An ^{18}O Double-Labeling Study of Haemoglobin Catabolism in the Rat

By STANLEY B. BROWN and RODERICK F. G. J. KING
Department of Biochemistry, University of Leeds, Leeds LS2 9LS, U.K.

(Received 25 June 1975)

The degradation of haemoglobin to bilirubin in the rat was investigated by ^{18}O labelling of the molecular oxygen consumed by the animal. The oxygen atoms incorporated into bilirubin were derived from two different oxygen molecules. Implications of this finding for the mechanism of haem catabolism *in vivo* are discussed; both verdohaem and a dioxygen-bridged compound appear to be excluded as intermediates.

The detailed nature of the catabolism of haem to bilirubin as it occurs in mammals is not yet understood. Intermediates such as oxyferrihaem, verdohaem and dioxygen-bridged species (Fig. 1) have been postulated, but, with the exception of studies in model systems (see, e.g., Besecke & Fuhrhop, 1974), direct evidence for their involvement is lacking (see Jackson, 1974, for review). It is well established that molecular oxygen is involved in the degradation of haemoglobin (Lathé, 1972; Jackson, 1974). During this process, two oxygen atoms not present in the original haem are incorporated into bilirubin at the terminal pyrrole rings (Fig. 1). Determination of the origin of these atoms and the way in which incorporation occurs is clearly important in assessing the mechanism of haem cleavage and the possible role of various intermediates. There are three possible ways in which oxygen incorporation into bile pigment may occur. The first (designated the 'Hydrolytic Mechanism') involves the incorporation of one atom from molecular oxygen and the other from solvent water, presumably by a hydrolysis step. In the second (designated the 'One-Molecule Mechanism'), both atoms are derived from the same molecule of molecular oxygen, whereas in the third (designated the 'Two-Molecule Mechanism'), again both atoms are derived from molecular oxygen, but in this case from two different oxygen molecules. In a study of haem degradation in a spleen microsomal system *in vitro*, Tenhunen *et al.* (1972) have shown, by ^{18}O labelling, that both oxygen atoms incorporated into bilirubin are derived from molecular oxygen. From their results, however, it was not possible to distinguish between the One-Molecule Mechanism and the Two-Molecule Mechanism. Intact-animal studies, have not yet been carried out.

These three possible mechanisms may be distinguished experimentally by use of an ^{18}O double-labelling technique, with the label in the molecular oxygen. The basis of the method is that the oxygen used for oxidation contains unlabelled molecules and doubly labelled molecules, but no mixed molecules. For example, at a total enrichment of 15 atoms % ^{18}O , 85% of the oxygen molecules would be $^{16,16}\text{O}_2$, 15%

would be $^{18,18}\text{O}_2$, with a negligible amount of $^{18,16}\text{O}_2$. This composition may be achieved experimentally by dilution of 100% $^{18,18}\text{O}_2$ with naturally occurring $^{16,16}\text{O}_2$. Under normal conditions (ambient temperature, absence of catalysts), no scrambling of oxygen atoms occurs in such mixtures. Bilirubin obtained by reaction of haem with this labelled oxygen may be analysed mass-spectrometrically to determine ^{18}O incorporation. The mass spectrum of bilirubin (Jackson *et al.*, 1967) shows a molecular ion at m/e 584. After making allowances for the natural abundance of ^{13}C , ^2H and ^{15}N , residual peaks at m/e 586 and m/e 588 correspond to incorporation of one or two heavy oxygen atoms respectively. A simple statistical approach shows that the three mechanisms lead to different predictions of ^{18}O incorporation into bilirubin. This is illustrated in Table 1 for a total ^{18}O enrichment of 15 atoms %. Notably, only the Two-Molecule Mechanism predicts incorporation at both m/e 586 and m/e 588.

The results presented here refer to an ^{18}O double-labelling experiment using an intact rat. Haemoglobin was administered intravenously before exposure of the animal to ^{18}O , in order to increase the yield of bilirubin and thereby facilitate mass spectrometry.

Experimental

Materials. Oxygen gas containing 99.8 atoms % ^{18}O was obtained from Prochem, The British Oxygen Company Ltd., Deer Park Road, London SW19 3UF, U.K. Rat haemoglobin was prepared by collecting 10 ml of fresh rat blood, rapidly mixing with 0.6 ml of 7.6% (w/v) sodium citrate solution and collecting the erythrocytes by centrifugation. The cells were lysed by resuspension in 30 ml of water for 20 min, followed by addition of 2 ml of 10% (w/v) NaCl. Haemoglobin was separated from cell debris by centrifugation at 105000g for 1 h. The supernatant was made up to 40 ml with 0.1 M-phosphate buffer ($\text{Na}_2\text{HPO}_4\text{--KH}_2\text{PO}_4$), pH 7.8, and the solution deep-frozen until required. Solvents used in bilirubin extraction were

Fig. 1. Structures of some postulated intermediates in haem catabolism
 $M = CH_3$; $V = CH=CH_2$; $P = CH_2-CH_2-CO_2H$.

redistilled Laboratory-Reagent Grade, and ether used during surgical operation was Anaesthetic Grade. Other materials were Analytical-Reagent Grade. Nylon tubing for bile-duct cannulation was supplied by Portland Plastics Ltd., Hythe, Kent, U.K.

Bile collection. A male Wistar rat (325 g) was anaesthetized with ether. The common bile duct was exposed and an incision made through about half its diameter. Cannulation was effected with Type 00 nylon tubing, which was secured with a double ligature. The cannula was routed subcutaneously and exteriorized behind the left ear. Immediately after cannulation, 1 ml of the haemoglobin solution (containing 0.94 mg of haem) was injected into the tail

vein. After the rat had been allowed to recover (approx. 0.5 h) it was placed in a specially designed glass chamber which could be isolated from the atmosphere and into which labelled oxygen could be introduced. The cannula through which bile was flowing emerged from this apparatus via a rubber seal and was led to a fraction collector. CO_2 was absorbed by a soda-lime trap. To initiate the experiment, ^{18}O -labelled oxygen was introduced and, by supplementing with natural oxygen, an enrichment of 15 atoms % ^{18}O was maintained over a 2 h period.

Isolation of bilirubin. Bilirubin was isolated from the bile by the method of Ostrow *et al.* (1961). This procedure is reported to involve a very low

Table 1. Predicted and observed ^{18}O -labelling patterns for various mechanisms

The experiment used 15% enrichment in molecular oxygen. For a Hydrolytic Mechanism, 1 oxygen atom is always derived from solvent. No peak at m/e 588 is therefore possible. The mol % predicted at m/e 586 is the same as in the oxygen gas, i.e. 15%. For a One-Molecule Mechanism, the oxygen atoms incorporated are either both ^{18}O or both ^{16}O . The mol % predicted at m/e 588 is therefore the same as in the oxygen gas, i.e. 15%, and that at m/e 586 is zero. For a Two-Molecule Mechanism the mol % values predicted are derived as follows.

Fraction of total oxygen as ^{18}O = 0.15.

Probability of incorporation of two ^{18}O atoms (to give peak at m/e 588) = $0.15 \times 0.15 = 0.023$.

Probability of incorporation of two ^{16}O atoms (to give peak at m/e 584) = $0.85 \times 0.85 = 0.722$.

Probability of incorporation of one ^{18}O atom and one ^{16}O atom (to give peak at m/e 586) = $(0.15 \times 0.85) + (0.85 \times 0.15) = 0.255$.

m/e	Mol % found	Predicted (mol %)		
		Hydrolytic Mechanism	One-Molecule Mechanism	Two-Molecule Mechanism
584	70.3	85	85	72.2
586	25.5	15	Zero	25.5
588	3.4	Zero	15	2.3

extent of bilirubin isomerization (McDonagh & Assisi, 1972), and this was confirmed when t.l.c. analysis revealed only very small amounts of the $\text{III}\alpha$ and $\text{XIII}\alpha$ isomers. Such isomerization would render labelling experiments ambiguous. Various trial experiments (all using male Wistar rats) confirmed that, after haemoglobin administration, maximal bilirubin excretion occurred after about 2 h, in agreement with published data (Ostrow *et al.*, 1962). For the labelled run, the bilirubin from the bile sample collected between 1 h and 2 h after haemoglobin injection was therefore used for mass-spectral analysis.

Mass spectrometry. Mass spectra of bilirubin samples were run on an A.E.I. M.S.9 mass spectrometer. Spectra of unlabelled samples showed good agreement with published spectra.

Results and discussion

Results obtained from the mass spectrum of bilirubin collected between 1 and 2 h after haemoglobin administration are shown in Table 1. It is clear that incorporation of label occurred both at m/e 586 and m/e 588, consistent with a Two-Molecule Mechanism. Further, the results show good quantitative agreement with this mechanism. It appears on this basis therefore that both oxygen atoms in bili-

rubin are derived from molecular oxygen and that they come from two different oxygen molecules.

This finding suggests that current ideas regarding the mechanism of haem cleavage and the intermediates involved may require revision. The oxo-bridged species verdohaem, postulated by Foulkes *et al.* (1951), appears to be excluded as an intermediate on the basis of these results, since its conversion into bilirubin (via biliverdin) would require a hydrolytic step and insertion of an oxygen atom from solvent water. Similarly the dioxygen-bridged intermediate (Jackson, 1974) is unlikely to be involved, since its conversion into bilirubin would involve either a Hydrolytic Mechanism or a One-Molecule Mechanism. Our results are surprising in view of the evidence presented by Jackson (1974) in favour of the dioxygen-bridged species, largely by analogy with organic model systems. However, it appears that, even in these latter systems, direct proof of a dioxygen bridge (e.g. by the double-labelling technique) is lacking. The results presented here suggest that haem cleavage occurs by three oxidation steps of a mixed-function oxygenase type, at the α -methene carbon atom and its two nearest-neighbour carbon atoms. These reactions appear to be independent of one another so far as molecular oxygen is concerned. It should be emphasized, however, that these findings apply to haemoglobin catabolism in a living animal. The mechanism of haem cleavage *in vitro* would not necessarily be expected to be identical.

We gratefully acknowledge the technical assistance of Mrs. Margaret Shillcock and the expertise in mass spectrometry provided by Mr. P. Kelly, School of Chemistry, University of Newcastle upon Tyne, Newcastle upon Tyne NE1 7RU. We are also indebted to Professor G. H. Lathe for much helpful advice and criticism. This work was supported by a Project Grant awarded by the M.R.C.

- Besecke, S. & Fuhrhop, J. (1974) *Angew. Chem. Int. Ed.* **13**, 150–151
- Foulkes, E. C., Lemberg, R. & Purdom, P. (1951) *Proc. R. Soc. London Ser. B* **138**, 386–402
- Jackson, A. H. (1974) in *Iron in Biochemistry and Medicine* (Jacobs, A. & Worwood, M., eds.), pp. 145–182, Academic Press, London and New York
- Jackson, A. H., Kenner, G. W., Budzikiewicz, H., Kjerass, C. & Wilson, J. M. (1967) *Tetrahedron* **23**, 603–632
- Lathe, G. H. (1972) *Essays Biochem.* **8**, 107–121
- McDonagh, A. F. & Assisi, F. (1972) *Biochem. J.* **129**, 797–800
- Ostrow, J. D., Hammaker, L. & Schmid, R. (1961) *J. Clin. Invest.* **40**, 1442–1452
- Ostrow, J. D., Jandl, J. H. & Schmid, R. (1962) *J. Clin. Invest.* **41**, 1628–1637
- Tenhunen, R., Marver, H., Pinstone, N. R., Trager, W. F., Cooper, D. Y. & Schmidt, R. (1972) *Biochemistry* **11**, 1716–1720