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Methanogenic Fermentation of Benzoate

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A methanogenic enrichment culture decomposed small concentrations of ^{14}C-benzoate to $^{14}C_4$ and $^{14}CO_2$ under stringently anaerobic conditions with or without preceding exposure to benzoate.

Although Fina and Fiskin (4) reported that benzoate-*1*-^{14}C and -*7*^{14}C were converted anaerobically to methane and carbon dioxide, subsequent publications (1, 7) have not mentioned their work and have inferred that molecular oxygen is necessary for cleavage of the benzene ring. An exception has been found in *Rhodopseudomonas palustris* which has been shown (2, 3, 5, 8) to oxidize benzoate in the dark with oxygen and in the light without oxygen.

Availability of uniformly ring-labeled material prompted a reexamination of benzoate utilization in methanogenesis.

MATERIALS AND METHODS

A 1-liter anaerobic methanogenic laboratory digest, started initially with sewage sludge and fed nutrient broth plus yeast extract and glucose on a 10-day turnover time, was maintained in the laboratory for several weeks. The daily gas production became relatively constant. Small-volume enrichments were started by transferring 5 ml of the contents of the 1-liter digestor to each of several butyl rubber-stoppered test tubes (16 by 150-mm) from which all air was displaced with CO_2 gas freed of oxygen by passage through a column of hot (350 C) copper. Each day a 0.5-ml quantity was removed from these small cultures, and they were fed with 0.5 ml of NYG medium composed of 1% Nutrient Broth (Difco), 1% Yeast Extract (Difco), and 1% glucose in the mineral medium of Lawrence and McCarthy (6) containing (per liter): 20 ml of solution A [2.28% $(NH_4)_2HPO_4$] and 20 ml of solution B (3.73% KCl, 4.635% NH_4Cl, 5.075% $MgCl_2 \cdot 6H_2O$, 1.334% $FeCl_3 \cdot 6H_2O$, and 0.0783% $CoCl_2 \cdot 6H_2O$).

The routine cultures were incubated at 37 C in a slanting position on a shaker and the amount of gas produced each day was measured by inserting a needle attached to a 10-ml syringe (dead space filled with O_2-free CO_2 or digestor gas) and noting the volume of gas forced into the syringe as the needle penetrated the stopper. The gas in the syringe was analyzed for methane and carbon dioxide on a Perkin-Elmer Vapor Fractometer fitted with a silica gel column. After several weeks of subculturing on this 10-day cycle, 8 ml of gas was produced per day, composed of 53% CH_4 and 47% CO_2.

In the experimental cultures, gas produced was measured daily with the syringe or allowed to accumulate in the culture until finally analyzed.

Radioactivity in the CO_2 was determined by absorbing the collected gas in 0.1 N NaOH. An 0.5-ml amount of the solution was added to 15 ml of Bray's solution and counted on a Packard Tri Carb Series 3000 liquid scintillation counter. After absorption of CO_2 the gas was passed through CuO at 700 C to combust the methane to CO_2, which was then absorbed in sodium hydroxide; a sample was counted.

The uniformly ring-labeled benzoic acid, obtained from Amersham/Searle (97% radiopure), showed a count of 1.5×10^6 per min per μc.

RESULTS

In a first experiment, the nutrient of several cultures was changed from 0.5 ml of NYG medium per tube to: (i) 0.25 ml of NYG medium plus 0.25 ml of 1% sodium benzoate, (ii) 0.1 ml of NYG medium plus 0.4 ml of 1% benzoate, (iii) 0.5 ml of 1% benzoate containing 5 μmoles of sodium pyruvate, or (iv) the same as iii except that 0.4 ml of hydrogen gas was also added. Gas production in all the experimental tubes decreased as compared with controls fed 0.5 ml of NYG and after 5 days was almost nil.

In a second experiment, 0.02% sodium benzoate was added to the NYG medium, and an 0.5-ml amount was fed daily for 13 days to four tubes, controls receiving NYG medium without benzoate. Cultures with benzoate produced about 0.5 ml more gas per day than did the controls. After adaptation of the enrichments to benzoate, one pair of tube cultures was fed daily for 5 days with 0.5 ml of NYG medium containing 0.5 μc (3 μg) of the ^{14}C-benzoic acid. Gas produced was measured for 5 days. On the 6th day, the two cultures were fed 0.5 ml of mineral solution containing 2 μc of ^{14}C-benzoate and incubated without further additions of any substrate. After 9 days, the excess gas was analyzed for radioactivity

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in CO_2 and CH_4. After 5 additional days of incubation, 0.5 ml of 5 N HCl was added to each tube to convert bicarbonate to CO_2. The gas was measured and analyzed. In a sample of the residual liquid, the radioactivity (probably due chiefly to $^{14}CO_2$, since dissolved gas was not driven out of the liquid) was also counted.

The recovered counts in duplicate cultures (Table 1) were 70 and 74%, respectively, of the added 6.75×10^6 counts per min. Some label was lost in the 0.5 ml of medium discarded each day during the first 5 days of feeding labeled benzoate.

In another experiment, a single dose of labeled benzoate was added. Tubes 3 and 4, fed 0.5 ml of NYG medium containing 0.02% unlabeled benzoate each day for the preceding 18 days, were fed only salt solutions containing 2 μc of ^{14}C-benzoate. This same quantity of labeled benzoate was added to tubes 5 and 6, previously fed daily with 0.5 ml of NYG medium containing no benzoate. All the tubes were incubated for 14 days without addition of any substrate. At 9 days, the excess gas was measured and counted; on the 14th day, 0.5 ml of 5 N HCl was added to each tube and the total gas was measured and counted (Table 2). The initial scintillation count was 3×10^6 counts per min.

To confirm the identity of the labeled benzoate and its seeming conversion into methane and carbon dioxide, a concentration of the labeled benzoate similar to that in tubes 3 to 6 was spotted on a thin-layer silica gel plate and chromatographed in parallel with similar volumes of culture medium from tubes 3 to 6 at the end of incubation. The chromatogram was developed with a mixture of 100 ml of 96% ethyl alcohol–12 ml of water–16 ml of 25% ammonia. A sample of benzoic acid showed an R_F identical with the R_F of the radioactivity of the ^{14}C-benzoate as detected with photographic film placed against the silica gel. Tube 3 showed a trace of radioactivity with the R_F of benzoic acid, but media from the other tubes showed none at this point nor at any other location on the plate. No radioactivity was detected at the origin. The benzoic acid had been completely metabolized.

DISCUSSION

If sodium benzoate were converted to $NaHCO_3$ plus a total of six molecules of CH_4 and CO_2, the expected gas from 0.5 ml of 0.02% sodium benzoate would be 0.105 ml at 37 C and one atmosphere of pressure. The daily production of 0.5 ml of excess gas in the benzoate cultures is unexplained.

If ring-labeled benzoic acid were converted to methane and carbon dioxide without the participation of other substrates, the stoichiometry of the reaction would be: $4\ ^{14}C_6H_6CO_2 + 18\ H_2O \rightarrow 15\ ^{14}CH_4 + 9\ ^{14}CO_2 + 4\ CO_2$. From this equation, labeled methane would be expected to constitute 62.5% of the labeled gaseous products. From the results of experiments 3 to 6 (Table 2) it can be calculated that labeled methane constituted 59, 52, 59, and 58%, respectively, of the labeled products. These values suggest that the carbons of the benzoic acid were in part oxidized and in part reduced, in approximate agreement with the equation.

If the anaerobic decomposition of benzoate involved the metabolic activities of a number of bacteria, one of which removed hydrogen to oxidize the ring to acetate, a second which converted the acetate to methane and carbon dioxide, and a third which used the hydrogen to reduce carbon dioxide (chiefly unlabeled since the tubes contained much CO_2) to methane, half of the ring carbons would be in methane and half in carbon dioxide. The greater than 50% yield of radioactivity in methane and the fairly good agree-

TABLE 1. *Labeled products from the methanogenic fermentation of benzoate in duplicate cultures*

Incubation time	Gas produced in duplicate cultures		Scintillation count (10^6 counts/min)				Medium		Recovered label	
			CO_2		CH_4					
	A	B	A	B	A	B	A	B	A	B
hr	*ml*	*ml*								
1	9.8	9.0	0.00	0.00	0.01	0.01	—	—		
2	9.2	9.2	0.01	0.02	0.03	0.05	—	—		
3	8.4	8.2	0.01	0.02	0.03	0.05	—	—		
4	8.6	9.4	0.03	0.05	0.08	0.13	—	—		
5	9.3	8.6	0.04	0.05	0.08	0.13	—	—		
14	4.3	4.2	0.20	0.14	0.54	0.52				
19	32.5[a]	34.0[a]	1.93	1.60	1.85	1.82	0.15	0.13		
Sum			2.22	1.88	2.62	2.71	0.15	0.13	4.99	4.72

[a] After addition of 0.5 ml of 5 N HCl; includes gas remaining in culture tube.

TABLE 2. *Recovery of label from a single dose of ^{14}C-benzoic acid*

Tube	Incubation	Gas analyzed	Scintillation count (10^6 counts/min)			Total	Recovery of label
			CO_2	CH_4	Medium		
	days	*ml*					%
3	9	5.0	0.14	0.37	0.08		
	14	28.0[a]	0.83	1.15	0.07		
	Sum		0.97	1.52	0.15	2.64	88
4	9	4.3	0.13	0.33	0.09		
	14	29.0[a]	1.03	1.10	0.07		
	Sum		1.16	1.43	0.16	2.75	92
5	9	8.4	0.13	0.55	0.09		
	14	31.5[a]	0.86	1.10	0.06		
	Sum		0.99	1.65	0.15	2.79	93
6	9	8.6	0.13	0.54	0.09		
	14	31.0[a]	0.79	0.96	0.06		
	Sum		0.92	1.50	0.15	2.57	86

[a] After addition of 0.5 ml of 5 N HCl; includes gas remaining in culture tube.

ment with the stoichiometry of the above equation are consistent with the view that the hydrogens removed from certain carbons of the benzoic acid are transferred to other carbons from the same substrate. This might indicate that a single organism was responsible for the conversion of the benzoate. The presence of other substrates in the enrichment and the myriads of species in the mixed culture make interpretation difficult. However, regardless of the organisms concerned, the results of the experiments confirm that methanogenesis from benzoate can occur in the complete absence of oxygen.

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