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The Metabolism of 2-Fluorobenzoic Acid

II. STUDIES WITH $^{18}O_2$

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G. W. A. MILNE, PETER GOLDMAN, AND JORDAN L. HOLTZMAN

From the National Heart Institute, and National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland 20014, and the National Cancer Institute, Baltimore, Maryland 21200

SUMMARY

Studies with $^{18}O_2$ indicate that catechol formed from 2-fluorobenzoic acid by a pseudomonad contains 2 oxygen atoms derived from a single molecule of oxygen. This finding supports a defluorination reaction which proceeds through a cyclic peroxide intermediate. The formation of 2-fluorocatechol from 2-fluorobenzoic acid seems also to proceed through a cyclic peroxide intermediate.

The growth medium of a pseudomonad which utilizes 2-fluorobenzoic acid as a sole carbon source (1)[1] has now been found to contain small amounts of catechol as well as 3-fluorocatechol. This compound probably results from the defluorination which is the fate of 85% of the fluorobenzoate utilized by this organism (1). Two mechanisms might explain the release of fluoride (Diagram 1). One would be completely analogous to the oxidation of anthranilic acid by an enzyme isolated from a pseudomonad (2). In such a scheme fluorobenzoic acid (I) would react with molecular oxygen, presumably to form a cyclic peroxide (II), which would then be reduced to catechol (IV). In this mechanism a single molecule of oxygen supplies the 2 oxygen atoms of the catechol. Another possible scheme for the conversion of 2-fluorobenzoate to catechol is suggested by the conversion of 4-fluorophenylalanine to tyrosine catalyzed by the enzyme phenylalanine hydroxylase (3). An analogous defluorination of 2-fluorobenzoate might be expected to yield salicylate (III), which is known to be oxidized to catechol by a hydroxylase found in *Pseudomonas* (4). The successive action of two hydroxylases in the second scheme would be expected to produce catechol derived from 2 separate molecules of oxygen rather than 1 (Diagram 1).

This paper reports on the use of mass spectrometry to show that the catechol derived from 2-fluorobenzoate is formed from only 1 molecule of oxygen, a finding which suggests that a cyclic peroxide is involved in the reaction.

[1] Paper I of this series.

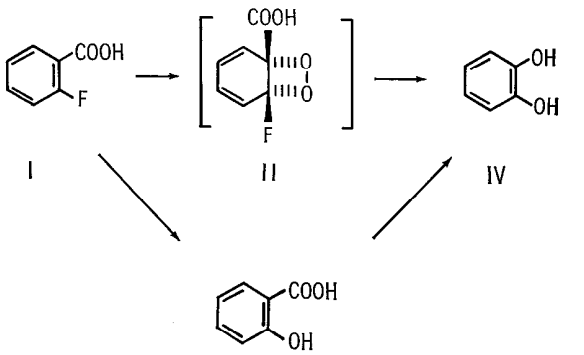

DIAGRAM 1

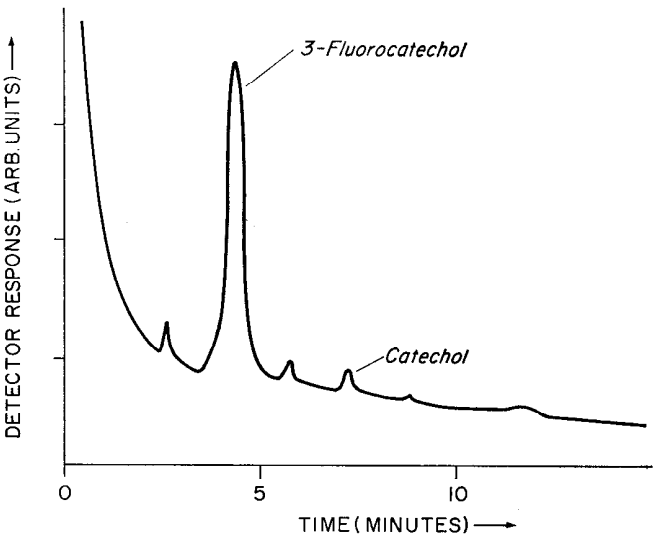

FIG. 1. Gas-liquid chromatogram of the crude catechol mixture. Chromatography was performed on a 6-foot column of 3% OV-17, maintained at 120°.

EXPERIMENTAL PROCEDURE

Materials—2-Fluorobenzoic acid (Aldrich Chemicals) was recrystallized from toluene and its purity was established by gas-

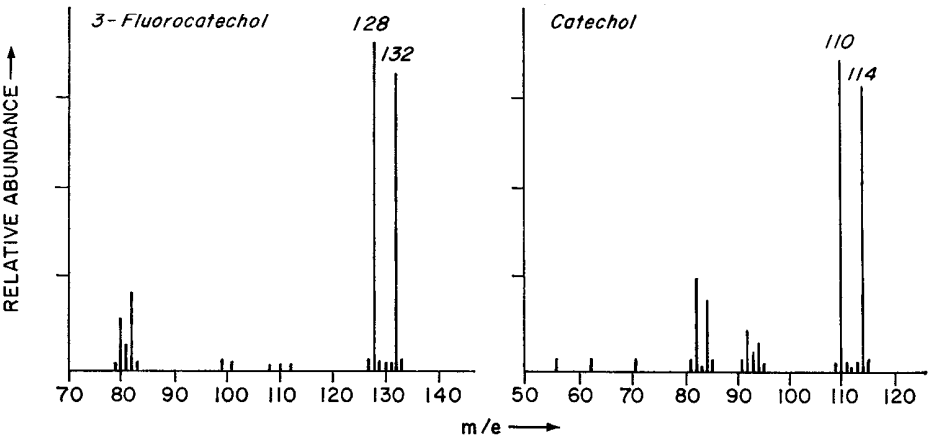

FIG. 2. Low resolution mass spectra of 3-fluorocatechol (*left*) and catechol (*right*). Spectra were recorded in an LKB-9000 spectrometer with an ionizing potential of 70 e.v. Catechols were isolated from an incubation mixture with initial gas composition of 85% N_2, 7.5% $^{18}O_2$ (91%), 7.5% $^{16}O_2$ provided by manometry, and a final composition of 87.5% N_2, 3.8% $^{18}O_2$, 8.0% $^{16}O_2$, and 0.6% O_{16}-O_{18} as analyzed by mass spectrometry. A slight air leak in the manometry presumably accounted for the increase in the percentage of $^{16}O_2$ and N_2 during the incubation.

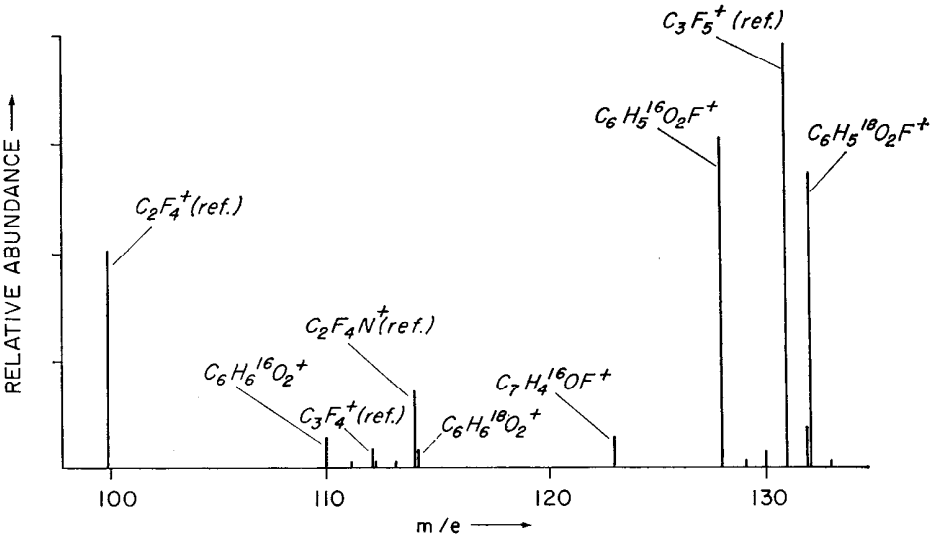

FIG. 3. Partial high resolution mass spectrum of the catechol mixture was determined on an AEI MS-9 double-focussing mass spectrometer at 70 e.v. and a source temperature of 100 ± 20°. Peak matching at a resolving power of 1 in 10,000 (10% valley) was carried out with reference to an internal standard of perfluorotributylamine.

TABLE I

Accurate masses of ions from catechol mixture

Observed *m/e*	Formula	Calculated *m/e*
110.0374	$C_6H_6{}^{16}O_2$	110.0368
114.0457	$C_6H_6{}^{18}O_2$	114.0453
123.0240	$C_7H_4{}^{16}OF$	123.0246
128.0277	$C_6H_5{}^{16}O_2F$	128.0274
132.0360	$C_6H_5{}^{18}O_2F$	132.0358

liquid chromatography of its methyl ester (3% OV-17 at 120°, Applied Science Laboratories Inc., State College, Pennsylvania). These conditions would have enabled the detection of 0.1% benzoic acid and no evidence of this contaminant was found. 3-Fluorocatechol was synthesized by the method of Corse and Ingraham (5). $^{18}O_2$ (91%) was obtained from Miles Laboratories, Inc., Elkhart, Illinois.

Bacterial Growth—A pseudomonad which can utilize 2-fluorobenzoic acid as a sole carbon source was allowed to grow at 24–30° with aeration in a medium with the following composition per liter: salts A, 5 ml; salts B, 1.5 ml (6); $K_2HPO_4 \cdot 3H_2O$, 1.2 g; KH_2PO_4, 0.5 g; sodium 2-fluorobenzoate, 1.0 g; sodium succinate, 1.0 g; and NH_4NO_3, 10 g. Cells were harvested by centrifugation 4 to 7 hours after the end of the logarithmic growth and were stored at −15° until used. The yield was approximately 0.5 g of cells (wet weight) per liter of medium.

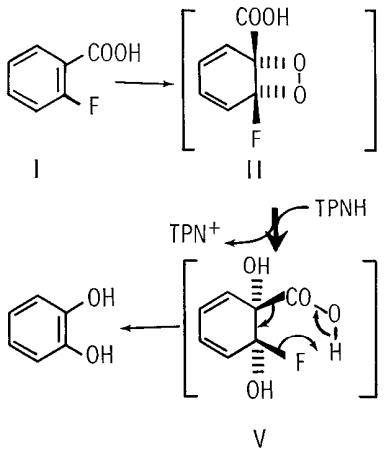

DIAGRAM 2

Incubation Conditions and Extraction of Product—In 125 ml of 0.01 M potassium phosphate buffer, pH 6.5, containing 8×10^{-3} M sodium 2-fluorobenzoate, 4 g of cells (wet weight) were suspended.

The incubation was carried out in a reaction flask as described by Rothberg and Hayaishi (7) in an atmosphere consisting of approximately 85% N_2, 7.5% $^{16}O_2$, and 7.5% $^{18}O_2$. After the completion of the incubation the composition of the gas phase was analyzed by mass spectrometry. During incubation at 25° with shaking, aliquots were removed from a parallel reaction mixture and analyzed for catechol content (8). When successive analyses at 30-min intervals revealed no further increase in catechol (2 to 3 hours) (1), the cells were removed by centrifugation at 0–4° and the medium was extracted three times with half volumes of ether. The ether extracts were pooled, dried over sodium sulfate, and evaporated at 0° under reduced pressure.

RESULTS

Gas-liquid chromatography of the extracted growth medium in comparison with authentic standards indicates that there is approximately 5% as much catechol as fluorocatechol (Fig. 1). On the LKB-9000 gas chromatograph-mass spectrometer a similar separation of these compounds was effected; in this instrument the low resolution mass spectra of the resolved compounds were recorded in 10-sec scans of the column effluent and these mass spectra confirmed the identity of the two catechols. Fig. 2 presents the spectra of catechols derived from 2-fluorobenzoate during an incubation with a mixture of $^{18}O_2$ and $^{16}O_2$ (where the amount of ^{18}O-^{16}O molecules was negligible). In addition to the ion at *m/e* 110 (the molecular weight of catechol), there is an ion at *m/e* 114, and the relative heights of these peaks are in accord with the composition of $^{18}O_2$-$^{16}O_2$ in the gas phase of the incubation. Of particular interest is the absence of a peak at *m/e* 112, which indicates that there is no mixed isotope incorporation. These results favor the incorporation of a whole molecule of oxygen rather than 2 separate atoms, and are thus consistent with the cyclic peroxide intermediate (Diagram 1, II) rather than the successive action of two hydroxylases in the conversion of 2-fluorobenzoate to catechol. It should be noted, however, that this evidence does not preclude the intermediacy of a noncyclic peroxide.

The mass spectrum of 3-fluorocatechol is comparable to that of catechol, where, in addition to the peak at *m/e* 128 (the molecular weight of 3-fluorocatechol), there is a slightly smaller peak at *m/e* 132. Again, there is no evidence for mixed isotope incorporation, which would yield a peak at *m/e* 130.

Since the amount of catechol was small and could not be completely separated from 3-fluorocatechol, high resolution mass spectrometry was performed on the mixture. The significant ions (those above *m/e* 100) were measured accurately by peak matching, and this region of the spectrum is shown in Fig. 3. The formula assignments of Fig. 3 are based on the accurate masses of the ions given in Table I.

The ion at *m/e* 123.0240 is a fragment ion from the 2-fluorobenzoic acid present in the mixture. It is noteworthy that the high resolution mass spectral data confirm that the ions at *m/e* 110 and 114 are not merely fragmentation ions of the 3-fluorocatechols. These data therefore support the conclusions of the low resolution mass spectra.

DISCUSSION

These experimental results indicate that catechol and 3-fluorocatechol formed from 2-fluorobenzoic acid incorporate either 2 atoms of ^{16}O or 2 atoms of ^{18}O from an atmosphere consisting of a mixture of $^{16}O_2$ and $^{18}O_2$. The data rule out the introduction of oxygen atoms by successive hydroxylation reactions in which one would expect to find a random mixture of the two oxygen isotopes in the catechols. The incorporation of both oxygen atoms from a single molecule of oxygen can be explained in several ways but a cyclic peroxide intermediate analogous to that offered to explain similar data in the oxidation of anthranilic acid (2) is particularly attractive. In this mechanism of oxygenation a *cis* configuration of the reduced diol (V) is required and it is this stereochemistry which permits a low energy transition state leading to the spontaneous elimination HF and CO_2 (Diagram 2). This is analogous to the elimination of NH_3 and CO_2 from anthranilic acid in its conversion to catechol (2).

In the conversion of 2-fluorobenzoic acid to 3-fluorocatechol the fluorine probably plays no significant role, which suggests that this reaction is analogous to the conversion of benzoic acid to catechol (9).

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