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The Anaerobic Decomposition of Benzoic Acid during Methane Fermentation

III. The Fate of Carbon Four and the Identification of Propanoic Acid*

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Abstract. Anaerobic rupture of the benzoic acid ring was investigated. Carbon 4 was converted primarily to carbon dioxide. Following ring rupture during methane fermentation, propanoic acid is an intermediate, and carbon 4 of benzoate becomes its carboxyl.

Key words: Anaerobic – Benzoate – Degradation – Methane – Propanoate.

Anaerobic utilization of benzoic acid during methane fermentation was first reported by Tarvin and Buswell (1934); confirmed by Clark and Fina (1952); and later by Nottingham and Hungate (1969). Fina and Fiskin (1960) established that carbon 1 of benzoic acid was converted to methane, and carbon 7, primarily to carbon dioxide. They also established that carboxyl carbons are converted to CO_2 and some may subsequently be reduced to methane.

Recent work on the anaerobic assimilation of benzoic acid has been done with systems other than methane producers. Dutton and Evans (1969) showed that the photoassimilation of benzoate by *Rhodopseudomonas palustris* involved reducing the aromatic ring to cyclohex-1-ene-1-carboxylate, followed by hydration to 2-hydroxycyclohexanecarboxylate. After dehydrogenation to 2-oxycyclohexanecarboxylate, further hydration results in ring fission and produces pimelic acid. They also detected some cyclohexane carboxylic acid, but did not clarify its role. A different path for assimilation of benzoate has been proposed for a *Moraxella* sp. during nitrate respiration (Williams and Evans, 1975). Ring reduction steps are the same, but the ring rupture product for the *Moraxella* sp. is adipic acid instead of pimelic acid. In this path 2-oxycyclohexanecarboxylate is decarboxylated to cyclohexanone. Subsequent dehydrogenations and additions of water result in adipic acid.

We investigated the metabolism of benzoic acid by methanogenic enrichment cultures because of the extreme anaerobic conditions necessary and apparent difference from previous pathways proposed. Methane-forming bacteria cannot function at oxidation-reduction potentials above −330 mv (Smith and Hungate, 1958). At −330 mv the amount of oxygen present is calculated at 1.48×10^{-56} molecules/l. Obviously, methane production precludes any oxygen being present (Hungate, 1969). The objectives were to identify products formed during benzoic acid degradation and determine the possible rupture point(s) on the aromatic ring. The fate of carbon 4 was of prime interest.

Cultural Methods and Procedures

In general, cultures and procedures were those of Fina and Fiskin (1960). The culture-consortiums used were subcultures of highly enriched stocks obtained from sewage sludge and maintained solely on benzoic acid for more than ten years. They were kept in carbon balance; i.e., for each 0.3–0.5 mmole of benzoic acid fed daily (normal feed), equivalent amounts of gas were produced (2.1–3.5 mmoles per 24 h). All gas fermentors were used with positive inside pressure to insure anaerobiosis. Methane and carbon dioxide produced were collected manometrically under lithium chloride solution (Boell et al., 1939) and periodically released. The ^{14}C was added as [4-^{14}C] benzoic acid[1], or as commercially obtained [7-^{14}C] benzoic acid or [1-^{14}C] benzoic acid. The ^{14}C of methane and carbon dioxide collected was analyzed as described by Fina and Fiskin (1960).

In preparation for isolation of intermediates, we gave three times normal feed (force feeding) containing 0.1–0.5 mCi of appropriate label. After 2–3 h, approximately 50 ml of the 100 ml culture was

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Table 1. Fate of carbon 4 during anaerobic decomposition of benzoic acid

Time h	CH_4		CO_2		Ratio of specific activities CH_4 to CO_2
	Specific activity[a]	mmoles gas collected	Specific activity[a]	mmoles gas collected	
3.5	1.15	0.500	4.44	0.490	1:3.9
7.0	6.15	0.628	19.07	0.516	1:3.1
10.75	9.56	0.608	32.50	0.385	1:3.4
14.50	26.00	0.569	93.50	0.569	1:3.6
21.75	27.00	0.616	113.70	0.437	1:4.2

[a] Counts per min per mmole $\times 10^{-3}$

removed and centrifuged to remove any asbestos nidus and other particulate matter. Clear supernatant was acidified with H_3PO_4 and direct distilled. The first 10 ml of distillate was used to form the hydroxamates by the method of Block et al. (1955). The hydroxamates were formed, and propanohydroxamic acid isolated on Whatman 1 paper by a solvent system of amyl alcohol-acetic acid-water (4:1:5 v/v). Located by development with ferric chloride, the propanohydroxamic acid spot was cut out and its ^{14}C activity measured in a windowless gas-flow, α, β, γ, proportional counter.

Determination of the location of radioactivity in propanoic acid was done with samples obtained by isotopic trapping. The following procedure was used: 14 h after a normal feed of 0.5 mmole benzoate (containing 1–3 μCi of label), 2 mmoles of neutralized nonlabeled propanoic acid was added to a 400 ml culture; after an additional 5 h incubation, most (300–350 ml) of the culture fluid was removed, acidified, and direct distilled; the distillate (90% of the culture fluid) was then neutralized and evaporated to a volume of 3 ml. Benzoate was removed by precipitation at pH 4 and centrifuged at 4° C. Gas chromatography showed propanoic acid to be the only volatile fatty acid in the sample. This propanoic acid was then decarboxylated by the Phares-Schmidt procedure (Arnoff, 1956). Phenethylamine was substituted for NaOH to absorb CO_2 (Woeller, 1961). Radioactivity of the CO_2 was assayed in a liquid scintillation counter.

New Barker's solution "A" (Fina and Fiskin, 1960), containing cold benzoate substrate, was added to nidus-culture-consortium used for isotopic trapping experiments, to replace fluid removed. Residual radioactivity in culture fluid was determined by periodically removing 1 ml and assaying the ^{14}C directly in a liquid scintillation counter.

Results

Carbon 4 of benzoic acid was converted primarily to carbon dioxide (Table 1). Part was reduced to methane; however, this was not favored. Determined by liquid scintillation, the specific activity ratio of CH_4 to CO_2 was 1:4.2 after 21 3/4 h, and in a subsequent experiment the ratio was 1:4.8 after 24 h.

Propanoic acid was isolated and identified as its hydroxamate when cold benzoic acid was the substrate. Benzoate-using cultures fed propanoic acid yielded gas at a comparable rate and with no lag, if quantities of substrates were adjusted to contain equal total carbon. The propanohydroxamate spots isolated from cultures fed [1-^{14}C] or [7-^{14}C] benzoic acid contained no activity above background, but when [4-^{14}C] benzoic acid was fed, the spot had five times the activity of background. Background was approximately 125 counts per min.

The carboxyl carbon of propanoic acid obtained from the isotopic trapping trials was radioactive when [4-^{14}C] benzoic acid was the substrate (Table 2). No further degradation was done as there was no radioactivity in the remaining reaction mixture. Tests indicated

Table 2. Fate of benzoic acid Carbons revealed by Phares Schmidt-degradation of propanoate

Labeled substrate	$^{14}CO_2$ trapping solution	Disintegrations per min[a]
ϕCOOH-1-^{14}C	0.5 N NaOH	0
ϕCOOH-1-^{14}C	Phenethylamine	57
ϕCOOH-4-^{14}C	0.5 N NaOH	6400
ϕCOOH-4-^{14}C	Phenethylamine	14600

[a] Average of two determinations, calculated as disintegrations per min above background for the entire quantity of culture liquid

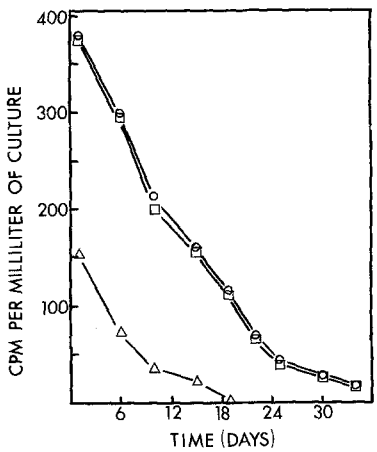

Fig. 1. Disappearance of residual radioactivity in representative experimental cultures fed labeled benzoic acid. At the end of each isotopic trapping trial, most of the culture fluid was removed for analysis and subsequently replaced with new fluid containing cold substrate. Gas yields continued unabated. Symbols: (△) benzoic [1-^{14}C] acid; (□) benzoic [7-^{14}C] acid; (○) benzoic [4-^{14}C] acid

that Schmidt degradation would not rupture the aromatic ring but could decarboxylate benzoic acid.

Residual radioactivity remained more than 33 days in cultures given [7-^{14}C] or [4-^{14}C] benzoic acid as substrate. Radioactivity in cultures given [1-^{14}C]benzoate as substrate was eliminated by 20 days (Fig. 1).

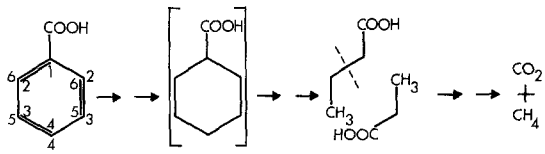

Fig. 2. Proposed scheme for the anaerobic decomposition of benzoic acid during methane fermentation

Discussion

The fates of benzoate ring carbons 1, 7 (Fina and Fiskin, 1960) and now of carbon 4 are known; in addition, evidence presented suggests strongly that propanoic acid is an intermediate following the rupture of the benzoic acid molecule in the pathway producing methane and carbon dioxide. Carbon 4 of benzoic acid is converted to the carboxyl carbon of propanoic acid, as shown by the Phares-Schmidt procedure. The carboxyl is then converted primarily to CO_2, as indicated by the final CH_4-to-CO_2 ratio of specific activities, which is comparable to the cases of carbon 7 of benzoic acid and exogenous $^{14}CO_2$ added as $Na_2\ ^{14}CO_3$ (Fina and Fiskin, 1960). The similarity of the metabolic fates of carbons 4 and 7 is also indicated by the nearly identical retention of radioactivity in the fluids of cultures used for isotopic trapping (Fig. 1). One hot feed was followed by successive cold feedings in cultures fed [4-^{14}C] or [7-^{14}C] benzoic acid. Exchange between exogenous CO_2 and the carbonate buffer used account for the prolonged retention of radioactivity. However, as carbon 1 goes directly to CH_4, exchange is quite limited.

From the evidence presented, the direct production of propanoic acid can be explained if the ring is first cleaved between carbons 1 and 6. The molecule then undergoes β-oxidation to release carbons 4, 5, and 6 as propanoate. However, since the ring is symmetrical and may be numbered either way, the propanoic acid could contain carbons 2, 3, and 4 if the first cleavage was between carbons 1 and 2. In either case, carbon 4 will still be the carboxyl of propanoate. We hypothesize that propanoic acid comes directly from the ring skeleton because carbons 1 and 7 are not found in the propanoate as would be the case if the molecule were synthesized from other degradative products. The production of methane from propanoic acid has been studied by Buswell et al. (1951) and Stadtman and Barker (1951). Incidentally, the isotopic trapping technique was necessary to locate the label in propanoic acid. The acid is found in trace amounts as would be expected for a potential intermediate. A large amount of a suspected intermediate would indicate a side path, as Dagley and Nicholson (1970) suggest.

It is proposed that the events shown in Figure 2 occur in the anaerobic rupture of benzoic acid by methanogenic cultures. The dearomatization of the benzene nucleus to cyclohexane carboxylic acid is proposed. Unlike other paths reported, hydroxylated intermediates do not appear likely. Cultures adapted to use benzoic acid will not produce methane and carbon dioxide from hydroxylated derivatives of benzoic acid except after a prolonged delay (Clark and Fina, 1952). In addition, these cultures failed to use pimelic acid; isobutyric acid, a branched chain fatty acid; or fatty acids containing double bonds.

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