question how it is coupled to the four-electron transfer required for the reduction of molecular oxygen to water (eqn. 2).

Oxygen is a good oxidizing agent only from a thermodynamic point of view; kinetically it is rather inert. These properties form part of the concept of 'the fitness of oxygen' (George, 1965), but also pose severe mechanistic problems. Reduction in four consecutive one-electron steps would be expected to be extremely slow in view of the high free-energy barrier for the first step. This could be overcome by a stabilization of the O_2^- intermediate. However, in cases having water as the final product of reduction, there is another difficulty with the step-wise mechanism: the last free-radical intermediate, $OH\cdot$, is very reactive, and this could easily lead to undesirable side reactions.

The two difficulties just mentioned would be overcome in mechanisms involving multi-electron steps, e.g. two double-electron transfers, which could be made possible by the co-operation of several electron acceptors in the enzymes. Such a concept receives support from some simple considerations (Malmström, 1969). For example, the cytochrome oxidase unit contains four potential electron acceptors (haems α and α_3 plus two copper ions), and the 'blue' oxidases all contain at least four copper ions/molecule. Further, proteins that contain only one prosthetic group of a similar nature in each molecule, e.g. cytochrome *c* (haem) or azurin (copper), can carry out reactions of the type in eqn. (1) rapidly, but the reduced proteins are not autooxidizable (eqn. 2). Recent studies have now given a direct demonstration of the correctness of the multi-electron-step mechanism for one enzyme, namely laccase from the fungus *Polyporus versicolor*.

Laccase contains four copper ions/molecule. These represent three distinct forms of the metal: (1) type 1 Cu^{2+} ; (2) type 2 Cu^{2+} ; (3) two diamagnetic copper ions (Malmström, Reinhammar & Vänngård, 1968). Type 1 Cu^{2+} is responsible for the strong blue colour of the enzyme, and it also has unusual electron-paramagnetic-resonance parameters. Type 2 Cu^{2+} represents a spectrally more normal form of the metal. However, it has an exceptionally high affinity for F^- , which also acts as an inhibitor of the enzyme by interfering with the reaction in eqn. (2), whereas type 1 Cu^{2+} and its reduction (eqn. 1) are not affected. The two diamagnetic copper ions have been suggested to be a binuclear pair of spin-coupled Cu^{2+} (Fee, Malkin, Malmström & Vänngård, 1969). This pair is responsible for the strong absorption at about 330 nm found in all 'blue' oxidases (Malkin, Malmström & Vänngård, 1969).

In anaerobic redox titrations it has been found that the type 1 Cu^{2+} and the diamagnetic pair are reduced together in a linear fashion on the addition of about 3.5 electron equivalents. (Type 2 Cu^{2+} is only

partially reduced under these conditions, accounting for the non-integral end point.) All the electron-accepting sites have a potential greater than 500 mV. The linearity of the titration curve can be explained on the basis that the diamagnetic copper ions act as a co-operative two-electron-accepting unit, and that the type 1 Cu^{2+} has the same standard potential as this pair.

Kinetic studies (Malmström, Finazzi Agrò & Antonini, 1969) show that the type 1 Cu^{2+} and the diamagnetic pair are reduced by substrates rapidly enough to involve such reductive steps in the catalytic mechanism, although this does not seem to be true for the type 2 Cu^{2+} . Rapid reoxidation with molecular oxygen, on the other hand, occurs only when the two-electron-accepting unit is reduced. A mechanism involving two double-electron transfers to oxygen is consistent with all experimental results. The function of the type 2 Cu^{2+} in this mechanism may be to stabilize the HO_2^- intermediate.

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Mixed-Function Oxidation

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Enzymes that catalyse the introduction of molecular oxygen into a substrate are termed oxygenases. There are various types of oxygenase, and this account will be concerned with mono-oxygenases in which one atom of molecular oxygen is incorporated into the substrate as follows:

In these reactions there is no evidence as yet to indicate that a peroxide ($S\cdot O\cdot OH$) is an intermediate. Similarly there is as yet no evidence to suggest that the oxygen molecule is cleaved before incorporation into the substrate.

Most mono-oxygenases require also an external electron donor and the reaction is formally written:

These external mono-oxygenases are therefore involved in oxygenation and oxidation reactions. Hence the reaction was termed by Mason (1957) a mixed-function oxidation. In certain mixed-function oxidases it has been possible to demonstrate the precise stoichiometry of the above reaction sequence. The electron donor in many mammalian mixed-function oxidases is NADPH, but in some cases NADH may also serve in this capacity. This account is concerned with some of the characteristics of a few mammalian mixed-function oxidases; one aspect of this subject, namely hydroxylases, has been reviewed by Hayaishi (1969).

In mammals the mixed-function oxidases are localized in certain specific organs and subcellular particles. Many of the enzymes involved in the hydroxylation of physiological substrates and enzymes that participate in the hydroxylation (detoxification) of foreign compounds are located in the liver endoplasmic reticulum (microsomes). In addition, certain selective hydroxylations are carried out by liver mitochondria.

Similarly in the steroid-hormone-producing tissues (adrenals, gonads and placenta) some mixed-function oxidases are found in the mitochondria and others occur in the microsomal fraction of these tissues. There is evidence that mixed-function oxidase activity may be present also in other tissues (cf. Mason, 1957).

The adrenal mitochondrial steroid 11β -hydroxylase system has been intensively studied. This multi-enzyme complex was resolved into three separate proteins by Omura, Sato, Cooper, Rosenthal & Estabrook (1965). These workers suggested that in certain mixed-function oxidases the electrons pass from NADPH to a flavoprotein (Fp) and hence to a non-haem-iron-containing protein (NHI) and from there to cytochrome *P*-450 before reacting with oxygen as follows:

Similarly in adrenal mitochondria the cholesterol side-chain-cleavage enzyme system was shown to involve the same electron-transport chain in the hydroxylations and cleavage of cholesterol to pregnenolone and isocaproic (4-methylpentanoic) aldehyde (Simpson & Boyd, 1967; Bryson & Sweat, 1967).

These hydroxylase enzymes occurring in the mitochondria of steroid-producing tissues are thus cytochrome *P*-450-dependent, and proof of this dependence is obtained by spectral identification of this cytochrome, inhibition of the enzyme by CO

and release of this CO inhibition by irradiation with light of 450nm.

A simplified type of electron-transfer chain from NADPH to cytochrome *P*-450 also exists in liver endoplasmic reticulum and is the enzyme system involved in the hydroxylation of physiological and foreign compounds. The evidence that has accumulated to date from inhibition, spectral and kinetic studies suggests that cytochrome *P*-450 is the 'reaction centre' of mixed-function oxidations. Thus the electrons, the substrate and the molecular oxygen appear to interact at this site.

Although considerable advances have been made in the extraction and purification of cytochrome *P*-450 this haemoprotein, which has certain lipoprotein characteristics, has proved extremely difficult to resolve into a single native enzymically active protein (Cooper, Schleyer & Rosenthal, 1968). For example, the molecular weights that have been reported for cytochrome *P*-450 range from 40 000 to 100 000. The redox potential is possibly in the range of -0.4 to 0.0 V. This cytochrome may exist in two states (Imai & Sato, 1966) that are energetically not too dissimilar; one is termed a 'high-spin' state and the other a 'low-spin' state. Methods have been developed for the quantitative determination of these different forms of cytochrome *P*-450. Thus Hildebrandt, Remmer & Estabrook (1968) demonstrated differences in the reduced complexes of the two forms of cytochrome *P*-450 with CO, and Jefcoate, Gaylor & Calabrese (1969) showed that certain amines bind to the different forms of oxidized cytochrome *P*-450 to give characteristic spectra. This haemoprotein exhibits an electron-spin-resonance signal with absorption at 1.9, 2.2 and 2.4 G (Mason, North & Vanneste, 1965). The reduced cytochrome *P*-450 exhibits a characteristic absorption band at about 450nm when reduced and exposed to CO. Various reagents such as *p*-chloromercuribenzoate, certain inorganic anions and cations can convert cytochrome *P*-450 into cytochrome *P*-420, which is enzymically inactive and the reduced complex of which with CO shows an absorption maximum at 420nm. The molecule has possibly two haem groups/molecule (Imai & Sato, 1968), and there is evidence also for the participation of thiols in the reaction complex.

Mixed-function oxidations have certain interesting and unusual enzymic properties. The hydroxylation reaction is virtually irreversible and this gives these hydroxylase enzymes considerable significance as control steps in metabolism. The hydroxylation reaction is 'energetically expensive' because 1mol of NADPH is consumed/mol of substrate hydroxylated. Thus this biological hydroxylation involves the coupling of two spontaneous reactions:

and

The liver microsomal cholesterol 7 α -hydroxylase has been found to have a K_m for O₂ about 20 μ M and a K_m for NADPH about 300 μ M. The activation energy of this hydroxylase has been shown to be 22 $\pm$ 4 kcal/mol, which is of the same order as those of certain other sterol hydroxylases determined in this laboratory (J. I. Mason & G. S. Boyd, unpublished work). The cholesterol 7 α -hydroxylase system requires the presence of certain thiols, such as β -mercaptoethylamine, for optimum activity. In the absence of these specific thiols an 'autoxidative attack' occurs on the substrate, somewhat similar to a photosensitized reaction. This moderating effect of β -mercaptoethylamine, which is known to be efficient in scavenging radicals as a radiobiological protecting agent, suggests that thiols or thiyls may play an important part in the generation and steering of the reaction complex.

Until we have a highly purified sample of cytochrome P-450 and possibly also developments in physical methods for the investigations of these complex structures our concepts of the nature of the oxygen-attacking species in hydroxylation reactions will be largely conjecture. The oxygen molecule has to accept two electrons in the 'activation' process, and as this process is likely to occur stepwise it seems that O₂⁻ will be an intermediate (cf. Bray, 1970). It has been suggested that biological hydroxylation reactions have much in common with oxene ($\cdot\ddot{O}\cdot$) hydroxylations (Ullrich & Staudinger, 1966, 1969). On the above evidence it is at least possible to speculate that the second electron could be donated to the oxygen via a thiol, either as part of the cyto-

chrome P-450 molecule or from an external source. The absence of certain specific thiols may be the reason for certain biological hydroxylation reactions yielding aberrant products (cf. Scholan & Boyd, 1968).

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COMMUNICATIONS

The Subunits of Methionyl-Transfer-Ribonucleic Acid Synthetase

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The methionyl-tRNA synthetase of *Escherichia coli* has a molecular weight of 180 000 in its native form (Heinrikson & Hartley, 1967) and consists of four subunits (Lemoine, Waller & van Rapenbusch, 1968). Enzymically active monomers and dimers have been prepared (Cassio & Waller, 1968; Bruton & Hartley, 1968), but it has not been shown whether the subunits are identical, nor has the nature of the interconversion of the tetramer into dimer or monomer been established.

We have purified methionyl-tRNA synthetase in its dimeric form as described previously (Bruton &

Hartley, 1968) and in the tetrameric form by omitting the autolytic stage in the purification procedure. Both preparations were homogeneous as judged by ultracentrifugation and polyacrylamide-gel disc electrophoresis. Sedimentation-equilibrium studies showed that the molecular weights of the native and maleylated tetramer were 179 000 and 45 000 respectively.

[¹⁴C]Maleyl-protein was prepared from both the preparations and digested with thermolysin. The digests were acidified and applied to Dowex 50 columns equilibrated with 0.1M-acetic acid. The columns were eluted with 0.1M-acetic acid and the breakthrough tubes pooled. High-voltage paper ionophoresis at pH 6.5 revealed that these fractions contained three radioactive components, which for the dimeric form were identified as maleic acid, maleamic acid and a maleyl-peptide. From the tetramer three maleyl-peptides were found, but one