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Degradation of Rutin by *Aspergillus flavus*

STUDIES WITH OXYGEN 18 ON THE ACTION OF A DIOXYGENASE ON QUERCETIN*

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SUMMARY

The enzymatic oxygenation and cleavage of quercetin by quercetinase, an oxygenase produced by *Aspergillus flavus*, yield carbon monoxide and a depside, 2-protocatechuoylphloroglucinolcarboxylic acid. The enzyme does not incorporate oxygen from $^{18}O_2$ into carbon monoxide, but incorporates both atoms of $^{18}O_2$ into the depside. One atom of $^{18}O_2$ is incorporated into the carboxyl group and the other into the ester carbonyl; thus quercetinase is a dioxygenase. Oxygen from $H_2{}^{18}O$ is not incorporated into either the depside or carbon monoxide. Both the carbon and oxygen of carbon monoxide are derived from the hydroxylated carbon 3 of quercetin. The reaction probably involves a direct and concerted addition of "activated oxygen" to quercetin at positions 2 and 4 to form an unstable cyclic peroxide intermediate which rapidly decomposes to give the products.

Aspergillus flavus when grown on rutin excretes an inducible glycosidase, rutinase, that hydrolyzes rutin and an inducible oxygenase, quercetinase, that oxidatively cleaves the heterocyclic ring of the aglycone, quercetin (Fig. 5, *V*), to yield carbon monoxide and depside, 2-protocatechuoylphloroglucinolcarboxylic acid (Fig. 5, *X*) (1). Several other fungi such as species of *Alternia*, *Cephalosporium*, *Diaporthe*, *Fusarium*, *Penicillium*, and *Pullularia* are capable of carrying out this reaction (2–4). Previous work revealed that quercetinase acts on chromones that possess a substituent such as a methyl or a phenyl group on carbon 2, a free hydroxyl on carbon 3, and a double bond between carbons 2 and 3 (4, 5). When quercetin-3-^{14}C was used as the substrate for the enzyme, all of the radioactive carbon was present in the carbon of carbon monoxide (1). This paper presents the results of studies on the distribution of oxygen 18 in the products and a possible mechanism of the reaction.

METHODS AND RESULTS

The oxygen 18 gas and the oxygen 18-enriched water, normalized, were purchased from Miles Laboratories, Elkhart, Indiana.

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The ^{18}O concentration in CO_2 samples was measured with an AEI mass spectrometer type MS3 with the formula $R/2 + R$ where R is the ratio of the peak heights at mass 46 to mass 44. When the ^{18}O concentration was around 50%, the formula used was $^{18}O = (A/3.10 + A)$ where $A = (46/44) - (0.011 + (B/4))B$ and $B = (45/44) - 0.011$ (6). Samples of water were analyzed for ^{18}O by equilibration with a measured amount of CO_2 (7). The mass spectra of the depside and its derivatives were kindly measured by Dr. W. D. Jamieson of the Atlantic Regional Laboratory on a CEC 21-110B instrument. Samples were introduced directly into the ionization chamber (140–145°) with a 70-volt electron beam energy. The mass measurements were obtained with electrical detection by the peak-matching technique at a resolution of about 15,000. At this resolution all ions of measured mass were singlet signals.

The enzyme produced by *A. flavus* was purified by modification and further development of a procedure previously reported (5). This procedure, to be described in a subsequent publication, yields a preparation containing a single protein. The enzymatic degradation of quercetin in aqueous buffer is an extremely slow reaction because of the low solubility of the substrate. Usually the more soluble rutin with an excess of rutinase is used (5), but in this work we preferred to use quercetin as the substrate. Several solvents and experimental conditions were tested to increase the rate of the reaction. Quercetin is soluble in dimethyl sulfoxide. At concentrations of 10% or less, dimethyl sulfoxide does not inhibit the enzyme although higher concentrations are progressively inhibitory. The addition of 10% dimethyl sulfoxide to the reaction mixture accelerated the rate of the reaction, but the effect was most marked if the quercetin was first dissolved in dimethyl sulfoxide even though a suspension was formed immediately on adding the buffer. High partial pressures of oxygen also reduced the reaction time.

Iodine pentoxide was sieved through a 10 mesh screen to obtain small granular particles. Quinoline (synthetic) was dried over KOH and distilled. Quercetin dihydrate, chromatographically pure, was purchased from Mann. Nitrogen gas of 99.999% purity was further purified by passing through a column of reduced BTS catalyst (BASF, Ludwingshafen am Rhein, Germany) (8), through a trap, and then through soda asbestos and anhydrone before admission to the reaction vessels.

Exchange of Oxygen between Quercetin and Water—The reaction mixture consisted of quercetin dihydrate (20 mg), 0.1 ml of $H_2{}^{18}O$ (62.79 atom %), 0.2 ml of enzyme (1000 units), 0.2 ml of dimethyl sulfoxide, and 1.5 ml of phosphate buffer (pH 6.6, 1

M) and $^{16}O_2$. After 30 min, when the reaction was about half-completed, the mixture was filtered and the recovered quercetin was crystallized from ethanol. Its mass spectrum showed the parent peak at 302 ($C_{15}H_{10}{}^{16}O_7$) which is the base peak. There was no difference between this spectrum and that of quercetin crystallized from ethanol.

Examination of Carbon Monoxide—The experiments were designed to test whether or not the oxygen of carbon monoxide was labeled with ^{18}O when the reaction was conducted in the presence of either $^{18}O_2$ or $H_2{}^{18}O$. The first experiments were performed with $^{16}O_2$ and $H_2{}^{16}O$ to test the apparatus and procedures. The averaged data (two determinations) are recorded as No. 1 in Table I. This was followed by two experiments with $^{18}O_2$ and $H_2{}^{16}O$.

Quercetin dihydrate (0.1 g) was dissolved in dimethyl sulfoxide (6 ml) in a 250-ml round bottom flask. Potassium phosphate buffer (93 ml, 0.1 M pH 6.6) previously boiled and purged with pure nitrogen was added. The suspension was frozen and 1 ml (5,000 units) of quercetinase was added and frozen. This flask was attached to a manifold that permitted the addition of either N_2, $^{16}O_2$, or $^{18}O_2$. For experiments with $^{18}O_2$, a sealed tube containing 100 ml of enriched oxygen was attached to the manifold. The system was evacuated and then filled with pure nitrogen and this procedure was repeated four times. After the last evacuation, the system was closed and either $^{16}O_2$ was admitted to a maximum pressure of 700 mm Hg or $^{18}O_2$ was admitted by breaking, with a magnet, the seal of the tube containing $^{18}O_2$. The contents of the reaction flask were then brought to 30° and vigorously stirred with a magnetic stirrer. The suspended substrate disappeared within 2 hours. The carbon monoxide and the excess oxygen were swept out of the reaction vessel with purified nitrogen for 30 min (flow rate approximately 10 ml per min) into two wash bottles in series, each containing 50 ml of Fieser's solution (9) to remove oxygen, and then through a U-tube, the first half of which was packed with anhydrone and the other half with soda asbestos. The purified carbon monoxide was then oxidized by passage over iodine pentoxide at 119–120° (10, 11). The liberated iodine was trapped in a tube cooled in a Dry Ice-acetone bath and the carbon dioxide was collected in a U-tube cooled in liquid nitrogen. This complete train was thoroughly flushed with purified dry nitrogen before each experiment. The trap in the liquid nitrogen, containing the carbon dioxide, was evacuated to less than 1 mμ to remove condensed nitrogen. The carbon dioxide was then purified by distillation on a vacuum line three times before a sample was introduced into the mass spectrometer.

The same procedure was followed in Experiments 4 and 5 with $^{16}O_2$ and $H_2{}^{18}O$. The reaction mixture consisted of quercetin (20 mg), $H_2{}^{18}O$ (0.4 ml), enzyme (0.4 ml, 2000 units), dimethyl sulfoxide (0.4 ml), and phosphate buffer (2.8 ml, 1.0 M, pH 6.6). The reaction was complete within 1 hour. The reaction flask was swept with nitrogen and the carbon monoxide was oxidized to CO_2 and recovered as before.

TABLE I

Results of ^{18}O analysis of CO and recovered water

No. and isotope source	Atom %	Atom % in CO_2 from CO	Atom % in CO_2 from tank	Atom % in recovered water
1. None		0.201	0.206	0.204
2. $^{18}O_2$	92.3	0.259	0.206	0.224
3. $^{18}O_2$	97.0	0.204	0.205	0.230
4. $H_2{}^{18}O$	6.279	0.193	0.199	—[a]
5. $H_2{}^{18}O$	6.279	0.203	0.207	6.264

[a] Sample of CO_2 was too small.

In each of the above experiments the solution remaining in the flask was filtered and part lyophylized to recover the water. A sample of this was taken to determine the atom % O_2 by equilibrating with a measured amount of carbon dioxide (7). The carbon dioxide was purified twice on a separate vacuum line and its ^{18}O concentration was determined.

The oxygen of carbon monoxide was not labeled with ^{18}O when the enzymatic reaction was conducted either in the presence of $^{18}O_2$ or $H_2{}^{18}O$ (Table I). Moreover, under the conditions of these experiments in the presence of enzyme and substrate, no exchange of ^{18}O could be observed between $^{18}O_2$ and water, nor did a significant reduction in the enrichment of $H_2{}^{18}O$ occur (Table I).

Examination of Depside—The reaction system, the recovery, and analytical procedures developed to examine the depside were tested with material from reactions conducted in the presence of $^{16}O_2$ and then repeated with $^{18}O_2$. Since larger amounts of the depside than of carbon monoxide were required to determine the distribution of ^{18}O, the reaction was conducted in a round bottom flask of 500-ml capacity. This was attached to a glass manifold possessing a three-way vacuum stopcock for evacuating or adding purified gases. The manifold contained two additional ports to which could be attached two 100-ml tubes containing $^{18}O_2$ and provided with break seals and magnets.

Quercetin dihydrate (1.0 g) was first dissolved in 20 ml of dimethyl sulfoxide followed by the addition of 200 ml of phosphate buffer (0.3 M, pH 6.4) which had been previously purged with nitrogen. The contents of the reaction flask were frozen and 5 ml of quercetinase (50,000 units) were added and frozen. The flask was connected to the manifold and the apparatus was evacuated and filled four times with purified N_2. After the final evacuation to less than 1 mm Hg, $^{16}O_2$ was supplied to the system from a lecture bottle or $^{18}O_2$ from the sealed tubes. The reaction mixture was then thawed and brought to 30°. The reaction was complete in 2 hours as indicated by the disappearance of the insoluble quercetin. The flask was disconnected, and the solution was filtered and taken to dryness under reduced pressure at room temperature. The solid residue was cooled and macerated with ice-cold dilute HCl and the depside was recovered quickly by filtration in the cold and washed with ice-cold water. The product (0.65 g) was crystallized twice from dilute alcohol. Chromatography of samples from the filtrates indicated that negligible hydrolysis occurred. The depside crystallized from dilute ethanol as the dihydrate, m.p. 175–177°.

$C_{14}H_{10}O_8 2H_2O$

Calculated: C 49.12, H 4.09
Found: C 49.09, H 4.20

The anhydrous depside was obtained by drying overnight under high vacuum at 100°.

$C_{14}H_{10}O_8$

Calculated: C 54.91, H 3.29
Found: C 54.64, H 3.42

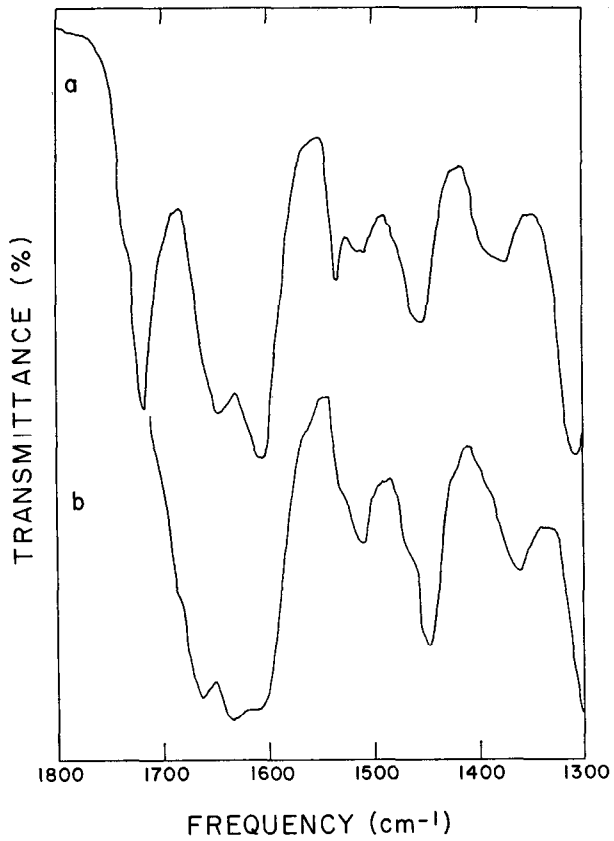

FIG. 1. Infrared spectra (KBr) of (*a*) ^{16}O-depside and (*b*) ^{18}O-depside.

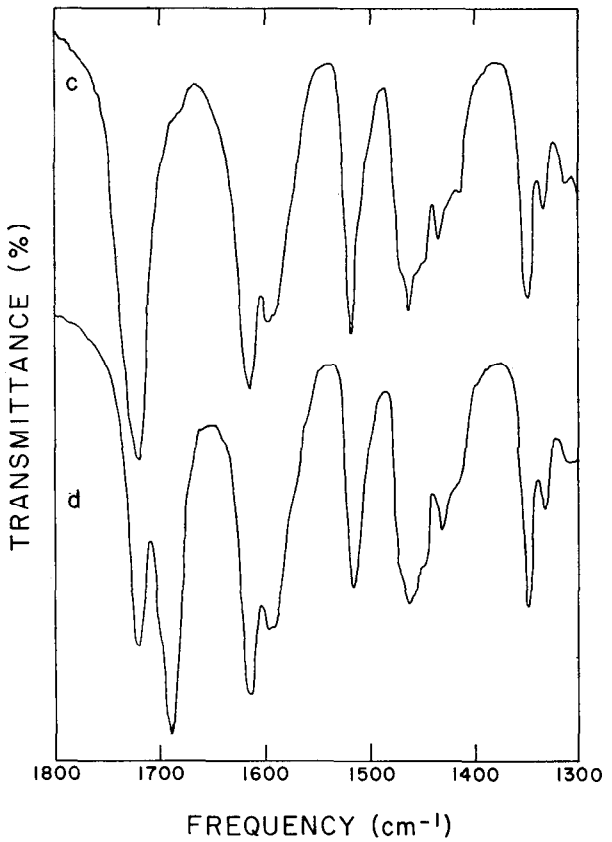

FIG. 2. Infrared spectra (KBr) of the tetramethyl ether methyl esters prepared from (*c*) ^{16}O-depside and (*d*) ^{18}O-depside.

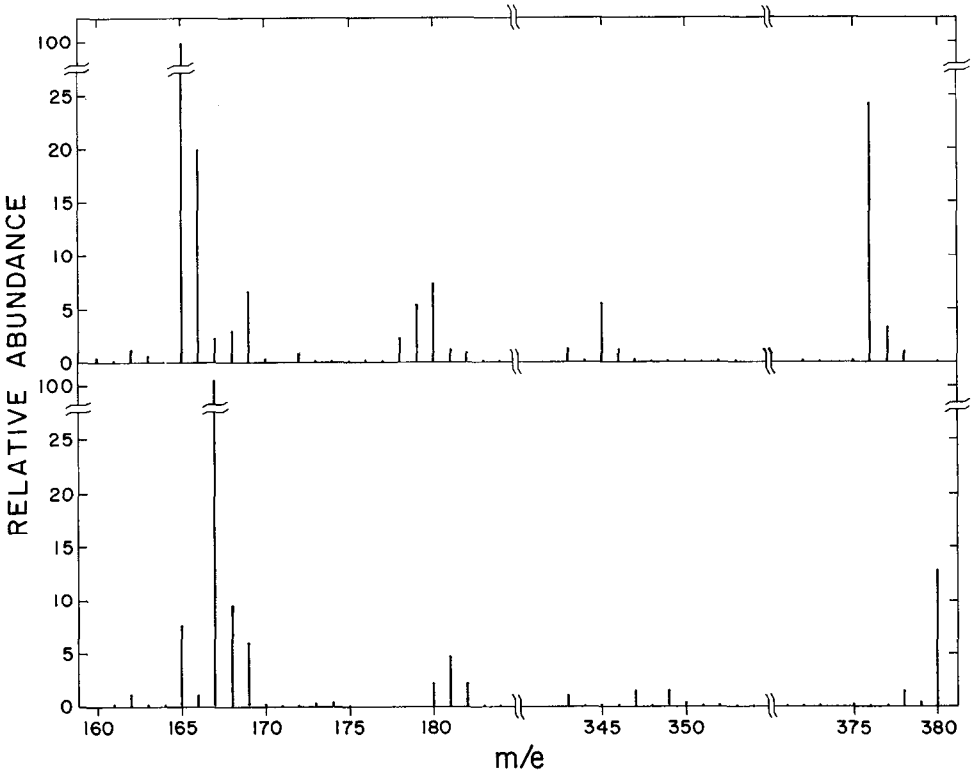

FIG. 3. Selected areas of the mass spectra of the tetramethyl ether methyl esters of the depsides obtained from reaction mixtures containing either $^{16}O_2$ or $^{18}O_2$.

Infrared spectra are shown in Fig. 1. The ^{16}O- or ^{18}O-depside did not give satisfactory mass spectra and thus it was necessary to prepare the methyl derivative.

Preparation of Tetramethyl Ether Methyl Ester of Depside—The foregoing depside (0.300 g) was methylated with excess diazomethane in a dry ether-methanol mixture at 4° for 24 hours. The product was crystallized thrice from methanol. Both labeled and unlabeled derivatives of the depside had the same melting point (144°) as previously reported (3) and identical thin layer chromatographic behavior. The infrared spectra of the tetramethyl ether methyl ester of the ^{16}O-depside showed strong absorptions at 1720 cm^{-1} and 1615 cm^{-1} and an inflection at 1680 cm^{-1}, whereas the ^{18}O-depside showed strong absorptions at 1720 and 1615 cm^{-1}, but a stronger absorption at 1688 cm^{-1} (Fig. 2). The mass spectra are shown in Fig. 3.

Decarboxylation of Depside—Decarboxylation of the depside was studied under various conditions (such as dry heating, heating in solvents at different temperatures) to determine the best procedure for releasing CO_2. Decarboxylation of the labeled depside with 2 N H_2SO_4 at 100° for 20 min under bubbling N_2 yielded carbon dioxide containing only 1.285% ^{18}O. Obviously there was rapid exchange of ^{18}O in the carboxyl group under acidic conditions. Satisfactory results were obtained only when the decarboxylation was done with the anhydrous depside and heating it in anhydrous quinoline at 125–130° for 25 to 30 min. Reaction in quinoline at higher temperature or by dry heating above its melting point (175°) led to the formation of catechol, protocatechuic acid, and phloroglucinol.

The apparatus used for the decarboxylation was a modification of that described by Beroza (12) and consisted of Beroza's reaction flask with the gas outlet attached in series to a condensor, a carbon dioxide trap immersible in a bath of liquid N_2, and a U-tube containing anhydrone in the first half and soda asbestos in the second half. The apparatus was assembled with a glass stopper in the port for the spoon. Quinoline (1 ml) was placed in the reaction flask and a stream of pure and dry N_2 was bubbled (10 ml per min) through the system. Any dissolved CO_2 in the quinoline was removed by heating the quinoline to the boiling point and then cooling to room temperature while flushing with N_2. The glass stopper was then replaced with a stopper containing the platinum spoon in which about 30 mg of the anhydrous depside was placed. After a further 15 min of flushing the reaction was begun by inverting the spoon and the carbon dioxide trap was immediately immersed in liquid nitrogen. The reaction flask was warmed to 135° and the reaction was allowed to proceed for 20 min. The CO_2 trap was then removed; the gas was purified and analyzed in the mass spectrometer. The atom % used in the enzymatic reaction mixture was 94.15 ^{18}O and 0.43 ^{17}O. The values obtained on analysis of the carbon dioxide released from the depside were 49.00, 54.84, and 53.90 atom % ^{18}O. The calculated value for incorporation of 1 atom of ^{18}O in the carboxyl group is 47.05% atom excess.

The decarboxylated ^{16}O-depside (phloroglucinol monoprotocatechuate was crystallized from benzene-ethanol, m.p. 195–199°.

$C_{13}H_{10}O_6$

Calculated: C 59.26, H 4.16
Found: C 59.54, H 3.82

DISCUSSION

The data in Table I reveal that, when the enzymatic reduction was conducted in the presence of either $^{18}O_2$ or $H_2{}^{18}O$, the oxygen of the carbon monoxide released was not enriched with ^{18}O. Similarly, the relative abundance of ^{18}O in the water before and after the enzyme reaction was almost identical. The carbon monoxide has been previously shown to be derived from carbon 3 (1). Therefore, it is likely that both the carbon and oxygen of carbon monoxide arise from the C—OH group at position 3 of quercetin. These results also indicate that position 3 is not

TABLE II

Accurate mass to charge ratios of selected ions in mass spectrum of ^{18}O-depside tetromethyl ether methyl ester

m/e	Formula	Found	Calculated
380	$C_{19}H_{20}{}^{16}O_6{}^{18}O_2$	380.1242	380.1243
376	$C_{19}H_{20}{}^{16}O_8$	376.1211	376.1158
349	$C_{18}H_{17}{}^{16}O_5{}^{18}O_2$	349.1047	349.1059
347	$C_{18}H_{17}{}^{16}O_6{}^{18}O_1$	347.0992	347.1008
345	$C_{18}H_{17}O_7$	345.0976	345.0994
167	$C_9H_9{}^{16}O_2{}^{18}O_1$	167.0588	167.0594
165	$C_9H_9{}^{16}O_3$	165.0548	165.0552

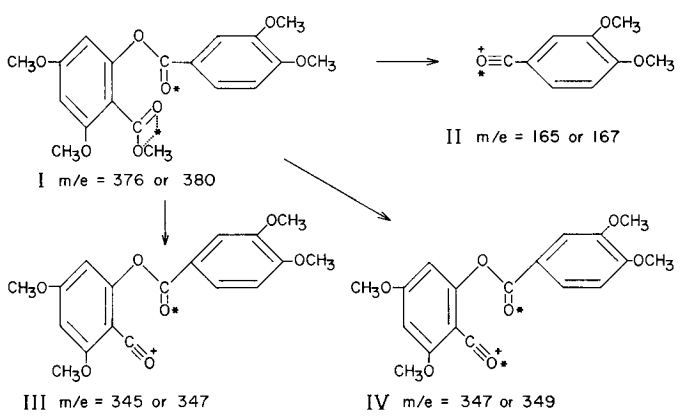

FIG. 4. Assignment of structures of fragment ions in the mass spectrum of the tetramethyl ether methyl ester of the depside. Positions enriched with ^{18}O are marked with *.

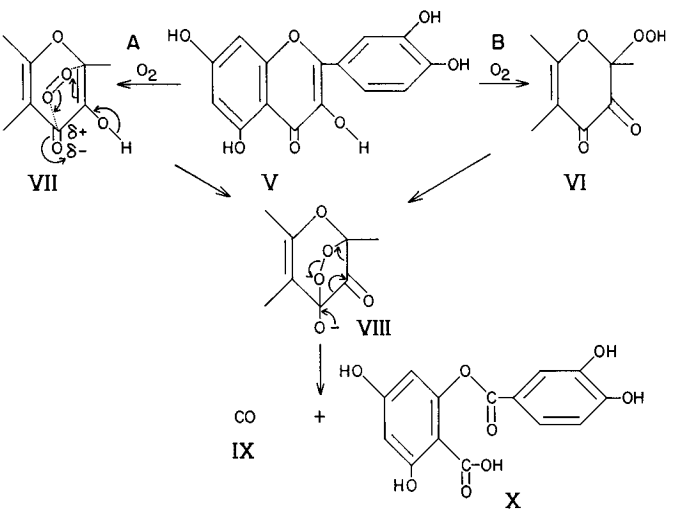

FIG. 5. Possible mechanisms of the oxygenation followed by cleavage of heterocyclic ring of quercetin.

attacked by either oxygen or by water and that exchange reactions are negligible.

The mass spectra of the tetramethyl ether methyl ester (Fig. 4, *I*) of the ^{16}O-depside and ^{18}O-depside are shown in Fig. 3. They unequivocally established the positions of the isotope. The mass spectrum of the unlabeled compound showed molecular ion at *m/e* 376 and a base peak at *m/e* 165. The other important ion in the spectrum, for the purpose of the present discussion, is at *m/e* 345 (M-31). It is possible to assign structures to these fragment ions from the general fragmentation pattern of esters as shown in Fig. 4. The ^{18}O-depside derivative gave a molecular ion at *m/e* 380 (376 + 4; $C_{19}H_{20}{}^{16}O_6{}^{18}O_2$), showing that 2 atoms of ^{18}O are incorporated into the depside from molecular oxygen. The relative abundance of *m/e* 380 to that at *m/e* 376 parallels the $^{18}O_2$ enrichment used in the experiment. The base peak is at *m/e* 167 (165 + 2; $C_9H_9{}^{16}O_2{}^{18}O$) which clearly shows 1 atom of ^{18}O in the (M-213) ion of the structure shown as Formula *II*, that is, in the ester carbonyl. The peak at 345 (III, M-31) is insignificant and, instead, peaks at *m/e* 347 (345 + 2; $C_{18}H_{17}{}^{16}O_6{}^{18}O$) and *m/e* 349 (345 + 4; $C_{18}H_{17}{}^{16}O_5{}^{18}O_2$) of exactly equal abundance are present (*IV*). Their appearance can be accounted for as follows. One of the 2 oxygen atoms of the carboxyl group of phloroglucinol carboxylic acid originates from the carbonyl oxygen originally present and the other from the $^{18}O_2$ molecule. Methylation to the ester with diazomethane gives two methyl esters depending on which oxygen the esterification takes place, thus producing two molecular species, one in which the ^{18}O is present in the ester carboxyl and the other in which the ^{18}O is present in the methoxyl oxygen. Both species (*III* and *IV*) contain ^{18}O in the ester carbonyl group (Fig. 4). The partial high resolution mass spectra of the ^{18}O-depside tetramethyl ether methyl ester shown in Table II confirm the structural assignments of the formulas discussed above.

The decarboxylation of the ^{18}O depside and estimation of atom % excess in the carbon dioxide released gave values between 49 and 54%. The theoretical value based on the incorporation of 1 atom of oxygen from $^{18}O_2$ is 47%. Although the observed values are higher than the calculated value, the result can be construed as evidence for the incorporation of 1 atom oxygen into the carboxyl group. This observation is in agreement with the mass spectral data.

The infrared spectra of the ^{18}O-depside and its methyl derivative in the region 2000 to 1600 cm^{-1} are shown in Fig. 2. In the ^{16}O-depside (Fig. 1) the ester carbonyl occurs at 1717 cm^{-1}, the chelated carboxyl at 1645 cm^{-1}, and aromatic double bond stretch at 1605 cm^{-1}. The ^{18}O-depside showed the ester carbonyl at 1662 and the carboxyl at 1630 cm^{-1}. Similarly, the tetramethyl ether methyl esters of the ^{16}O-depside showed only a single unresolved ester band at 1720 cm^{-1} but the ^{18}O-depside derivative showed two bands at 1720 and 1688 cm^{-1}. Such shifts in frequency of C = ^{16}O to C = ^{18}O are to be expected and have been recorded in the case of aldehydes, ketones, and amides (13, 14). These infrared data corroborate the mass spectral data.

The foregoing results clearly show that quercetinase is a dioxygenase. A molecule of oxygen is incorporated intact across positions 2 and 4 of quercetin to give the depside with the elimination of carbon monoxide. Any mechanism, therefore, necessitates the formation of a cyclic peroxide intermediate (Fig. 5).

Matsuura, Matsushima, and Nakashima[1] (15) recently proposed a chemical model for the enzyme reaction and a pathway for the formation of the cyclic peroxide (Fig. 5, *VIII*). They found that photosensitized oxygenation of quercetin produced, among other products, the depside, CO, and CO_2. Quercetin 3′,4′,5,7-tetramethyl ether gave in good yield the *O*-methyl derivative of the depside, CO and CO_2. Since photooxygenations are considered to involve singlet excited oxygen (electrophilic) (15), they proposed that singlet oxygen reacts to form the hydroperoxide (*VI*) by a process of concerted addition to the ene-ol system of quercetin (*Path B*). The next step is rearrangement of (*VI*) to (*VIII*) which then gives the products. A second mode of formation of the cyclic peroxide (*VIII*) by quercetinase can now be suggested (*Path A*). A keto hydroperoxide may not be involved. A direct and concerted addition of "activated oxygen" at carbons 2 and 4 with concomitant ketonization as depicted in Structure (*VII*) may occur while bound to the enzyme. This is followed by rapid collapse of the five-membered cyclic peroxide to yield the products.

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REFERENCES

1. Simpson, F. J., Talbot, G., and Westlake, D. W. S., *Biochem. Biophys. Res. Commun.*, **2**, 15 (1960).
2. Westlake, D. W. S., Roxburgh, J. M., and Talbot, G., *Nature*, **189**, 510 (1961).
3. Hattori, S., and Noguchi, I., *Nature*, **184**, 1145 (1959).
4. Padron, J., Grist, K. L., Clark, J. B., and Wender, S. H., *Biochem. Biophys. Res. Commun.*, **3**, 412 (1960).
5. Simpson, F. J., Narasimhachari, N., and Westlake, D. W. S., *Can. J. Microbiol.*, **9**, 14 (1963).
6. Klein, F. S., *Weizmann Institute of Science Communication to Yeda Research and Development Company, Ltd., Rehovoth, Israel*, 1963.
7. Falcone, A. B., *Anal. Biochem.*, **2**, 147 (1961).
8. Broadbent, A. D., *J. Chem. Educ.*, **44**, 145 (1967).
9. Fieser, L. F., and Fieser, M., in *Reagents for organic synthesis*, John Wiley and Sons, Inc., New York, 1967, p. 393.
10. Doering, W. von E., and Dorman, E., *J. Amer. Chem. Soc.*, **75**, 5595 (1953).
11. Unterzaucher, J., *Analyst*, **77**, 584 (1952).
12. Beroza, M., *Anal. Chem.*, **25**, 177 (1953).
13. Pinchas, S., Samuel, D., and Weiss-Broday, M., *J. Chem. Soc.*, 2666 (1961).
14. Byrn, M., and Calvin, M., *J. Amer. Chem. Soc.*, **88**, 1916 (1966).
15. Matsuura, T., Matsushima, H., and Sakamoto, H., *J. Amer. Chem. Soc.*, **89**, 6370 (1967).

[1] T. Matsuura, H. Matsushima, and R. Nakashima, personal communication.