The Mechanism of Coupled Oxidation of Octaethylhaem to Octaethylbiliverdin

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The normal pathway for haem catabolism involves the oxidation of the macrocyclic ring system at a methene-bridge carbon atom to produce biliverdin (Scheme 1b). In mammals, biliverdin does not accumulate, but is enzymically reduced to the yellow-orange pigment bilirubin (Scheme 1c), which is excreted in the bile (Schmid, 1977). The first step in this process is probably hydroxylation at the appropriate methene bridge to produce the oxyhaem intermediate (iron-oxophorin). Tenhunen et al. (1969) have shown that haem breakdown is catalysed by the microsomal enzyme haem oxygenase. Although the stage at which haem oxygenase acts is not known with certainty, it seems likely that the initial hydroxylation step is enzymic and the subsequent reactions to produce biliverdin occur spontaneously. Haem degradation may also be studied in non-biological model systems. For example, treatment of haem in aqueous pyridine with ascorbate followed by hydrolysis readily yields biliverdin. This so-called coupled-oxidation reaction occurs also with iron complexes of synthetic porphyrins. Thus octaethylhaem (Scheme 1d) yields octaethylbiliverdin (Scheme 1e) although in this case the reaction is usually carried out in systems containing very little water.

The precise nature of the ring-cleavage steps are not well understood, but they involve loss of the Fe atom, elimination of the methene carbon atom as CO and incorporation of two oxygen atoms not present in the original haem. Information on this reaction may be obtained by $^{18}$O labelling of the molecular oxygen used in the oxidation.

Scheme 1. Haem oxidation to bile pigment

Key to substituents: M, -CH$_3$; E, -CH$_2$CH$_3$; V, -CH=CH$_2$; P, -CH$_2$CH$_3$CO$_2$H.
Table 1. $^{18}O$ incorporation into octaethylbiliverdin

The data refer to coupled oxidation of octaethylbiliverdin with sodium ascorbate in pyridine (3%, v/v, of water added to aid solution of ascorbate). A specially constructed apparatus permitted the introduction of $^{18,18}O_2$, such that the molar percentage of $^{18}O$ was 25.9. The mass spectrum of octaethylbiliverdin shows a molecular ion at $m/e$ 554. Predictions were obtained as described by Brown & King (1975)

<table>
<thead>
<tr>
<th>Octaethylbiliverdin (mol %)</th>
<th>m/e 554</th>
<th>556</th>
<th>558</th>
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</thead>
<tbody>
<tr>
<td>Double-Hydrolytic</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Hydrolytic</td>
<td>74.1</td>
<td>25.9</td>
<td>0</td>
</tr>
<tr>
<td>One-Molecule</td>
<td>74.1</td>
<td>0</td>
<td>25.9</td>
</tr>
<tr>
<td>Two-Molecule (Observed)</td>
<td>54.9</td>
<td>38.4</td>
<td>6.7</td>
</tr>
<tr>
<td></td>
<td>54.6</td>
<td>38.4</td>
<td>7.0</td>
</tr>
</tbody>
</table>

process. For haem degradation by spleen microsomal fractions in vitro, Tenhunen et al. (1972) showed that both terminal oxygen atoms of bilirubin were derived from molecular oxygen. Brown & King (1975) have developed a somewhat different technique to examine the same problem, also by using labelled molecular oxygen. Four general mechanisms may be defined. In the Double-Hydrolytic Mechanism, both terminal-lactam oxygen atoms in bile pigment are derived from solvent water, whereas in the Hydrolytic Mechanism one atom comes from water and the other from molecular oxygen. In the One-Molecule Mechanism, both atoms are derived from a single oxygen molecule, whereas in the Two-Molecule Mechanism, they originate in different oxygen molecules. These mechanisms may be distinguished by carrying out haem oxidation in the presence of molecular oxygen containing $^{18,18}O_2$ and $^{16,16}O_2$, but not $^{18,18}O_2$. Brown & King (1975) showed that living rats degraded their haem by a Two-Molecule Mechanism. The same mechanism was found when the technique was applied to the haem oxygenase system and to the degradation of myoglobin in vitro with ascorbate (Brown & King, 1976). The observation of a Two-Molecule Mechanism rules out oxygen insertion by hydrolytic reactions during ring cleavage, and also renders unlikely a dioxygen-bridged intermediate (Jackson, 1974).

Chemical degradation of haem compounds in solvent systems containing little water would not necessarily be expected to proceed via the same mechanism as the biological oxidations. In the present work we report results obtained by the application of the $^{18}O$ technique to coupled oxidation of octaethylhaem to octaethylbiliverdin. Octaethylporphyrin was kindly supplied by Professor K. M. Smith (Department of Chemistry, University of Liverpool). Iron insertion was carried out by standard procedures (Bonnett & Dimsdale, 1972). Mass spectra of $^{18}O$-labelled octaethylbiliverdin were obtained on an AE1 MS9 mass spectrometer at the School of Chemistry, University of Newcastle upon Tyne. The results are shown in Table 1 along with the predictions for the various mechanisms. It is clear that there is good quantitative agreement with the predictions of the Two-Molecule Mechanism, and consequently there seems no doubt that the terminal oxygen atoms of octaethylbiliverdin are derived from two different oxygen molecules.

These findings strongly suggest that the mechanism of breakdown of this synthetic haem derivative in a largely non-aqueous system is the same as that of the natural haem derivatives in aqueous biological systems. This conclusion therefore supports the validity of extrapolation of mechanistic data from chemical model systems to biological systems and suggests that there may be a single mechanism by which the ring-cleavage oxidation of haem compounds proceeds.

We are grateful to Mr. P. Kelly for providing expertise in mass spectrometry, and to the Medical Research Council for the award of a project grant to S. B. B.
Amounts of Iron, Haem and Related Compounds in 
*Mycobacterium smegmatis* grown in Various Concentrations of Iron

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Although the mechanism of iron transport in *Mycobacterium smegmatis* is now known in broad outline (Ratledge & Marshall, 1972; Macham et al., 1977), the fate of iron within the cell and its distribution between the various iron-containing components has not been extensively studied. We have now determined the amounts of total iron and its distribution between the major compounds of the cell after growth of *M. smegmatis* N.C.I.B. 8548 with iron at either 0.05 µg/ml for iron-deficient growth or 2.0 µg/ml for iron-sufficient growth (see Ratledge & Hall, 1971).

Concentrations of metabolites (see Table 1)

When growth was limited by the availability of iron, a substantial decrease in the iron content of the cells occurred. Therefore either some of the iron in iron-sufficient cells is redundant or cells are able to reorganize their metabolism when they become iron-deficient. The minimal iron requirement was about 15-17 µg of iron/g dry wt., below which further growth could not occur. This value is appreciably lower than the 64 µg of iron/g dry wt. recorded by Winder & O'Hara (1966) for the same organism (although, admittedly, a different strain).

The proportion of iron as non-haem iron was always high, in keeping with previous results for *Mycobacterium phlei* (Kurup & Brodie, 1967), but was slightly less in older iron-deficient cultures than in iron-deficient cultures of the same age; i.e. when the effects of iron deprivation were beginning to show.

Amounts of cytochromes *a* and *b* were substantially depleted in iron-deficient cultures, although amounts of cytochrome *c* and flavoprotein(s) were not significantly altered. Somewhat similar changes have been observed in other organisms grown under conditions of iron deficiency (Light & Clegg, 1974). The disproportionate changes in some respiratory proteins probably indicate a lack of co-ordinated control over their biosynthesis or else an alteration in the pathways of electron transport under conditions of iron deficiency.

Unlike most other micro-organisms (see Light & Clegg, 1974), *M. smegmatis* showed a dramatic decrease in porphyrin under conditions of iron deficiency. Normally, in the absence of iron to produce haem from porphyrin, control over porphyrin biosynthesis becomes relaxed and high concentrations of porphyrins result. *M. smegmatis* must therefore regulate porphyrin biosynthesis not through haem, but through a more subtle mechanism, and clearly this is of advantage to the cells since it prevents needless diversion of carbon into unwanted compounds. We were unfortunately unable to detect δ-aminolaevulinate acid synthase in cell-free extracts, which is probably the controlling enzyme of porphyrin biosynthesis. Aminolaevulinate dehydratase (EC 4.2.1.24), the second enzyme in the pathway, however, was unaffected by the iron status of the cells.