Cleavage of Ether-Oxygen Bond in Phenoxyacetic Acid by an Arthrobacter Species

Phenoxy-^{18}O-acetic acid is metabolized by resting cells and cell-free extracts of an *Arthrobacter* sp. to phenol-^{18}O. The complete conversion by intact cells and the quantitative retention of the ^{18}O in the phenol demonstrates that the cleavage occurs between the aliphatic side chain and the ether-oxygen atom.

The metabolism of phenoxyalkanoic acids, an important class of herbicides, has been extensively studied in microbial, plant, and animal systems (Freed and Montgomery, 1963; Kearney *et al.*, 1967; Menzie, 1966). Breakdown generally proceeds with bacteria by loss of the alkanoic side chain, followed by hydroxylation and aromatic ring cleavage. With an *Arthrobacter* sp. the first product after the removal of the side chain is the corresponding phenol (Loos *et al.*, 1967a, b). No information exists which indicates the ether-oxygen bond that is cleaved.

Phenoxyacetic acid, the simplest analog of the phenoxy herbicides, was selected because the chosen organism is unable to degrade further the phenol produced (Loos *et al.*, 1967b).

MATERIALS AND METHODS

Labeled phenoxyacetic acid was synthesized by reaction of phenol-^{18}O (Isomet Corp., Palisades Park, N. J.; 10.3 atom % ^{18}O) with chloroacetic acid in aqueous NaOH. The product was recovered by acidification, extraction into diethyl ether, re-extraction into aqueous 5% $NaHCO_3$, and acidification. Identity of the acid was confirmed, after recrystallization from hot water, by its melting point, gas chromatographic characteristics, and infrared spectrum. Phenol contamination, determined by gas chromatography, was negligible.

An *Arthrobacter* sp. isolated from soil and used for the study of 2,4-D (2,4-dichlorophenoxyacetic acid), 2- and 4-chlorophenoxyacetic acid, and MCPA (4-chloro-2-methylphenoxyacetic acid) metabolism (Bollag *et al.*, 1967; Loos *et al.*, 1967a) was cultured with MCPA as sole carbon source using the conditions previously described (Bollag *et al.*, 1967). For the preparation of resting cell suspensions or cell extracts, cells were collected near their peak of exponential growth and were washed three times with chilled 0.02*M* phosphate buffer, pH 7.0. Essentially complete conversion of the substrate to phenol was achieved by incubating 350 mg. (dry weight) of cells with 250 ml. of 10^{-3}*M* phenoxy-^{18}O-acetic acid for 3 hours on a rotary shaker at 25° C. The reaction was terminated by addition of tungstic acid, and cells and cellular debris were removed by centrifugation. The acidified supernatant of a resting cell experiment was extracted twice with diethyl ether; the combined extracts were dried with Na_2SO_4 and concentrated to ca. 5 ml. in vacuo.

Phenol contents of the aqueous incubate and of the ether extracts were determined by the 4-aminoantipyrine colorimetric method (Loos *et al.*, 1967b) and by gas chromatography, respectively. An Aerograph Model A-700 chromatograph equipped with a flame ionization detector was used. It had a 3.0-meter × 7.5-mm. I.D. column containing 10% DC 200 on Gas-Chrom Q (80- to 100-mesh). Column, injector, and detector temperatures were, respectively, 170°, 225°, and 250° C. Nitrogen flow was ca. 135 ml. per minute.

Mass spectra of unlabeled phenoxyacetic acid and phenoxy-^{18}O-acetic acid were obtained by direct inlet (70° C.) into an Hitachi-Perkin-Elmer mass spectrometer. Unlabeled and metabolite phenol, as ether solutions, were first injected into a Perkin-Elmer gas chromatograph. The column used was 61-meter × 0.5-mm. I.D. and the liquid phase was Carbowax 20M maintained at 200° C. The carrier gas was He and the flow rate was 10 ml. per minute. The column effluent was monitored by a flame ionization detector and a portion passed through a separator at 170° C. into the spectrometer.

RESULTS AND DISCUSSION

Production of phenol from phenoxyacetic acid by freshly harvested cells was 100% after 2 hours, but more than 3 hours were necessary for total conversion if the cells were stored for 3 days at −15° C. The considerable lability of the phenoxyalkanoic acid-active enzyme was also obvious if cell-free extracts were prepared with a French pressure cell as previously described (Bollag *et al.*, 1967). The enzyme preparation was able to convert phenoxyacetic acid to phenol at a high rate (80% turnover) with a new preparation but its activity was lost after a few days' storage in the cold. No activity of the cell-free extract was observed if the enzyme assay was performed in Thunberg tubes from which the air was initially removed and replaced by nitrogen. This suggests that the reaction requires oxygen.

Mass spectra were recorded at three points during phenol appearance. All three spectra were consistent. This indicates satisfactory isolation of metabolite phenol from residual phenoxyacetic acid, which would have produced a phenol peak by fragmentation when ionized. Mass spectra of all samples were recorded at ionizing voltage, 80 volts, and the three reference samples additionally at 14 volts. The former value emphasized fragmentation of the molecule introduced and the latter value suppressed fragmentation, thus emphasizing parent molecular ion formation.

Table I. Labeling of Phenol Produced from Phenoxy-^{18}O-Acetic Acid Incubated with *Arthrobacter* sp.

Sample	$\frac{P+2^a}{P} \times 10^3$	^{18}O Excess, Atom %
	THEORY	
Phenoxyacetic acid	9.50	
Phenol	3.85	
	FOUND	
Phenoxyacetic acid	10.0 ± 0.5	
Phenoxy-^{18}O-acetic acid	17.5 ± 0.5	0.8 ± 0.1
Phenol (reference)	3.5 ± 0.2	
Phenol (metabolite)	12.3 ± 0.7	0.9 ± 0.1

[a] Ratio values represent the relative intensity of the ^{18}O-enriched ($P + 2$) to normal (P) parent molecular ion.

The correlation of theoretical (Beynon and Williams, 1963) and observed ratios of parent molecular ion intensities (Table I) for unlabeled standards indicates that the mass spectral data are reliable. If the small differences presented are real, they could have been caused by use of average corrections for unknown constituents due to column bleed which were observed to vary with sample solvent, column packing, and temperature. It is apparent that ^{18}O was totally retained by metabolite phenol, indicating cleavage occurs between ether-oxygen and the aliphatic group.

Indirect evidence of this cleavage mechanism was obtained by Renson (1964), who found only unlabeled *p*-hydroxyacetanilide when *p*-methoxyacetanilide was metabolized by liver microsomes in the presence of $^{18}O_2$ or $H_2{}^{18}O$; formaldehyde-^{18}O was trapped in the system containing $^{18}O_2$. Formation of formaldehyde or acetaldehyde from methoxy or ethoxy aromatic ethers has also been demonstrated with microorganisms (Woodings, 1961). Using similar conditions, Axelrod (1956) earlier established the requirement for O_2 and NADPH in ether cleavage, suggesting operation of a mixed function oxidase enzyme.

One mechanism of cleavage, that of attack on aromatic ring carbon 1, as occurs during periodate oxidation of certain anisoles (Adler *et al.*, 1962), would lead to partial or total loss of the label. This mechanism is therefore eliminated. A more likely pathway is prior oxidation of the methylene carbon since its protons are activated by both carboxyl and phenoxy groups. If hydroxylation occurs, the resultant phenoxy-^{18}O-glycolic acid would, by analogy with other hemiacetals, probably decompose to phenol-^{18}O and glyoxylic acid. On the other hand, if further oxidation occurs to form phenyl oxalate, phenol-^{18}O should also be found since ester hydrolysis, chemically and biologically, usually proceeds by cleavage adjacent to the acyl carbon (conditions known to cause chemical alkyl-oxygen cleavage include acid catalysis and formation of a stable carbonium ion) (Ingraham, 1962; Ingold, 1953).

Although this experiment used only phenoxyacetic acid, the result should be applicable to the various phenoxyacetic herbicides, because the same organism also degraded 2,4-D, MCPA, 2-chlorophenoxyacetic and 4-chlorophenoxyacetic acid to their corresponding phenol.

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