A study of the mechanism of quercetin oxygenation by ^{18}O labelling

A comparison of the mechanism with that of haem degradation

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The mechanism of quercetin oxygenation, which is formally analogous to haem degradation, was studied by using ^{18}O labelling. In both the enzymic oxygenation (catalysed by quercetinase) and the non-enzymic reaction (base-catalysed), both oxygen atoms incorporated into product were derived from a single oxygen molecule. Quercetin oxygenation therefore occurs by a classical dioxygenase mechanism and is not an appropriate model for study of the mechanism of haem catabolism.

The flavonol quercetin (Fig. 1, *a*) occurs as a pigment of higher plants and fungi (Harborne, 1976), where it is present as the 3-*O*-glycoside rutin (Fig. 1, *a*). Quercetin may be oxidatively decarboxylated to the corresponding depside, 2-(3',4'-dihydroxybenzoyloxy)-4,6-dihydroxybenzoic acid (Fig. 1, *b*), during which reaction the C-3 atom is lost as CO (Simpson *et al.*, 1960) and two atoms of oxygen (asterisked in Fig. 1) are introduced at C-2 and C-4 (Westlake *et al.*, 1959). The biological oxygenation of quercetin is catalysed by the enzyme quercetinase (Simpson *et al.*, 1963), but the reaction can also occur non-enzymically by treatment of quercetin with strong base in an oxygenated non-aqueous solvent (Nishinaga & Matsuura, 1973).

Quercetinase is conveniently prepared from cultures of *Aspergillus flavus* grown in medium containing rutin, when the enzyme is excreted into the suspending medium (Simpson *et al.*, 1963). Under these conditions, two other enzymes, rutinase (Hay *et al.*, 1961) and an esterase (Child *et al.*, 1963), are excreted into the medium. Rutinase hydrolyses rutin to quercetin and the disaccharide rutinose, and the esterase catalyses hydrolysis of the depside (Fig. 1, *b*) to produce phloroglucinol carboxylic acid (Fig. 1, *c*) and protocathechuic acid (Fig. 1, *d*). A combination of all three enzymes results in the successive degradation of rutin to phloroglucinol carboxylic acid and protocathechuic acid. The purification of quercetinase has been described by Oka *et al.* (1971).

Catabolism of the haem (Fig. 2, *a*) of haemoglobin and other haemoproteins is initiated by the hydroxylation of a methine-bridge carbon atom to yield hydroxyhaem (Fig. 2, *b*; for reviews, see Jackson, 1974; Schmid & McDonagh, 1975; Brown & Troxler, 1982). The macrocyclic ring-cleavage step

that subsequently occurs to produce biliverdin (Fig. 2, *c*) is strikingly similar to that occurring during quercetin oxygenation. In both cases a carbon atom is eliminated as CO (an unusual biological reaction) and two oxygen atoms become attached to the carbon atoms originally adjacent to that eliminated. It was originally thought that this step in haem catabolism involved attack by a single molecule of molecular oxygen, in which case the lactam oxygen atoms of biliverdin would both be derived from the same oxygen molecule (One-Molecule Mechanism). However, ^{18}O -labelling experiments in a variety of haem-degrading systems (e.g., Brown & King, 1978; King & Brown, 1978; Brown *et al.*, 1980) have shown that these atoms are derived from two different oxygen molecules (Two-Molecule Mechanism). No other dioxygenase reactions with this mechanism have yet been found. The molecular pathway by which the Two-Molecule Mechanism is achieved has not yet been elucidated, due in part to the difficulty in isolating the hydroxyhaem intermediate (Fig. 2, *b*). Since the oxygenation of quercetin and the macrocyclic-ring-cleavage step in haem degradation are so similar, it seems possible that the former might also occur via a Two-Molecule Mechanism. An investigation of the mechanism of quercetin oxygenation is potentially important therefore, both in terms of a general interest in the mechanism of action of dioxygenases and also, more specifically, as a possible model for the macrocyclic-ring-cleavage step in haem catabolism. A previous study by Krishnamurthy & Simpson (1970) showed that both oxygen atoms incorporated into the depside produced by the action of quercetinase on quercetin were derived from molecular oxygen, but it was not possible to distinguish between a One-Molecule Mechanism and a Two-Molecule Mech-

Fig. 1. Pathway of quercetin oxygenation

Fig. 2. Pathway of haem catabolism
 M = $-\text{CH}_3$; P = $-\text{CH}_2\text{CH}_2\text{CO}_2\text{H}$; V = $-\text{CH}=\text{CH}_2$.

anism. A preliminary study (Rajananda & Brown, 1981) suggested that the non-enzymic reaction occurred by a One-Molecule Mechanism. In the present work we report a study of both enzymic and non-enzymic oxygenation of quercetin by ^{18}O labelling.

Experimental

Materials and measurements

Oxygen gas containing 99.0 atom% ^{18}O was obtained from Prochem, The British Oxygen Co., London SW19 3UF, U.K. Rutin, quercetin, Mes (4-

morpholine-ethanesulphonic acid) and *N*-methyl-*N*-nitroso-*p*-toluenesulphonamide were obtained from the Sigma Chemical Co., St. Louis, MO, U.S.A. Potassium *t*-butoxide was obtained from Fluka A.G., Buchs, Switzerland, via Fluorochem, Glossop SK13 9NU, U.K. Buffer components, solvents and other reagents were obtained from BDH Chemicals, Poole, Dorset, U.K., and were of A.R. grade. Solvents were redistilled before use. Sephadex G-50 and G-100 were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Preparative t.l.c. was performed on 20 cm × 20 cm × 0.1 cm plates using silica gel GF₂₅₄ (E. Merck, Darmstadt, Germany) and bands were visualized in u.v. light.

A solution of diazomethane in diethyl ether was prepared from *N*-methyl-*N*-nitroso-*p*-toluenesulphonamide as described by Vogel (1974).

Electronic absorption spectra were recorded on a Pye-Unicam SP.1800 spectrophotometer. Mass spectra of solid depside samples were obtained on an AEI MS9-50 mass spectrometer. Mass spectra of gaseous samples were obtained on an AEI MS10 mass spectrometer.

Preparation and partial purification of quercetinase

Aspergillus flavus was grown at 30°C in 7 × 1 litre-batch cultures on medium containing rutin as sole carbon source, by the method described by Oka *et al.* (1971). From time to time, samples of the culture were taken, mycelium was removed by filtration and the cell-free filtrate was assayed for quercetinase activity as described by Oka *et al.* (1971). This method depends on the rate of breakdown of quercetin (decrease in A_{367}) by a known volume of the enzyme solution (0.1 M-Mes buffer, pH 6.0). One unit of enzyme activity represents the amount of enzyme required to cleave one micromole of quercetin per hour under standard conditions. Maximum activity (60 000 units) was obtained after about 5 days incubation. Cells from the seven separate cultures were removed by filtration and the cell-free filtrates were combined. The volume of the combined filtrate was reduced to about 100 ml by freeze-drying.

For ¹⁸O-labelling studies, sufficient product is required to permit formation of derivatives and quantitative mass spectrometry. A high total activity is therefore desirable, rather than a highly purified preparation with consequent reduction in total activity. For this reason, the quercetinase was only partly purified, broadly following the initial steps of the method described by Oka *et al.* (1971). However, it was essential to obtain a preparation free from esterase activity, since further reaction of the depside to phloroglucinol carboxylic acid and protocatechuic acid (Fig. 1), with consequent separation of the newly incorporated oxygen atoms, would

render distinction between the One-Molecule Mechanism and Two-Molecule Mechanism impossible.

The concentrated filtrate was divided into four portions and each was applied, in turn, to a Sephadex G-50 column (2 cm × 40 cm) and eluted with water. The fractions of the eluates containing quercetinase activity from the four runs were pooled and the combined solution was concentrated to 100 ml (total quercetinase activity, 20 000 units). At this stage, the preparation contained significant esterase activity (as determined by the detection of phloroglucinol carboxylate and protocatechuate by paper chromatography, after reaction with quercetin) and further purification was necessary. The concentrate was again divided into four portions and each was applied to a Sephadex G-100 column (2 cm × 40 cm) and eluted with potassium phosphate buffer (0.03 M, pH 7.0). The eluates containing quercetinase activity (total, 16 000 units) were again pooled and tested for esterase activity. At this stage, there was no significant esterase activity. Further purification with consequent loss in activity was therefore considered undesirable. The quercetinase preparation was stored at 4°C, under which condition it was stable for several weeks.

Enzymic oxygenation of quercetin

Distinction between the One-Molecule Mechanism and the Two-Molecule Mechanism requires mass-spectral analysis of the depside (or an appropriate derivative) produced by oxygenation of quercetin by a mixture of ¹⁸,¹⁸O₂ and ¹⁶,¹⁶O₂. Such a mixture was readily prepared on a specially constructed vacuum line by mixing pure (99%) ¹⁸,¹⁸O₂ with unlabelled oxygen (Brown & King, 1978). The reaction was carried out in a 25 ml round-bottomed flask fitted with a side-arm and high-vacuum tap.

Quercetin (50 mg) was placed in the side-arm and a solution containing the enzyme (60 units) in 2 ml of potassium phosphate buffer (0.03 M, pH 7.0) and dimethyl sulphoxide (0.2 ml) was placed in the flask. The reaction vessel was then attached to the vacuum line, which incorporated a manometer (to check for leaks), attachments to a vacuum pump, a gas sampling vessel and a glass reservoir containing a pre-determined mixture of ¹⁸,¹⁸O₂ and ¹⁶,¹⁶O₂.

The contents of the reaction vessel were degassed on the vacuum line by freezing and thawing. The mixture of ¹⁶,¹⁶O₂ and ¹⁸,¹⁸O₂ was introduced into the reaction flask containing the unmixed, degassed reactants and a small sample of the gaseous mixture was removed into the gas-sampling vessel for subsequent analysis by mass spectrometry. This analysis and that performed on a gas sample taken after the reaction served to confirm the pre-determined composition of the gas mixture and also to indicate the absence of leakage during the various manipulations. The high-vacuum tap on the reaction flask was

closed and the vessel removed from the vacuum line. The reagents were mixed by inverting the flask and the mixture was incubated with stirring at 37°C for 2 h. After this time, the reaction was stopped by cooling in ice and a further gas sample was removed for mass-spectral analysis. The reaction vessel was opened to the atmosphere and the reaction mixture diluted with 20 ml of ice-cold 0.04 M-HCl. The cold acidified mixture was extracted three times with ice-cold diethyl ether and dried over Na_2SO_4 .

The diethyl ether extract was separated by preparative paper chromatography [Whatman 3MM; acetic acid/water (3:7, v/v) as solvent; descending]. The depside (R_F 0.50) was detectable by short-wave u.v. light (blue fluorescence) and by its positive colour-reaction (orange) to diazotized sulphanic acid. The depside was eluted from the paper chromatogram with methanol.

Non-enzymic (base-catalysed) oxygenation of quercetin

A solution of quercetin (70 mg) of dimethylformamide (0.1 ml) was introduced into the side-arm of the 25 ml reaction flask, containing potassium *t*-butoxide (35 mg), suspended in dimethylformamide (2 ml). The contents of the flask were degassed by freezing and thawing and an $^{18,18}\text{O}_2/^{16,16}\text{O}_2$ mixture of known composition was introduced into the system. A sample of this gaseous mixture was removed for mass-spectral analysis.

The reaction flask was sealed, the reactants mixed by inversion and the reaction mixture incubated at 37°C for 3 h. The reaction was stopped by cooling in ice and a gas sample was removed for analysis. The depside was isolated from the reaction mixture by using paper chromatography as described above.

Methylation of the depside

The depside itself was not sufficiently volatile for mass-spectral analysis and an appropriate derivative was required. This was obtained by complete methylation (of the carboxylic acid group and the four hydroxy groups; see Fig. 1) with diazomethane in diethyl ether, to yield the depside tetramethyl ether methyl ester (mol.wt. 376). The methylated product was purified by preparative t.l.c. [silica gel GF₂₅₄; ethyl acetate/chloroform (1:9, v/v) as solvent system; R_F 0.53] and was eluted from the silica with methanol/acetone (1:9, v/v). Rotary evaporation yielded a white solid that was retained for mass-spectral analysis.

Results and discussion

Determination of mechanism

Provided that a mixture of $^{18,18}\text{O}_2$ and $^{16,16}\text{O}_2$ is used in the oxygenation, the labelling of the product permits identification of the mechanism. The One-

Molecule Mechanism predicts incorporation of label only at m/z 380. No incorporation at m/z 378 is possible since a labelled-oxygen molecule must add 4 mass units to the molecular ion. For this mechanism, the theoretical incorporation at m/z 380 is equal to the ^{18}O enrichment used in the gas phase (see Table 1). The Two-Molecule Mechanism, however, predicts incorporation both at m/z 378 and at m/z 380. Since two independent oxygen molecules each donate an oxygen atom, one may be labelled and the other unlabelled (m/z 378) or both may be labelled (m/z 380). The quantitative incorporation expected for this mechanism may be predicted from the known ^{18}O enrichment in the gas phase (Brown & King, 1978) as shown in Table 1. The two mechanisms are therefore readily distinguished by quantitative measurement of the ^{18}O incorporation into the depside derivative at m/z 378 and m/z 380.

^{18}O incorporation from non-enzymic oxygenation of quercetin

The methylated depside gave a good mass spectrum with a clear molecular ion at m/z 376. A partial mass spectrum for material prepared non-enzymically under an unlabelled oxygen atmosphere is shown in Fig. 3(a). This spectrum appeared to be identical with that of the methylated depside prepared from quercetin enzymically. The peaks appearing at m/z 377 and m/z 378 are due almost entirely to the naturally abundant isotope ^{13}C . Clearly a correction must be made for these naturally abundant isotope peaks in the interpretation of mass spectra from ^{18}O -labelling experiments. This correction has previously been discussed in detail (Brown & King, 1978). After allowance for such peaks, residual peaks at m/z 378 and m/z 380 correspond to incorporation of one or two ^{18}O atoms respectively. Fig. 3(b) shows the partial mass spectrum of methylated depside obtained by the non-enzymic oxygenation of quercetin under an atmosphere containing 99% $^{18,18}\text{O}_2$. Although such an experiment could not distinguish between the One-Molecule Mechanism and the Two-Molecule Mechanism, it is an important control to determine whether any oxygen exchange has occurred (for example, with solvent) either during quercetin oxygenation or during the formation of derivatives. Clearly, the mass spectrum shown in Fig. 3(b) reveals almost complete incorporation of ^{18}O atoms (to give the peak at m/z 380) and thereby demonstrates the absence of significant exchange.

Fig. 3(c) show the partial mass spectrum of methylated depside prepared non-enzymically under an atmosphere containing 48% $^{18,18}\text{O}_2$. The spectrum clearly shows incorporation of label at m/z 380 but little or none at m/z 378 (after allowance for the ^{13}C natural-abundance peak). This spectrum immediately suggests, therefore, the One-Molecule

Table 1. *Oxygenation of quercetin under $^{18}\text{O}_2$*

Reactions were carried out in a closed system either enzymically or non-enzymically as shown. Gas-phase enrichment of $^{18}\text{O}_2$ was achieved by mixing pure (99%) $^{18}\text{O}_2$ with $^{16}\text{O}_2$. Observed mass spectra were corrected for naturally abundant ^{13}C as previously described (Brown & King, 1978).

Conditions	^{18}O enrichment in gas phase (%)	Mass number	Proportion of methylated depside at various mass numbers (mole %)		
			Observed	Predicted from One-Molecule Mechanism	Predicted from Two-Molecule Mechanism
Non-enzymic	48	<i>m/z</i> 376	52.6	52	27
		<i>m/z</i> 378	0	0	50
		<i>m/z</i> 380	47.4	48	23
Non-enzymic	48	<i>m/z</i> 376	57.8	52	27
		<i>m/z</i> 378	0	0	50
		<i>m/z</i> 380	42.2	48	23
Enzymic	36	<i>m/z</i> 376	66.0	64	41
		<i>m/z</i> 378	0	0	46
		<i>m/z</i> 380	34.0	36	13

Fig. 3. *Partial mass spectra of methylated depside prepared from oxygenation of quercetin*

(a) Base-catalysed oxygenation under $^{16,16}\text{O}_2$; (b) base-catalysed oxygenation under 99% $^{18,18}\text{O}_2$; (c) base-catalysed oxygenation under 48% $^{18,18}\text{O}_2$; (d) enzymic oxygenation under 36% $^{18,18}\text{O}_2$.

One-Molecule Mechanism operates in this system also. The data from this experiment are also shown in Table 1 along with the predictions for both mechanisms. Again, the results are in good quantitative agreement with the One-Molecule Mechanism.

Discussion

The results presented here show that both enzymic and non-enzymic degradation of quercetin proceeds via a One-Molecule Mechanism, in contrast with the mechanism of haem degradation. This fundamental difference between the two degradation pathways rules out the possibility of quercetin oxygenation being used as a chemical model for the degradation of haem. Moreover this difference in mechanism, in the face of the superficial similarity between the two pathways, further demonstrates the singularity of haem catabolism compared with other dioxygenations. The reason for this unique mechanism for haem oxygenation is not clear. However, it seems likely that it is associated with the presence of the iron atom. In this context, it is noteworthy that iron is essential for the macrocyclic-ring-opening step, i.e. metal-free hydroxyporphyrins (oxophlorins) do not undergo a corresponding oxidative decarboxylation. It seems possible that in the hydroxyhaem ring-cleavage step, iron binds and activates two oxygen molecules, which successively attack the appropriate carbon atoms in intramolecular reactions (Brown & Troxler, 1982). If this is so, then true models for haem degradation, capable of undergoing oxygenation by the Two-Molecule Mechanism, would require an appropriately located iron atom, capable of binding and activating molecular oxygen.

Mechanism. These data (and those from a duplicate experiment) have been analysed quantitatively, after correction for the ^{13}C peaks, and are shown in Table 1 along with predictions for both mechanisms. Clearly, there is good quantitative agreement with the One-Molecule Mechanism.

^{18}O incorporation from enzymic oxygenation of quercetin

A corresponding experiment was carried out on the enzymic oxygenation of quercetin using an enrichment of 36 atom % ^{18}O . The mass spectrum obtained is shown in Fig. 3(d), indicating that the

Fig. 4. Postulated mechanism for both base- and enzyme-catalysed oxygenation of quercetin

Our results suggest that the oxygenation of quercetin involves the concerted addition of dioxygen to the enol system of quercetin (Nishinaga *et al.*, 1979). Such a mechanism could lead to formation of a oxohydroperoxy intermediate (Fig. 4) followed by nucleophilic attack on the C-4 carbonyl group to give the depside and releasing CO .

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