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^{18}O INVESTIGATION OF PYROCATECHASE REACTION: MODE OF ATTACK OF MOLECULAR OXYGEN

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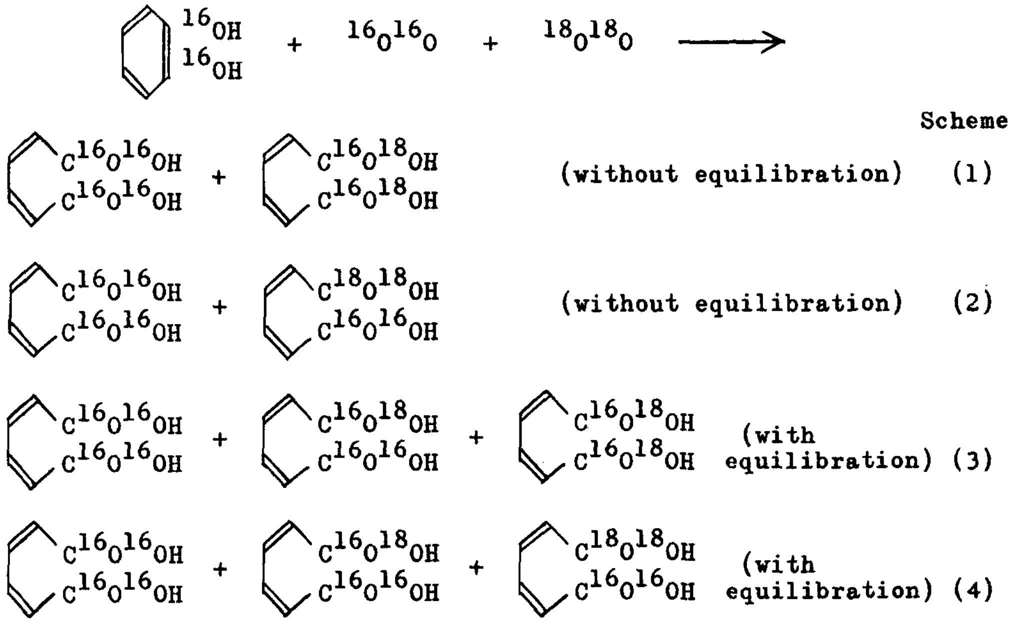
In the net reaction catalyzed by pyrocatechase of a pseudomonad, two oxygen atoms are added to catechol resulting in the formation of _cis,cis_-muconic acid. Hayaishi _et al_. (1955) demonstrated that two atoms of oxygen in the product were derived from molecular oxygen, by incubating the enzyme with its substrate in the presence of $^{18}O_2$. Since then, similar reactions have been reported by several groups of workers using ^{18}O as a tracer. However, these studies do not extend beyond the overall incorporation of ^{18}O in the product; for, in previous studies, ^{18}O-abundance of the product was determined in the form of $C^{18}O_2$, mostly according to the procedure of Rittenberg and Ponticorvo (1956).

In the cleavage of catechol to _cis,cis_-muconic acid by oxygen, two questions can be raised: (1) whether the incorporated two oxygen atoms are located in one carboxyl or two, and the other (2) whether the oxygenation involves the equilibration of the oxygen among the three molecular species, $^{16}O^{16}O$, $^{16}O^{18}O$ and $^{18}O^{18}O$, by cleavage of O-O bond. Thus, one could expect four possible mechanisms which bring about the incorporation of two oxygens from non-equilibrated $^{18}O_2$ into the product:



Scheme

(without equilibration) (1)

(without equilibration) (2)

(with equilibration) (3)

(with equilibration) (4)

Appropriate instrumentation and experimental conditions have now made it possible to answer these questions. In the present study, catechol was oxidized in a gas mixture which consisted predominantly of $^{16}O^{16}O$ and $^{18}O^{18}O$, and the product was isolated from the reaction mixture under carefully controlled conditions in order to avoid the chemical exchange of carboxylate oxygen with the medium and a cis,trans- or trans,trans-conversion. Since cis,cis-muconic acid did not have enough vapor pressure for a conventional mass spectrometric analysis, and since veratrole or diethylcatechol was not oxidized by the enzyme at all, the muconic acid was converted to its dimethylester by treating it with an ether solution of diazomethane, and the product was subjected to mass spectrometric analysis.

Fig. 1 illustrates mass spectra of cis,cis-muconic acid dimethylester and its fragments resulting from electron impact, from mass 111 ($M-COOCH_3$) to mass 170 (parent ion, M), for normal and

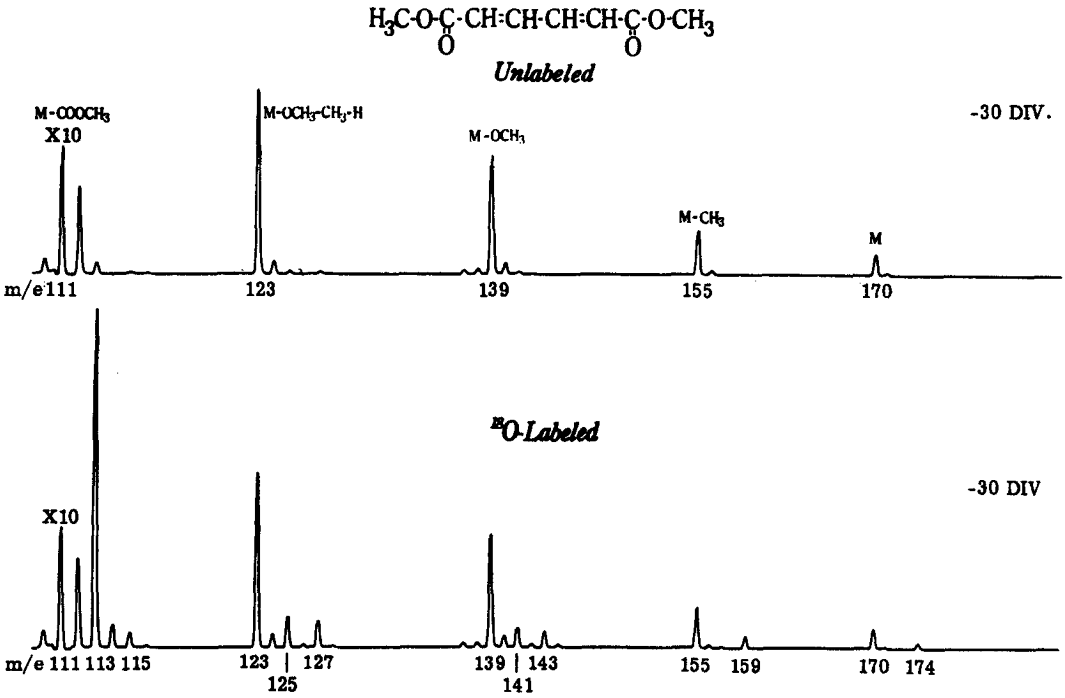


FIG.1 THE MASS SPECTRA OF DIMETHYL *cis,cis*-MUCONIC ACID

^{18}O-products, both of which were prepared and analyzed under the same conditions. The ^{18}O-isotopic pattern of the parent, M, and the fragment, M-CH_3, is identical with the molecular oxygen used in the gas phase of the reaction ($^{16}O^{16}O:^{16}O^{18}O:^{18}O^{18}O = 100:3.26:26.9$), indicating non-equilibration.

Accurate calculation of relative intensities of isotope peaks was made under appropriate attenuation and by repeated scanning. The results of parent (M), M-OCH_3, and M-$COOCH_3$ peaks are given in Table I. The data unequivocally support scheme (1), *i.e.*, only one molecule of oxygen contributes its oxygen atoms to form the two carboxyl groups of one molecule of product. Isotopic patterns of the other fragment peaks give further evidence to support this mechanism.

Table I. ^{18}O-Enrichment of *cis,cis*-Muconic Acid

m/e		Un-labeled	^{18}O-labeled	Relative Enrich-ment	Expected for			
					(1)	(2)	(3)	(4)
Parent	170	100	100	100	100	100	100	100
	172	2.15	4.56	2.41	2.86	2.86	56.1	56.1
	174	0.01	26.8	26.7	26.9	26.9	8.09	8.09
M-OCH_3	139	100	100	100	100	100	100	100
	141	1.95	17.4	15.4	15.5	15.5	40.3	40.3
	143	0.17	13.7	13.5	13.3	13.3	3.54	3.54
M-$COOCH_3$	111	100	100	100	100	100	100	100
	113	0.86	27.4	26.5	27.8	1.19	28.1	21.1
	115	0.13	1.25	1.12	0	11.7	0	3.05

Non-equilibrated $^{18}O_2$ was prepared by mixing natural tank oxygen and $^{18}O_2$ obtained by the electrolysis of $H_2{}^{18}O$ (98% ^{18}O, 0.45% ^{17}O). The relative intensities of the gas phase oxygen (mean of initial and final smaples) at m/e 32 ($^{16}O^{16}O$), 33 ($^{16}O^{17}O$), 34 ($^{16}O^{18}O$, $^{17}O^{17}O$), 35 ($^{17}O^{18}O$), and 36 ($^{18}O^{18}O$) were 100:0.0880: 3.26:0.249:26.9. From these peak heights, atom fractions of ^{16}O ($1-\alpha_1-\alpha_2$), ^{17}O (α_1), and ^{18}O (α_2) were calculated to be 0.779, 0.001, and 0.220, respectively. If these isotopes equilibrate to form O_2, the pattern at m/e 32, 34 and 36 should be 100:56.5:7.98. For natural tank oxygen, $\alpha_1 = 0.033 \times 10^{-2}$, and $\alpha_2 = 0.201 \times 10^{-2}$.

The reaction mixture (150 ml) consisted of (in mmoles); catechol 1.8, glutathione 1.8, potassium phosphate buffer (pH 7.5) 15, and 500 units (Hayaishi *et al*., 1957) of pyrocatechase. After a 45 minute incubation at 22°, *cis,cis*-muconic acid was isolated from the reaction mixture, and converted to the dimethylester by treating it with a small excess of diazomethane in ether. Ether and diazomethane were removed by evaporation, and the ester was further purified by sublimation. The purity of *cis,cis*-muconic acid was established by melting points of the acid (185°) and of its dimethylester (74°), UV spectrum, and elementary analyses. The ^{18}O-enrichment was determined in an Atlas CH4 mass spectrometer, with a 70eV ionizing potential. Temperature of the inlet system was maintained at 130°.

The results indicate that the cleavage of O-O bond does not take place prior to forming the intermediary oxygenated complex, and therefore two oxygen atoms which are incorporated in *cis,cis*-muconic acid originate from a single molecule. Another finding is that only one oxygen in a carboxylic group is labeled with ^{18}O. This points the fact that the oxygenation is completely irreversible.

A plausible mechanism to account for the observed findings is that the oxygenated intermediate might have a peroxide structure as proposed earlier (Hayaishi, 1957) and the active form of oxygen might be a perferryl ion as suggested by Ingraham (1956).

Furthermore, it is of considerable interest that the methylation of carboxylate by diazomethane in an ether solution does not cause any appreciable exchange of oxygen with the medium. This makes it possible to detect and determine ^{18}O separately in oxygenated groups in non-volatile carboxylic acids, such as keto, hydroxyl or carboxylic group at different positions in a molecule, or even to differentiate ^{18}O in the two atoms of oxygen in one carboxylic group.

The details of the experiments and calculations will be published elsewhere.

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