hydroxylase. Yet NADH dehydrogenase can be replaced by a different dehydrogenase, in the electron-transfer chain of the camphor-hydroxylating system, without loss of enzymic activity. Physicochemical investigation of putidaredoxin (by magnetic susceptibility, electron spin resonance, electron–nuclear double resonance, Mössbauer spectra, and circular dichroism spectra) indicates the presence of two iron atoms in the oxidised form in a high-spin state ($S = 5/2$). Thus it has been established that in several cases a non-haemin protein takes part in the transfer of electrons during the hydroxylation of a substrate, although its presence is not necessary in a paraffin-acidifying system.

Non-haemin proteins are preceded in the electron-transfer chain by protein reductases, usually flavoproteins capable of transferring two electrons. The reductase isolated from a system effecting the enzymic ω-oxidation of aliphatic acids contains FAD as prosthetic group (1 mole $mole^{-1}$ protein) and has molecular weight 55 000. [84] The reductase is very specific, interacting with rubredoxin to give a complex of dissociation constant $2.1 \times 10^{-7}$ M. The activation energy of the enzyme-catalysed reduction of rubredoxin by NADH is 5.4 kcal $mole^{-1}$.

Thus hydroxylating enzyme systems are composite multicomponent protein structures. Every component effects the transfer of electrons. Many obscurities and mysteries still remain in the structure and properties of individual parts of enzyme systems.

## V. KINETICS AND MECHANISM OF THE ACTION OF MONO-OXYGENASES

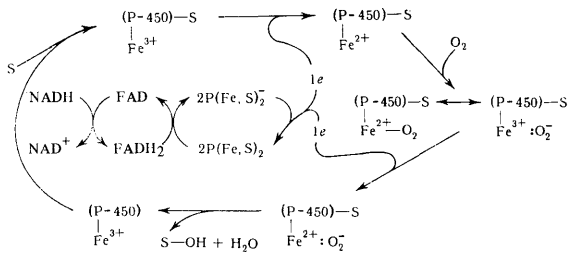
Kinetic study of the reactions of mono-oxygenases and measurement of the rates of individual stages in the composite enzymic process will provide valuable information on the mechanism of the action of these enzymes. It must be remembered, however, that study of the mechanism on extracts and even on purified preparations almost always yields only tentative conclusions on the mechanism of the oxidising stage.

Quantitative data have now been obtained for various stages in the oxidation of camphor in the presence of methylene hydroxylase [118,121,123,124]. Cytochrome P-450 reacts at very high rates with molecular oxygen to give a complex. The rate constant of formation of the camphor complex of this enzyme is $3.7 \times 10^6$ $M^{-1}$ $s^{-1}$ at 8°C [123], and the equilibrium constant of the complex is $0.47 \times 10^6$ $M^{-1}$. The enzyme–substrate complex itself has been isolated in the crystalline state [124]. It has been firmly established that oxidised cytochrome adds camphor, after which it acquires an electron. The enzyme–substrate complex then adds oxygen to form an enzyme–substrate–$O_2$ ternary complex, which acquires a second electron to form oxidised camphor and oxidised cytochrome. The rate of addition of camphor and oxygen to the enzyme exceeds hundredfold the rate of formation of the product. From the kinetic measurements and analysis of the functions of the component parts Scheme 2 has been suggested for the action of the methylene-hydroxylating system (S is the substrate, and S–OH the oxidised substrate). A distinguishing feature of Scheme 2 [121] is that both electrons for the reduction of oxygen are supplied by putidaredoxin.

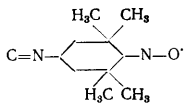
Study of the redox cycle of cytochrome P-450 from liver microsomes has shown that it involves formation of an oxygenated intermediate of the reduced cytochrome. This intermediate was detected by differential spectrophotometry, and the kinetics of its appearance has been studied. The rate constant of the reaction between cytochrome and oxygen has recently been measured, by stopped-flow flash photolysis, as $4.4 \times 10^6$ $M^{-1}$ $s^{-1}$ at 25°C. [120] Scheme 3 shows the course of electron transfer

in liver microsomes during hydroxylation of the substrate[103]. Here the electron donor for the oxygenated form of reduced cytochrome P-450 is cytochrome $b_5$.

Scheme 2

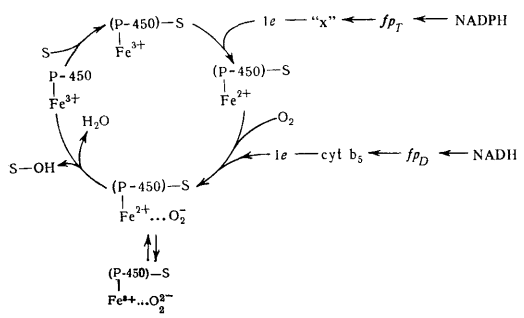


The spin-label method[110-112] has recently been used to study the molecular structure and the mechanism of the action of cytochrome P-450. The enzyme from liver microsomes interacted with the isocyanide spin-label



with the appearance of absorption bands around 435 and 455 nm characteristic of isocyanide derivatives of cytochrome. The e.s.r. spectra of liver microsomes treated with the spin-label suggest considerable retardation of the molecular motion of the latter, caused by its hydrophobic environment. The frequency of molecular rotation of the label increases on addition of the substrate to be hydroxylated (hexobarbital). The form of the e.s.r. spectra of the label indicates that the haem molecules to which it is attached are far apart and do not form dimeric structures. The active centre of cytochrome P-450 is assumed to be a haem group surrounded by non-polar amino-acid residues, whose spatial arrangement relative to the haem changes in definite fashion on interaction of the cytochrome with substrates.

Scheme 3



Considering now the mechanism of the action of metal-free mono-oxygenases, we note that the stopped-flow technique has been used to elucidate certain aspects of the reaction mechanism of salicylic hydroxylase from *Pseudomonas putida*[92]. With pH 7.0 at 25°C the rate constant of attachment of the enzyme to the substrate is $1.8 \times 10^7$ $M^{-1}$ $s^{-1}$, while that of the reverse reaction is 62 $s^{-1}$. Denoting the enzyme, flavin, and substrate as E, F, and S respectively, we can write the reaction

$$EF{-}S + NADH + H^+ \rightarrow X_1 \xrightarrow{k=230\ s^{-1}} EFH_2{-}S + NAD^+$$
$$EFH_2{-}S + O_2 \rightarrow X_2 \xrightarrow{k=21\ s^{-1}} EF + \text{catechol} + CO_2 + H_2O$$

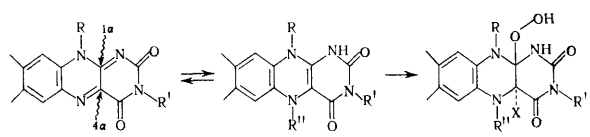
in which EF–S and $EFH_2$–S are the oxidised and the reduced complexes respectively, while $X_1$ and $X_2$ are kinetically active species. The rate of reduction of flavin in the presence of salicylate exceeds the rate in its absence by a factor exceeding 2000. The limiting stage of the whole process is oxidation of the reduced complex by an oxygen molecule.

Use of the stopped-flow method to study the kinetics of the formation of the enzyme–substrate complex of *p*-hydroxybenzoic hydroxylase shows[89] that the rate of complex formation is very high, and the reaction itself takes place in two stages

$$EF + S \underset{k_2}{\overset{k_1}{\rightleftarrows}} EF \sim S \underset{k_4}{\overset{k_3}{\rightleftarrows}} EF{-}S$$

the first of which is far more rapid than the second ($k_3$ = 480 $s^{-1}$ and $k_4$ = 140 $s^{-1}$). The multistage character may be due to isomerisation of the enzyme–substrate complex or to conformational changes in the enzyme. Examples of the multistage formation of complexes with substrates are known also for enzymes of other classes[125-128]. Thus multistage interaction of enzyme with substrate has been found in ribonuclease–cytidine 3-phosphate, chymotrypsin–proflavin, lysozyme–oligosaccharide, etc. systems. The reaction of *p*-hydroxybenzoic hydroxylase from the microorganism *Pseudomonas fluorescens* with a 2,4-dihydroxybenzoate in the presence of NADH and molecular oxygen has now been studied in stages. The anaerobic reduction of the complex of *p*-hydroxybenzoic hydroxylase with the 2,4-dihydroxybenzoate by NADH has been studied spectrophotometrically by the stopped-flow technique, and formation of at least three intermediate compounds has been shown during the process. All three intermediates absorb in the long-wavelength region, and exhibit no e.s.r. signals. Oxidation of the reduced enzyme–substrate complex by oxygen has also been studied, and an intermediate—an oxygenated form of the flavin prosthetic group attached to the enzyme—has been clearly recorded. The second intermediate was identified as an oxidised "enzyme–product" complex; it undergoes conversion at a rate close to the maximum rate of an enzymic process. The suggested[90] oxygenated intermediate of flavins has been detected quite recently when completely reduced flavin and the flavoprotein interact with singlet molecular oxygen[129]. The structure of the oxygenated flavin was established from the absorption spectra and by means of a high-resolution mass spectrometer fitted with a computer. The oxygenated form is characterised by absorption at 500–680 nm and a peak at 375 nm.

Scheme 4

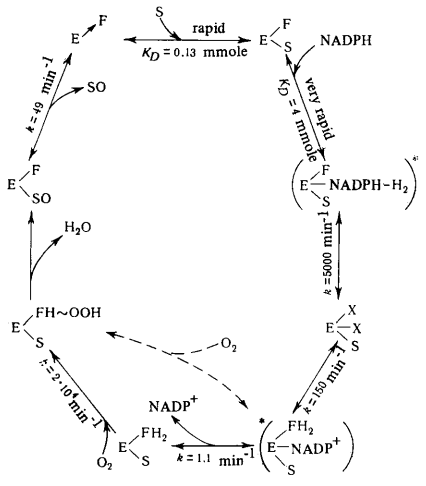


where X = $-OCH_3$; $-SCH_2CH(NH_2)COOCH_3$

Scheme 4 represents the formation of oxygenated flavin with oxygen in its first excited state ($^1\Delta g$). Oxygen attacks position 1$\alpha$, with formation of a hydroperoxy-derivative of flavin. The mechanism depicted in Scheme 5 was suggested[90,91] on the basis of kinetic investigations for the interaction of *p*-hydroxybenzoic hydroxylase with the 2,4-hydroxybenzoate substrate, F being flavin attached to the enzyme E, the X unidentified species, and SO 2,3,4-trihydroxybenzoate, while the asterisk denotes charge-transfer complexes. Scheme 5 shows that the slowest

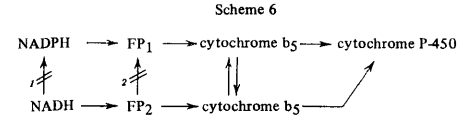
stage in the reaction is charge transfer in the enzyme–substrate–NADP complex ($k$ = 1.1 min$^{-1}$). The other stages occur much more rapidly. Formation of an oxygenated flavin derivative in the enzyme–substrate complex precedes transfer of an oxygen atom to the substrate.

Scheme 5




Investigation of the prestationary and steady-state kinetics of the reactions of mono-oxygenase enzymes provides indications of the limiting stage in the overall enzymic process and of the intermediate complexes responsible for activating molecular oxygen. Experience shows that hydrophobic interaction of the substrate with hydrophobic (e.g. lipid) portions of the enzyme molecules alone is insufficient for the enzymic reaction to occur[109–111]: the inert oxygen molecule must be activated during the reaction. This main function of mono-oxygenases is accomplished by cytochrome P-450, which forms an oxygenated intermediate compound with molecular oxygen, and by the flavin groups of mono-oxygenases free from metal ions, which form with molecular oxygen a 1$\alpha$-hydroperoxy-derivative of flavin, as has definitely been proved quite recently. Thus the oxygen molecule is activated by two-electron reduction either by an iron ion or by flavin reduced in the electron-transfer chain. The two-electron reduction of oxygen involving cytochrome P-450 in Scheme 3 contains many obscurities connected with the source of the second electron for reduction, since the interaction of NADPH and NADH-specific electron-transfer chains is still not clear[130]. Recently exchange of equivalents undergoing reduction, between NADPH and NADH-specific electron-transfer chains, has been discussed[131]. In Scheme 6 this exchange occurs with the aid of cytochrome $b_5$. Exchanges *1* and *2* do not take place.

Scheme 6




In spite of great achievements in the kinetic study of enzymic reactions of mono-oxygenases and their individual stages, the central problem—the mechanism of the activation of oxygen—cannot yet be regarded as solved, since the structure of the active oxygen in cytochrome P-450 is completely unknown. It is clear only that transfer of an oxygen atom to the substrate is not always the limiting stage. Since direct examination of the transition state is impossible with most enzymic reactions, it becomes necessary to study model systems and model reactions reflecting individual aspects of the complicated enzymic process. Only model reactions and model systems will enable details of the activation of molecular oxygen to be elucidated.

Three methods—theoretical considerations, investigation of the properties of enzymes, and study of the mechanisms of chemical reactions and their catalysis—are available for solving the problem of establishing the mechanism of enzymic action[132]. According to Jencks, "...to understand the mechanism of catalysis of an enzymatic reaction one would first like to understand the mechanism of the non-enzymatic reaction and the means by which this reaction can be accelerated". In Section VI we shall discuss in detail problems involved in modelling the action of mono-oxygenase enzymes.

## VI. METHODS OF SIMULATING MONO-OXYGENASE ENZYMES

With all the complexity of enzymes, they obey the same thermodynamic and kinetic laws as do ordinary catalysts, so that even simple catalysts simulate in some measure certain aspects of the action of enzymes. Concerning the modelling of enzymes it is Shilov's opinion that "this concept can most usefully be applied in the light of the purpose of the investigation rather than on the basis of the degree of similarity between the catalyst studied and the enzyme which this catalyst simulates". Many reviews have been published[132–138] on the simulation of enzymes. A combined approach may yield significant results, as occurred for the enzymic fixation of molecular nitrogen[137]. By learning from Nature and using chemical concepts, we can discover novel, sometimes surprising reactions.

The action of a mono-oxygenase transfers one atom of an oxygen molecule to a substrate molecule. It is therefore most advisable to study firstly ways in which oxygen is transferred to a substrate by various systems and their mechanisms. The investigation of mono-oxygenases has shown that an oxygen atom can be incorporated into a substrate only after the oxygen molecule has acquired two electrons. All systems which transfer oxygen to a substrate can be divided into two broad groups: the first group includes metal ions and complexes, whose oxidation provides two electrons for the oxygen; the second group comprises compounds of peroxide character, in which oxygen has already been activated, since it possesses these two electrons. In the former case oxidation of the substrate involves that of the metal ion or complex. In the latter case an oxygen atom is transferred directly to the substrate from the peroxy- or hydroperoxy-compound. Imitation of enzymic oxygen transfer will be more complete the wider the range of reactions which can be accomplished by the suggested model for mono-oxygenase (Table 3). *N*-Oxides, ozone, and oxo-anions of metal salts are also able to transfer oxygen to a substrate.

Henceforward we shall discuss in detail the reactions of each group of suggested model systems and the mechanism of their action. The most interesting are the first group of systems, containing a metal ion or complex together with molecular oxygen. Table 3 lists several such systems (with aromatic substrates the proportions of isomeric phenols formed are given in the sequence—*ortho* : *meta* : *para*).

**Table 3.** Systems containing a metal ion or complex and molecular oxygen.

| System | Substrate | Products | Ref. |
|---|---|---|---|
| $Fe^{2+}$ + ascorbate | dehydroepiandrosterone | 7-hydroxyandrostenones | 139 |
| $Fe^{2+}$ + ascorbate | cortexolone | cortisol + cortisone | 140,141 |
| $Fe^{2+}$ + ascorbate + EDTA | anisole | methoxyphenols 43 : 18 : 39 | 142 |
| $Fe^{2+}$ + 2,4,5-triamino-6-hydroxypyrimidine | anisole | methoxyphenols 49 : 13 : 38 | 143 |
| $Fe^{2+}$ + thiosalicylic acid | acetanilide | phenols 57 : 3 : 40 | 144 |
| $Fe^{2+}$ + thiosalicylic acid | toluene | cresols 61 : 13 : 16 | 144 |
| $Fe^{2+}$ + thiosalicylic acid | napthalene | 1-naphthol 11%<br>2-naphthol 9% | 144 |
| $Fe^{2+}$ + *N*-benzyl-1,4-dihydronicotinamide | arene | hydroxyarene | 145 |
| $Fe^{2+}$ + tetrahydropterin | phenylalanine | tyrosine 2 : 1 : 1 | 146 |
| $Fe^{2+}$ + tetrahydropterin | tryptophan | 5-hydroxytryptophan + melanin + kynurenine | 146,147 |
| Haemin + thiosalicylic acid | cyclohexane | cyclohexanol | 148 |
| $Fe^{2+}$ + EDTA | toluene | cresols 38 : 24 : 38 | 145 |
| $Fe^{2+}$ + EDTA | anisole | methoxyphenols 56 : 8 : 36 | 145 |
| $Fe^{2+}$ + EDTA | fluorobenzene | fluorophenols 2 : 45 : 53 | 145 |
| $FeCl_2$ (acetone or methanol) | cyclohexane | cyclohexanol | 149 |
| $TiCl_3$ | acetanilide | phenols 35 : 32 : 33 | 150 |
| $TiCl_3$ | toluene | cresols 61 : 16 : 23 | 145 |
| $TiCl_3$ | anisole | methoxyphenols 54 : 0 : 46 | 145 |
| $TiCl_3$ | fluorobenzene | fluorophenols 2 : 33 : 65 | 145 |
| $TiCl_3$ | nitrobenzene | nitrophenols 4 : 36 : 60 | 145 |
| $TiCl_3$ | cyclohexane | cyclohexanol + cyclohexanone | 151 |
| CuCl | toluene | cresols 29 : 29 : 49 | 145 |
| CuCl | anisole | methoxyphenols 75 : 8 : 17 | 145 |
| CuCl | fluorobenzene | fluorophenols 30 : 68 : 2 | 145 |
| $SnHPO_4$ | toluene | cresols 46 : 38 : 16 | 145 |
| $SnHPO_4$ | anisole | methoxyphenols 53 : 20 : 17 | 145 |
| $SnHPO_4$–organic solvent | acetanilide | phenols 34 : 44 : 22 | 152 |
| $SnCl_2$–acetonitrile | alkanes | isomeric alcohols | 153 |
| $SnCl_2$–acetone | cyclohexane | cyclohexanol | 154 |
| $MoCl_3$ | cyclohexane | cyclohexanol | 151 |
| $Mo(CO)_6$–acetonitrile | naphthalene | 1-naphthol | 155 |
| $Mo(CO)_6$–acetonitrile | benzene | phenol | 156 |
| $MoO(S_2CNR_2)_2$ | triphenylphosphine | triphenylphosphine oxide | 157 |

Table 4 sets out the reactions of peroxy-acids and of several peroxy-compounds [158–173] with a series of substrates, to which oxygen is transferred on the pattern of the action of mono-oxygenases containing flavoproteins but no metal ion or complex. Reactions of peroxy-acids with alkenes are omitted from Table 4, for their mechanism and kinetics have been thoroughly studied [174]. Aromatic substrates are treated as in Table 3.

**Table 4.** Reactions of peroxy-acids and peroxide compounds.

| Reagent | Substrate | Products and constants | Ref. |
|---|---|---|---|
| $CF_3CO_3H$ | benzene | phenol | 158 |
| $CF_3CO_3H$ | phenol ethers | phenoxyphenols | 159 |
| $CF_3CO_3H$ | anisole | methoxyphenols, quinones | 160 |
| $CF_3CO_3H$ | anisole | methoxyphenols 73 : 0 : 27 | 161 |
| $CF_3CO_3H$ | toluene | cresols 78 : 2 : 20 | 161 |
| $CF_3CO_3H$ | fluorobenzene | fluorophenols 17 : 0 : 83 | 161 |
| $CF_3CO_3H$ | acetanilide | phenols 43 : 1 : 56 | 56 |
| $CF_3CO_3H$ | *p*-deuterotoluene | *p*-methylphenol<br>2-deutero-4-methylphenol | 162 |
| $CF_3CO_3H$ | naphthalene | $K = 1.6 \times 10^8 \exp(-14400/RT)$ | 163 |
| $CF_3CO_3H$ | 2-methylbutane | isomeric butanols | 15 |
| $CF_3CO_3H$ | methylcyclohexane | isomeric methylcyclohexanols | 15 |
| $CH_3CO_3H$ | *p*-methylphenols | muconic acids and lactones | 164 |
| $CH_3CO_3H$ | hexamethylbenzene | | 165 |
| $CH_3CO_3H$, $HCO_3H$ | phenol | catechol-quinol | 166 |
| $CH_3CO_3H$ | naphthalene | $K = 6.2 \times 10^9 \exp(-21000/RT)$ | 167 |
| $CH_3CO_3H$ | anisole | $K = 6.3 \times 10^8 \exp(-17000/RT)$ | 167 |
| $CH_3CO_3H$ | phenol | quinones + muconic acid | 168 |
| $CH_3CO_3H$ | 1-methoxynaphthalene | muconic acids | 169 |
| $CH_3CO_3H$ | *p*-methylphenol | *cis, trans*-β-methylmuconic acid | 170 |
| *m*-Chloroperbenzoic acid | 4-methyl-2,6-di-t-butylphenol | | 108 |
| Autoxidising dihydroalloxazines | | | 171 |
| $MoO(O_2)_2L_1L_2$* | allyl alcohol | glycidol | 172 |
| $MoO(O_2)_2L_1$ | allyl alcohol | glycidol | 172 |
| $MoO(O_2)_2L_1$ | naphthalene | 1-naphthol + quinones | 173 |

*$L_1$ = Hexametapol; $L_2 = H_2O$.

Model systems of the first type (Table 3) can in turn be divided into two subgroups: the first subgroup—tin(II) hydrogen orthophosphate, tin(II) and molybdenum(III) chlorides, and hexacarbonylmolybdenum—contains a metal ion capable of transferring not one but two or more electrons; the second subgroup includes a metal ion capable of one-electron transfer and a redox-active ligand—ascorbic acid, thiosalicylic acid, tetrahydropterin, and pyrimidines. Table 3 shows that model systems of the first type effect the hydroxylation of aliphatic [139–141, 151, 154] and aromatic [142–147, 155, 156] compounds, and also oxidise triphenylphosphine to its oxide [157]. Furthermore, systems containing an $Fe^{2+}$ ion and a redox-active ligand can convert alkenes into epoxides [138]. Thus model systems of the first type accomplish many of the reactions catalysed by mono-oxygenases (Table 2). The most important task is to recognise the species containing active oxygen and capable of accomplishing all the reactions enumerated in Table 3. The radicals HO· and $HO_2$· are possible active species, but these free radicals cannot be the activated forms of oxygen in enzyme systems, since they would destroy the cell. They can be stabilised by metal ions: $HO_2$· is converted into $FeO_2^{2+}$ (complex III) on interaction with $Fe^{3+}$, but HO· forms $FeO^{2+}$ (complex II). In such a stabilised state the radicals HO· and $HO_2$· may be oxygen carriers in biological oxidation. An important characteristic of a species attacking an aromatic substrate is the distribution of isomers and the orienting influence of the substituent in the benzene ring. For all the cases listed in Table 3 the distribution of isomeric phenols differs significantly from that typical of HO· radicals generated by various methods—with Fenton's reagent,

radiolytically, etc.[2]—which means that these radicals are not the active intermediate for all the systems investigated. Several workers have suggested that the radical $HO_2^{\cdot}$ may act as attacking species for the $TiCl_3 + O_2$ and $Sn^{2+} + O_2$ systems[152]. The distribution of isomeric phenols indicates that the substrate is attacked by a less electrophilic species than HO· ; yet it is well known that $HO_2^{\cdot}$ is more electrophilic than HO· (with electron affinities respectively of 3.04 and 2.65 eV—Ref. 1, p. 207). Thus either the radical $HO_2^{\cdot}$ is unable to function as the active species or it does so in a less electrophilic state than that of HO· , as a consequence of interaction with the solvent (hydrogen bonding). It is noteworthy also that $HO_2^{\cdot}$ may be less active towards aliphatic and aromatic substrates than is HO· .[2,175] The hydroxylating ability of the former radical towards aromatic compounds has been convincingly proved by one of the Reviewers[2], yet the activity of HO· towards indigodisulphonate exceeds that of $HO_2^{\cdot}$ by factors of $10^6$.[175]

Study of the $Sn^{2+} + O_2$ system is of great value in the choice between the radical $HO_2^{\cdot}$ and other attacking species. It is well known that the $Sn^{2+}$ ion can be oxidised either successively through $Sn^{3+}$ or directly to $Sn^{4+}$ with simultaneous loss of two electrons[176]. In the two-electron oxidation oxygen is transferred to the substrate according to the equations

$$Sn^{2+} + O_2 \rightleftarrows [Sn^{2+}O_2]$$
$$[Sn^{2+}O_2] + S \rightarrow Sn^{4+}O + SO$$

a simple scheme suggested by Ullrich[152]. It has been proved, however, that in aqueous solution oxidation of the $Sn^{2+}$ ion by molecular oxygen has a radical-chain mechanism[176,177]:

$$Sn^{2+} + O_2 \xrightarrow{H^+} Sn^{3+} + HO_2^{\cdot}$$
$$Sn^{2+} + O_2 \xrightarrow{H^+} Sn^{4+} + HO_2^{\cdot}$$
$$Sn^{2+} + HO_2^{\cdot} \xrightarrow{H^+} Sn^{3+} + H_2O_2$$
$$Sn^{2+} + H_2O \xrightarrow{2H^+} Sn^{4+} + 2H_2O$$
$$Sn^{3+} + Sn^{3+} \rightarrow Sn^{2+} + Sn^{4+} .$$

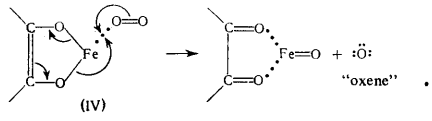
The retarding effect of inhibitors—4-methyl-3,6-di-*p*-tolyliminocyclohexa-1,4-dienylamine, potassium trioxalatocobaltate, etc.—confirms the radical-chain mechanism of the oxidation of tin(II) in aqeuous solution[176]. Thus in aqueous solutions or in aqueous-alcohol and aqueous-acetone mixtures the hydroxylating species may be either the $HO_2^{\cdot}$ radical or a complex of oxygen with the $Sn^{2+}$ or $Sn^{3+}$ ion. The kinetics of the oxidation of $Sn^{2+}$ by oxygen has been investigated in an aprotic solvent (acetone), as well as the hydroxylation of cyclohexane under these conditions, in order to choose between these possibilities[154]. The $HO_2^{\cdot}$ radical cannot be formed during the oxidation of $Sn^{2+}$ in acetone: thus cyclohexane is hydroxylated by tin-oxygen complexes, since the radical-ion $O_2^-$, formed by the transfer of one electron from the tin ion to oxygen, is devoid of hydroxylating power. The stoichiometry of the reaction in acetone is that one $Sn^{2+}$ ion oxidises two oxygen molecules, which means that simultaneous transfer of two electrons from the tin(II) ion to oxygen, as is assumed by Ullrich, is impossible. The $Sn^{2+}$ ion is oxidised by oxygen in two steps:

$$Sn^{2+} + O_2 \rightleftarrows \underset{(I)}{[Sn^{2+} \cdots O_2]} \rightleftarrows \underset{(II)}{[Sn^{3+} \cdots O_2^-]} \rightarrow Sn^{3+} + O_2^-$$
$$Sn^{3+} + O_2 \rightarrow \underset{(III)}{[Sn^{3+} \cdots O_2]} \rightarrow Sn^{4+} + O_2^-$$
$$O_2^- + O_2^- \rightarrow \text{Recombination} .$$

Such a scheme for the oxidation of $Sn^{2+}$ in acetone is supported by the observed stoichiometry and also by the high rate constant of 100 $M^{-1}$ $s^{-1}$ at 25°C for the recombination of $O_2^-$ radical-ions[178]. The $O_2^{2-}$ ion is not usually formed in aprotic media, since it is stable only in aqueous solutions and in crystals[179]. Tin-oxygen complexes—$[Sn^{2+} \ldots O_2]$, $[Sn^{3+} \ldots O_2^-]$, or $[Sn^{3+} \ldots O_2]$—may possess hydroxylating power in an aprotic medium. It is difficult to choose between these three forms of activated oxygen; it is clear only that the activated oxygen in the complex has a radical character, since the hydroxylation of cyclohexane in acetone is retarded by the addition of inhibitors of radical reactions.

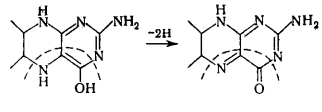
It is important to note that direct participation of the metal ion in hydroxylation and its effect on the distribution of isomers in the hydroxylation of aromatic compounds have been reliably established with other systems, e.g. $Fe^{2+}$–EDTA–$O_2$, so that the authors[145] speak of the hydroxylating power of the $[Fe^{2+} \ldots O_2]$ complex. Comparison of the distribution of isomers for a given substrate, e.g. toluene, obtained with oxygen and the $Fe^{2+}$, $Sn^{2+}$, $Ti^{3+}$, and $Cu^+$ ions shows that the isomeric composition of the cresols is strongly dependent on the metal ion[145]. If the radicals $HO_2^{\cdot}$, formed in the oxidation of all the above ions in aqueous solution, had been the hydroxylating species, the nature of the cation would not have influenced the distribution of isomers. Hence the hydroxylating species is a complex of the metal cation with oxygen, which shows variation in electrophilic strength on passing from one metal to another. The variation in the electrophilicity of species attacking the substrate is responsible for the difference in distribution of isomers. At high ionic concentrations the distribution becomes random—40 : 40 : 20—probably owing to a change in the mechanism of hydroxylation[145].

It is very important how an oxygen atom is transferred from the metal complex to the substrate. Hamilton noted that most reactions catalysed by mono-oxygenases and certain of their models are analogous to those of the very reactive carbenes $CR_2$ and nitrenes NR, which have six electrons around the carbon or nitrogen atom. Carbenes are incorporated at carbon-hydrogen bonds into aliphatic and aromatic compounds and add to alkenes to give cycloalkanes[138,143,180]. Reactions of the same type are accomplished by mono-oxygenases and their models. An oxygen atom, which has six electrons in the outer shell, may be the analogue of a carbene or a nitrene. However, oxygen cannot be generated in the enzymic processes. Hamilton therefore suggested that the oxygen atom is transferred to the substrate from an oxenoid complex, so called by analogy with carbenoids, which contain a combined carbene transferable to a substrate in the transition state. The oxygen molecule forms an oxenoid by interaction with a complex of the $Fe^{2+}$ ion and a redox-active ligand such as ascorbic acid, tetrahydropteridine, etc. :




(IV)

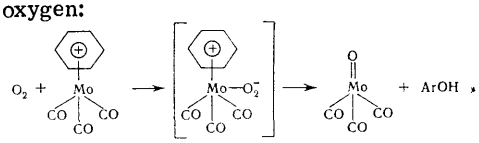
A complex similar to (IV) can be formed from tetrahydropteridine, involving the atoms segregated by the broken curve[138]:



The function of the $Fe^{2+}$ ion in the complex (IV) consists in combining in the transition state with the reducing agent and oxygen to transfer electrons to the latter. In this complex (IV) transfer of a singlet oxygen atom from the bound oxygen molecule to the substrate probably becomes possible in such a way that radical intermediates are not formed. Transfer of a triplet oxygen atom to the substrate is energetically less advantageous, since a triplet product SO, less stable than the singlet product, would then be formed.

Hamilton's suggested oxenoid mechanism of oxygen transfer to a substrate explains satisfactorily the ability of mono-oxygenases to catalyse hydroxylation and epoxidation. The oxenoid complex may vary in electrophilicity depending on the nature of the metal and the ligand. Moreover, this complex, which attacks the substrate, may be radical or even nucleophilic in character. This property of the oxenoid complex helps us to understand the different distribution of isomers and the different orienting influence of substituents in the benzene ring on the hydroxylation of aromatic substrates. In individual cases the electrophilic character of the active centre in an enzyme system transmitting oxygen to an aromatic substrate can be assessed on the basis of model experiments. Thus the microsomal hydroxylation of toluene yields the isomeric cresols in the proportions 59 : 13 : 28, almost identical with the distribution obtained on hydroxylation with molecular oxygen in the presence of thiosalicylic acid and $Fe^{2+}$ ions [144]. This suggests that the active centre of the microsomal hydroxylating system (cytochrome P-450) forms with molecular oxygen a complex which acts on the substrate as a moderately electrophilic reagent. The hydroxylation of toluene by such a strong electrophilic reagent as trifluoroperacetic acid gives the proportions 78 : 2 : 20 of isomeric cresols[161]. Hence the complex which attacks the substrate in the microsomal hydroxylating system is less electrophilic than trifluoroperacetic acid, which effects the same reaction.

A reaction has recently been investigated in which molecular oxygen and the substrate to be oxidised (benzene, naphthalene, or 2-methylnaphthalene) are activated simultaneously on the same active centre, a molybdenum atom[155, 156]. These aromatic compounds are oxidised by molecular oxygen neither under physiological conditions nor even at quite high temperatures. In the presence of a complex of molybdenum with carbon monoxide (hexacarbonylmolybdenum) in acetonitrile at 70–80°C, however, they undergo oxidation to phenols and naphthols. Arenes form with this carbonyl arenetricarbonyl complexes $ArHMo(CO)_3$ in which, as in hexacarbonylmolybdenum itself, the metal is present in the zerovalent state. The molybdenum atom is able to add an oxygen molecule and transmit to it one or more electrons, i.e. to activate molecular oxygen. At the same time the aromatic ligand in the arenetricarbonyl complex carries an effective positive charge, in contrast to the uncomplexed arene: i.e. it becomes capable of nucleophilic substitution. Thus the arene, having become more electrophilic, is attacked by oxygen, and the oxygen in the complex is more strongly nucleophilic than is free molecular oxygen:




The actual transfer of an oxygen atom to the aromatic molecule may be supposed to occur by Hamilton's "oxenoid" mechanism, with intermediate formation of a thermodynamically unstable arene epoxide, which isomerises into a phenol. Study of the kinetics of the hydroxylation of aromatic compounds in the $Mo(CO)_6$–$O_2$ system shows that oxygen is directly involved in the reaction: the rate of accumulation of phenols is directly proportional to the partial pressure of oxygen. With all the substrates the formation of phenols is preceded by that of the arenetricarbonyl complex $ArHMo(CO)_3$, which in the case of naphthalene limits the overall rate of the process. The $ArH$–$Mo(CO)_6$–$O_2$ system resembles "internal" mono-oxygenases in its type of action, since the oxygen atom not inserted into the substrate is stabilised by the metal, which is oxidised by the reaction. Unfortunately, the variation in reactivity of the aromatic molecules investigated in this system cannot be used to make deductions on the nature of the attacking species, since the complicated sequence of reactions is limited at different stages with different substrates.

It has recently been shown that peroxy-acids are capable of the oxygen-transfer reactions typical of mono-oxygenases [15, 108]. In several cases the products formed from the same substrate by the action of peroxy-acids are identical in character with those obtained by microsomal or microbiological oxidation [108, 167]. Thus identical products are obtained by the microsomal oxidation of 4-methyl-2,6-di-t-butylphenol and by its reaction with *m*-chloroperbenzoic acid. The sequence of changes undergone by naphthalene on oxidation with peracetic acid [167] is completely analogous to the microbiological transformation of this hydrocarbon by five different species of microorganisms [181]. As already noted, some mono-oxygenases do not contain metal ions, and activate oxygen by forming intermediates with the flavin components of the hydroxylating system. These intermediate compounds are essentially hydroperoxides [89–91, 129]. Peroxy-acids and certain other peroxy-compounds are therefore able to imitate the transfer of oxygen to a substrate characteristic of mono-oxygenases [108, 158–171, 173] (Table 4). It is very important to compare the rates of transfer of oxygen by peroxy-acids and by peroxy-derivatives of flavoproteins, which requires knowledge of the rate constants for reaction of such acids with various substrates. The reactions of peroxy-acids with alkenes have been characterised quantitatively [174], but no single rate constant had been measured until recently for their reactions with aromatic and aliphatic compounds. Only recently have determinations been made of the rate constants for the reactions of peracetic acid with naphthalene and with anisole [163, 167], which were measured in the presence of a mixture of acetic acid with dichloroethane or dichloromethane. The activation energies for peracetic acid with aromatic compounds are higher than in Prilezhaev's reaction, in good agreement with the increase in nucleophilic strength on passing from arenes to alkenes. An electrophilic peroxy-acid molecule undergoes a bimolecular reaction with a nucleophilic aromatic molecule, as with an alkene. No intermediate complex of a peroxy-acid with an aromatic molecule can be detected [163, 167]. The existence of such a complex has been proved kinetically for the reaction of peracetic acid with propene [182].

The bimolecular rate constants for the reactions of peroxy-acids with aromatic compounds show that the transfer of the oxygen atom from the acid to the substrate is quite rapid: thus at room temperature (20°C) the constant for trifluoroperacetic acid with naphthalene is $10^{-3}$ $M^{-1}$ $s^{-1}$ [163], while a molecule of 1-naphthol at the same temperature reacts with peracetic acid with a rate constant of ~0.33 $M^{-1}$ $s^{-1}$. [163] Roughly similar rates are

observed for the reaction of hydroperoxy-derivatives of the flavin part of *p*-hydroxybenzoic hydroxylase with a 2,4-dihydroxybenzoate [90,91].

The autoxidising dihydroalloxazines [171], which react with oxygen to form hydroperoxy-derivatives, are similar to metal-free mono-oxygenases in their action on substrates which can be hydroxylated.

Peroxo-complexes of sexivalent molybdenum



(where $L_1$ and $L_2$ are ligands), discovered in the laboratory by Sajus [183], have recently been used [172,173] to simulate the transfer of oxygen by mono-oxygenases. Kinetic study of the interaction of such complexes having two ligands ($L_1$ = Hexametapol and $L_2 = H_2O$) and only one ligand $L_1$ has shown that both complexes form an intermediate compound with the substrate, which breaks down with the formation of glycidol. One molecule of the peroxo-complex epoxidises two molecules of allyl alcohol. The rate constants $k_2$* of decomposition of the intermediate complex have been determined at 50°C, together with the Michaelis–Menten constants

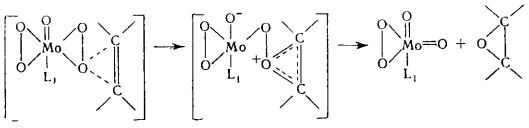
$$K_m = \frac{k_{-1} + k_2^*}{k_1}$$

where $k_1$ and $k_{-1}$ are the rate constants of formation and dissociation of the intermediate complex. Table 5 gives the constants which we have obtained for both complexes at 50°C. The value $k_2 = 2.4 \times 10^9 \exp(-18300/RT)$ gave the enthalpy of activation as $\Delta H^{\neq} = 17.7$ kcal mole$^{-1}$ and the entropy of activation as $\Delta S^{\neq} = -18.0$ cal mole$^{-1}$ deg$^{-1}$. [172]

**Table 5.**

| | $K_m$, M | $k_2^*$, s$^{-1}$ |
|---|---|---|
| $MoO(O_2)_2L_1$ | 0.81 | $9.71 \cdot 10^{-4}$ |
| $Mo(O_2)_2L_1L_2$ | 0.78 | $4.17 \cdot 10^{-4}$ |

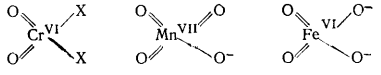
The closely similar quantitative data obtained for the two complexes and the steric hindrance to direct involvement of the molybdenum atom in interaction with the substrate suggest that the mechanism of the epoxidation of allyl alcohol by the molybdenum peroxo-complexes may be



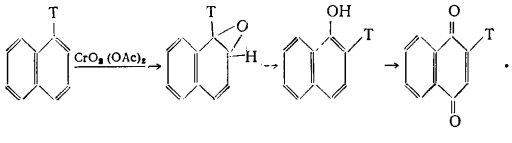
A significant feature of this mechanism is the involvement of the peroxo-oxygen atom in complex formation with allyl alcohol and the absence of direct involvement of the molybdenum atom in complex formation, which had been suggested by French workers [184]. The former mechanism is supported by the closely similar rate constants and values of $K_m$ for the complexes with one and with two ligands, as well as by the good agreement between the activation functions of the limiting stage of the reaction and the thermodynamic functions of the epoxidation of allyl alcohol by other peroxide reagents that form a complex by means of the peroxide oxygen (Ref. 174, p. 1761).

It may be supposed, as in the above reaction scheme, that transfer of the oxygen atom to the allyl alcohol molecule takes place by an "oxenoid" mechanism. The electrophilic character of the epoxidising species is indicated by the ability of the peroxo-complex $MoO(O_2)_2L_1$ to hydroxylate naphthalene to 1-naphthol [173], the reaction being conducted in dichloroethane at 70°C. 1-Naphthol is an intermediate, undergoing further reactions with the peroxo-complex.

A team of American workers have emphasised the similarity between oxygen transfer by mono-oxygenases and by several oxo-compounds of metals of variable valency [185]

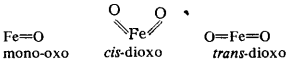


where X = OH, Cl, $OCOCH_3$. Indeed, chromyl acetate (X = $OCOCH_3$) epoxidises alkenes, potassium permanganate hydroxylates hydrocarbons [186], and chromyl chloride acetate accomplishes both reactions; oxo-compounds of lead $Pb(OCOCH_3)_{4-n}(N_3)_n$ are used for oxygenating the steroid androsta-4,9(11)-diene-3,17-dione, which is epoxidised and hydroxylated simultaneously [187]. It is well known also that the activity of a heterogeneous catalyst in oxidation is due to the presence of an M=O bond [188]. Oxygen transfer by such an oxo-compound as chromyl diacetate shows the surprising peculiarity that, in the hydroxylation of substituted aromatic substrates, this reagent can cause migration of a group from the position hydroxylated to a neighbouring position [185]:



Such migration of a substituent in an aromatic ring is very characteristic of the action of mono-oxygenases [189], and occurs in the hydroxylation of aromatic compounds by trifluoroperacetic acid [162]. Intermediate formation of arene oxides has been shown in the microsomal oxidation of aromatic compounds, without which the migration of substituents in aromatic compounds cannot be explained [162, 189–191].

Thus oxo-compounds of transition metals imitate an important feature of the action of mono-oxygenases. This suggested that in many other cases the species transferring oxygen to the substrate contains an oxo-group [185]; for example, the oxygen-transferring structures



are assumed for the iron ion. However, this hypothesis is not always confirmed in practice. The oxygen-carrying peroxo-complex of molybdenum $MoO(O_2)_2L_1$, where $L_1$ is hexametapol, contains two peroxo-groups and one oxo-group. Use for epoxidation of a complex labelled with the oxygen-18 isotope in the oxo-group gave an epoxide from which this isotope was absent: i.e. the oxo-group of the molybdenum(VI) complex plays no part in oxygen transfer [192]. Thus the oxo-form of the bond between metal and

oxygen is not universally present in all oxygen-transferring species.

All the reactions discussed in this Section simulate in some measure the transport of an oxygen atom to the substrate by mono-oxygenases. However, systems that generate free radicals can in no way be regarded as models, since active free radicals are incompatible with the living cell. Biochemical oxidations may involve not free radicals but their complexes with ions of metals of variable valency, e.g. complexes of HO· and $HO_2$· radicals with trebly charged iron ions. The study of models containing a metal ion or complex and oxygen shows that the most probable attacking species is a metal–oxygen complex. This is quite remarkable, since complex formation between oxygen and the active centre of mono-oxygenases (cytochrome P-450) has now been convincingly demonstrated. It has been shown by physical methods that the cytochrome binds the oxygen molecule by complex formation of the latter with an iron ion in the reduced state.

The study of model systems ($Fe^{2+} + O_2$, $Sn^{2+} + O_2$) has yielded numerous data confirming the hydroxylation of substrates by complexes of these ions with oxygen—involvement of the metal ion in hydroxylation, its effect on the distribution of isomers in the hydroxylation of aromatic substrates, absence of effect of added hydrogen peroxide on the reaction, the hydroxylation of arenes and the epoxidation of alkenes effected by the same system, which eliminates the HO· radical as attacking agent, the hydroxylation of substrates in aprotic media, where the formation of $HO_2$· is excluded, and other facts.

Hamilton's "oxenoid" mechanism appears the most probable and universal for transfer of an oxygen atom to the substrate, providing a satisfactory explanation of all experimental results obtained for model reactions and systems. The oxenoid mode of oxygen transfer most probably operates also with peroxide compounds such as peroxy-acids and peroxo-complexes of metals. These compounds well simulate oxygen transfer by mono-oxygenases having a flavin group in the active centre. Peroxy-acids, like mono-oxygenases, cause migration of a substituent from the position at which oxygen is inserted into the ring of an aromatic substrate to a neighbouring position.

The intense study of the reactions of mono-oxygenases, the isolation and purification of this group of enzymes, the investigation of the structure and the functions of component parts of the composite enzyme system, the wide use of physical methods in studying the state of the metal ion in the active centre: all this has in many cases established great certainty as to the mechanism of the action of these enzyme systems. Investigations of the prestationary and steady-state kinetics of the reactions of various mono-oxygenases have played a very important part in establishing the mechanisms discussed in this Review. However, even such a frontal attack by research workers in this field has not revealed the ultimate mechanism of the activation of molecular oxygen. Not only study of the action of the mono-oxygenases themselves but also detailed study of model systems have played a great part in elucidating individual features of this mechanism. Examination of models will enable us to ascertain the structure of the species transferring oxygen to the substrate, and perhaps indicate ways in which oxygen may be chemically activated under physiological conditions. The yields of reaction products obtained by means of model systems are still quite low, but systems have been found enabling us to follow the kinetics of the accumulation of reaction products and the valency changes undergone by metal ions occupying a leading role in these systems.

The practical value of mono-oxygenase enzymes is very great for the most diverse fields of toxicology, pharmacology, and oncology. Recent results threaten a revolution in the synthesis of several physiologically active substances. Thus 11 $\beta$-hydroxylase and $\Delta^{1-2}$-dehydrogenase have recently been used to effect the two-step transformation of the readily available Reichtein's substance S into prednisolone [193]. This conversion is achieved by means of enzymes fixed on gel matrices and placed in two columns connected in series. Hydroxylation of substance S to cortisol takes place in the first column, and dehydrogenation to prednisolone in the second column. It may be hoped that the number of examples of such transformations by means of mono-oxygenase enzymes will increase.

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