of xenobiotics catalysed by cytochrome P-450, but it differs from that mechanism in that
the haem substrate itself, not cytochrome P-450, acts as terminal oxidase. Although
several aspects of this postulated mechanism, including the nature of the haem-binding
protein(s), its apparent substrate inducibility and the identity of the hydroxylating
oxygen species, require further elucidation, the model accounts for most of the available
experimental evidence derived from studies in vitro. Moreover, a strong, albeit indirect,
case can be made for a physiological role of haem oxygenase in the conversion of haem
and haemoproteins into bile pigment in vivo.

The following comprehensive reviews have been published: Lemberg (1935); Lathe
(1972); Jackson (1974); Bissell (1975); Schmid & McDonagh (1975); O’Carra (1976).

Bissell, D. M. (1975) Gastroenterology 69, 519–538
O’Carra, P. (1976) in Porphyrins and Metalloporphyrins (Smith, K. M., ed.), Elsevier, Amsterdam,
in the press

18O Studies of Haem Catabolism

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A notable feature of studies of haem catabolism has been the variety of experimental
systems used. These have ranged from living animals through subcellular fractions and
partially purified protein preparations to chemical model systems [for reviews see
Lathe (1972), Jackson (1974) and O’Carra (1975)]. Although these various systems have
been investigated with the primary aim of evaluating the mechanism of haem cleavage
in vivo, there have been major differences in such factors as nature of the haem substrate
and nature of the products formed. It has therefore not yet been possible to deduce with
certainty whether all haem degradations proceed by the same mechanism or whether
the influence of specific enzymes ensures a different mechanism in vivo from that operat-
ing in chemical systems. As O’Carra (1975) has pointed out, however, a number of
factors, particularly the loss of the α-methene carbon atom as CO, are common to
degradations both in vivo and in vitro.

A significant reason for this uncertainty is the fact that a sizeable number of possible
intermediates can be postulated between the original haem and the product bilirubin,
some of which are shown in Scheme 1. Until recently it has been difficult to obtain
evidence for the existence or exclusion of any particular intermediate. However, recent
evidence from chemical synthesis and metabolic studies (Kondo et al., 1971) strongly
suggests that the oxyhaem (Scheme 1b) is the first degradation product in vivo, and this
also appears to be the case in chemical systems. (It is at this stage, i.e. the conversion
of haem to oxyhaem, that the specificity of cleavage is determined. The basis for the
introduction of α-specificity is not yet understood, but this problem will not be con-
sidered here.) It is also generally accepted that biliverdin (Scheme 1g) is formed in all
these haem-cleaving systems and that in biological systems the presence of the enzyme
biliverdin reductase ensures further reaction to bilirubin (Scheme 1h). However, the
molecular mechanism for the section of the pathway between the oxyhaem and biliverdin
remains poorly understood; it is to this problem that the present work is directed.
The degradation of oxyhaem to biliverdin involves loss of iron, elimination of the α-methene carbon atom bridge as CO and incorporation of two oxygen atoms, not present in the original haem, into the terminal carbonyl groups of the bile pigment. An understanding of the way in which the oxygen atoms are incorporated is virtually equivalent to an understanding of the cleavage mechanism. A notable contribution to the distinction between the various possible intermediates came when, by using 18O-labelling, Tenhunen et al. (1972) showed that in their spleen microsomal system both of the terminal oxygen atoms in bilirubin were derived from molecular O2. Therefore in this system verdohaem (Scheme 1d; formulation I) is not an intermediate. In the present work we have developed a somewhat different technique, again by using 18O-labelling as a probe to distinguish between the various possible mechanisms and intermediates. Our aim was to use this probe to obtain specific information on the intermediates involved, and to determine if the same mechanism operates in all the various haem-cleaving systems that have been studied. A particular feature of the approach is the application of the technique to living rats.

Scheme 1. Possible intermediates in haem catabolism

M represents-CH3, V represents-CH=CH and P represents-CH2CH2CO2H.
Principle of the $^{18,18}$O$_2$ double-labelling technique

Assuming that the terminal oxygen atoms of bilirubin are derived from either molecular O$_2$ or water, there are four possible ways in which incorporation can occur. These are outlined below.

- **Type A** (Double-hydrolytic mechanism)
  - Both oxygen atoms from solvent water.
- **Type B** (Hydrolytic mechanism)
  - One oxygen atom from solvent water and one from molecular O$_2$.
- **Type C** (One-molecule mechanism)
  - Both oxygen atoms from the same molecule of molecular O$_2$.
- **Type D** (Two-molecule mechanism)
  - Both oxygen atoms from molecular O$_2$, but from different O$_2$ molecules.

Type A, involving a double hydrolytic step, but no oxidation, would be most unlikely to occur, but is included for completeness. Type B, involving incorporation of one oxygen atom from molecular O$_2$ and the other from water, is compatible with the formation of verdohaem (Scheme 1d; formulation 1) as an intermediate. In view of the results of Tenhunen et al. (1972) this scheme is ruled out for the spleen microsomal system, but is a possibility in the chemical systems or (less likely) the whole animal system. Types C and D, where molecular O$_2$ supplies both atoms, might be regarded as the most likely to occur. From the results of Tenhunen et al. (1972), it is known with certainty that either Type C or Type D applies to the microsomal haem oxygenase system. These latter schemes differ in that in Type C both atoms are derived from the same O$_2$ molecules whereas in Type D they are derived from different O$_2$ molecules. These four reaction types may be experimentally distinguished by the use of $^{18}$O-labelling in molecular O$_2$. However, the basis of the distinction lies in the fact that the molecular O$_2$ is doubly labelled, i.e. it contains mostly $^{16,16}$O$_2$, some $^{18,18}$O$_2$, but no mixed molecules of $^{18,16}$O$_2$. This is achieved experimentally by dilution of 99.9% $^{18,18}$O$_2$ by unlabelled $^{16,16}$O$_2$. No exchange (scrambling) occurs under the conditions of the experiments. Enrichments of the order of 10–50 atom % $^{18}$O are used. In a typical experiment with 20 atom % $^{18}$O, the composition of the molecular O$_2$ would therefore be: $^{15,15}$O$_2$, 80%; $^{18,18}$O$_2$, 20%; $^{18,16}$O$_2$, 0%. The mass spectrum of bilirubin shows a strong molecular ion at $m/e$ 584. After making allowance for the natural abundancies of $^{13}$C, $^2$H and $^{15}$N, residual peaks at $m/e$ 586 and $m/e$ 588 correspond to incorporation of one or two $^{18}$O atoms respectively. A simple statistical approach shows that the four reaction types A, B, C and D lead to different predictions of $^{18}$O incorporation into bilirubin. This is illustrated in Table 1 for 20% enrichment. Notably, only the two-molecule mechanism predicts incorporation at both $m/e$ 586 and $m/e$ 588.

Experiments with living rats

Living male Wistar rats were used to study the $^{18}$O incorporation in vivo. The common bile duct of each rat was cannulated before it was placed in a specially designed apparatus that could be isolated from the atmosphere, and into which labelled O$_2$ could be introduced. Bile was collected and bilirubin isolated by the method of Ostrow et al. (1961). Some experiments were carried out after intravenous injection of haemoglobin to boost bilirubin output, whereas in others the endogenous bilirubin was sufficient. Experimental conditions were such that after commencing the admission of labelled O$_2$ into the apparatus the $^{18}$O enrichment of the atmosphere experienced by the rat rapidly increased to a constant value. This was maintained for 2–3h after which it decreased to zero. The results of two of these experiments are shown in Table 1, along with the predicted results for the various reaction types at appropriate enrichments. It is clear that the results are both qualitatively and quantitatively in good agreement with a two-molecule mechanism.
Table 1. Predicted and observed $^{18}$O-labelling patterns for various mechanisms

The following experimental systems were used: (1) a rat to which rat haemoglobin was administered immediately after cannulation; (2) a rat in which bile from endogenous haem breakdown was collected (i.e. no haem administration); (3) the system of Tenhunen et al. (1972); (4) myoglobin coupled oxidation. The predictions for a given enrichment are self-evident except for Type D. An example of the calculation in this case is given below. If the fraction of total oxygen as $^{18}$O is 20%, then the probability of the incorporation of two $^{18}$O atoms (to give a peak at m/e 588) = $0.2 \times 0.2 = 0.04$, the probability of the incorporation of two $^{16}$O atoms (to give a peak at m/e 584) = $0.8 \times 0.8 = 0.64$ and the probability of the incorporation of one $^{18}$O atom and one $^{16}$O atom (to give a peak at m/e 586) = $(0.2 \times 0.8) + (0.8 \times 0.2) = 0.32$.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>$^{18}$O enrichment (%)</th>
<th>m/e</th>
<th>Type...</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Rat</td>
<td>15</td>
<td>584</td>
<td>100</td>
<td>85</td>
<td>85</td>
<td>72.2</td>
<td>70.3</td>
<td></td>
</tr>
<tr>
<td>(1) Rat</td>
<td>20</td>
<td>584</td>
<td>100</td>
<td>80</td>
<td>80</td>
<td>64.0</td>
<td>65.4</td>
<td></td>
</tr>
<tr>
<td>(3) Spleen microsomes</td>
<td>30</td>
<td>584</td>
<td>100</td>
<td>70</td>
<td>70</td>
<td>49.0</td>
<td>56.2</td>
<td></td>
</tr>
<tr>
<td>(4) Coupled oxidation</td>
<td>48</td>
<td>584</td>
<td>100</td>
<td>52</td>
<td>52</td>
<td>27.0</td>
<td>70.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>586</td>
<td>0</td>
<td>48</td>
<td>0</td>
<td>49.9</td>
<td>17.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>588</td>
<td>0</td>
<td>0</td>
<td>48</td>
<td>23.0</td>
<td>9.9</td>
<td></td>
</tr>
</tbody>
</table>

Experiments with microsomal haem oxygenase

Similar experiments were carried out with the microsomal haem oxygenase system of Tenhunen et al. (1972). Results from one such experiment are shown in Table 1. The results clearly show that both terminal oxygen atoms are derived from molecular O$_2$, in agreement with the work of Tenhunen et al. (1972) referred to above. However, the results further show that in this system also a two-molecule mechanism is in operation. Also in agreement with the results of Tenhunen et al. (1972) was our observation of $^{18}$O label in the CO produced, manifested by a significant peak in the mass spectrum at m/e 30. However, accurate measurements revealed that this peak was some 10 times higher than that predicted on stoichiometric grounds. Indeed, the predicted amount of CO is so small as to be at the limit of detection. The explanation of this effect is not apparent.

A number of mass-spectral measurements were made on the O$_2$ atmosphere during these experiments to determine if any oxygen scrambling had occurred, i.e. the production of $^{18}{}_{16}$O$_2$ by an exchange between $^{18}{}_{16}$O$_2$ and $^{16}_{16}$O$_2$, promoted by the spleen microsomes. However, no trace of such scrambling was observed.

Coupled oxidations in chemical systems

Coupled oxidations with ascorbate, whether using myoglobin, haemoglobin or pyridine haemochrome as haem source, result in products readily hydrolysable to biliverdin (O’Carra, 1975). Bilirubin is not produced since these systems lack biliverdin reductase. Although the mass spectrum of biliverdin dimethyl ester has been well documented (Jackson & Kenner, 1968), we have been unable to obtain this spectrum from the products of coupled oxidation. Therefore it was decided to convert the coupled oxidation product directly into bilirubin, the mass spectrum of which could be readily
determined. For meaningful results it was necessary to carry out this reduction under conditions where neither bilirubin isomerization nor oxygen exchange between solvent and bilirubin terminal oxygen atoms could occur. Chemical reduction was avoided because of the danger of isomerization. Biliverdin reductase preparations were used in initial experiments, but proved inefficient owing to the very low $K_m$ value. Bilirubin isomerization was also a possible problem in these experiments. The most appropriate method proved to be the use of a living rat to convert intravenously injected biliverdin into bilirubin, which could be collected from the bile. Since the bilirubin was conjugated in situ, the isomerization problem was thereby overcome. A disadvantage of this method is the dilution of the bilirubin from administered biliverdin by bilirubin from endogenous haem catabolism. This factor must be considered in any quantitative treatment of the results.

Myoglobin was used as haem source since it is degraded with complete $\alpha$-specificity (O’Carra, 1975) ensuring maximum yield of bilirubin. Results of a typical experiment are shown in Table I. Clearly the $^{18}O$ enrichment in the gas phase is not directly relatable to the incorporation values, because of the dilution with endogenous bilirubin. Nevertheless, a hydrolytic mechanism can be excluded because of the significant incorporation observed at $m/e$ 588. To distinguish between a one- and two-molecule mechanism the precise dilution is required. Approximate values can be obtained by assuming a reasonable value for the endogenous bilirubin output, but this is variable and not sufficiently accurate. Also endogenous haem catabolism may itself be influenced by biliverdin administration. This problem is potentially capable of solution by use of $^{14}C$-labelled haem in the $^{18,18}O$ experiment. The $[^{14}C,^{18}O]$biliverdin produced may then be injected into the rat and the resultant radiolabelled bilirubin distinguished from the endogenous bilirubin. In this way the bilirubin dilution can be accurately measured.

Mechanism of haem degradation

Verdohaem (Scheme 1d, formulation I) is not formed in any of the haem-degrading systems. Although this result was anticipated by comparison with the data of Tenhunen et al. (1972) for the biological systems, it was not necessarily expected for coupled oxidation reactions. As O’Carra (1975) has pointed out, the alternative formulation for verdohaem (Scheme 1e; formulation I) is not necessarily excluded by the lack of a solvent oxygen atom in the product bile pigment. However, the observation of a two-molecule mechanism is hardly compatible with the presence of this structure in the degradative pathway. The most important (and surprising) conclusion of the present work is the apparent exclusion of the dioxygen-bridged species (Scheme 1c) as a possible intermediate, since such a structure would require either a one-molecule mechanism or possibly a hydrolytic mechanism.

A two-molecule mechanism suggests independent oxidation events occurring at the carbon atoms adjacent to the $\alpha$-methene carbon atom (C$_\alpha$) with consequent rupture of the C—C$_\alpha$ bonds. Therefore assuming that these reactions occur sequentially it is possible that intermediates which retain the $\alpha$-carbon atom, such as the one shown in Scheme 1(f), may be present.

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