[CONTRIBUTION FROM THE ILLINOIS STATE WATER SURVEY]

Use of C^{14} in Mechanism Studies of Methane Fermentation. II. Propionic Acid

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The action of anaerobic bacteria on propionates to yield carbon dioxide and methane quantitatively has been studied. When propionates having the carbons 1, 2 and 3 labeled, respectively, were fed to methane-producing bacterial cultures which had been adapted to and maintained on propionic acid, the distribution of radioactivity in the evolved gases indicated that propionic acid is split in such a manner that both gases may arise from all three carbons, and that carbon dioxide is, in part at least, a precursor of the methane formed. A preliminary hydrolysis of propionic acid to ethyl alcohol and formic acid is consistent with the results obtained. This is in part confirmed by simultaneous adaptation experiments. When labeled carbon dioxide was administered to cultures stabilized on formic acid and ethyl alcohol, respectively, labeled methane was found in both cases. The role of carbon dioxide in this reaction is significant.

The mechanism of the methane fermentation of propionic acid must be much more complicated than that of acetic acid,[1,2] since to balance the empirical equation requires 4 molecules of propionic acid and 2 molecules of water. With the recent availability of propionic acid labeled on the 3 different carbons, respectively, an attack on this problem became possible.

It appeared that, if decarboxylation were the first step with the oxidation of the alpha carbon to carboxyl, one might expect the following: From the carboxyl-labeled propionic acid, all of the activity should be recovered in the CO_2 and a negligible amount of labeled methane should be obtained. In the α-labeled compound, some of the radioactivity should be recovered in the methane and some in the carbon dioxide, empirically, perhaps, in the ratio of 1 to 3 (C^*O_2:C^*H_4).[3] In the case of the β-labeled compound, one might expect to recover all of the radioactivity in the methane with a negligible amount in the CO_2. As is shown below, the results obtained vary considerably from such a purely empirical analysis.

Culture Methods and Procedures.—The anaerobic fermentation of propionic acid to methane and carbon dioxide was carried out in fermenters similar to those used for the anaerobic utilization of acetic acid.[4] The cultures used in the fermentation studies were obtained from a stock fermenter (400-ml. volume), which had been adapted and maintained on propionic acid without any decrease in gas production. This stock culture had been purified and enriched by repeated transfers. A simple salt mixture[5] served as the basal medium and acid-washed asbestos was used as a nidus.[6] In all experimental studies, 35-ml. aliquots of the liquor and asbestos were transferred from the stock culture to the culture flasks of the all-glass fermenters used, which were then incubated at 37°. By means of the side arm fitted with a vaccine bottle stopper, feeds could be administered while maintaining anaerobic conditions. Non-radioactive propionic acid was fed until the approximate theoretical gas yields were obtained, after which the radioactive feeds were introduced. The evolved gases were then collected[1] and assayed for their activity. In some studies, aliquots of the evolved gases were collected and assayed. In other studies, the fermentation was permitted to go to completion after several feedings of the non-radioactive propionic acid. Upon final removal of the gases to be assayed, the culture was acidified with sulfuric acid (1:4) to release carbon dioxide from bicarbonates and carbonates.

Preparation of the Radioactive Feeds.—The feeds containing the isotope in the α-, β- and the carboxyl positions were prepared so that the activity in each mg. of carbon fed would be approximately 0.05 μc. This amount is in the range that can be conveniently detected by the method employed. The calculated amounts of the isotope and the non-radioactive propionic acid were accurately weighed into a volumetric flask and diluted to 50 ml. Each ml. of the solution contained an amount of carbon equivalent to approximately 10 ml. of gas. The feeds were assayed for their specific activity using 0.2-ml. aliquots, which were then combusted using the micro-combustion technique of Niederl and Niederl,[7] and the resulting carbon dioxide assayed as described under assay methods. Duplicate assays for each feed checked within 2%.

Separation and Conversion of the Gases.—A modified Burrell gas analysis apparatus was used for the separation of the gases. The carbon dioxide was absorbed in aqueous sodium hydroxide. Following this, the methane was converted to carbon dioxide by combusting in a catalytic methane-combusting furnace[8] and the carbon dioxide absorbed as above. The resulting sodium carbonate was converted to barium carbonate using the technique of Calvin, *et al.*[9]

The carbon dioxide was regenerated from the barium carbonate using perchloric acid and transferred to an ionization chamber by means of a high vacuum line,[10] essentially by the procedures of Calvin, *et al.*[9] The radioactivity of the carbon dioxide was measured with a vibrating reed electrometer which is especially designed to measure very small currents.[11]

Results and Discussion

The anaerobic fermentation of α, β, and carboxyl-labeled propionic acid to methane and carbon dioxide has shown that the decomposition of propionic acid does not follow a similar mechanism to that indicated for acetic acid by Buswell and Sollo,[1] and Stadtman and Barker.[2] If the decomposition of propionic acid had been analogous to that of acetic acid, as was first assumed, α-labeled propionic acid would have resulted in 1:3 activity ratio of carbon dioxide to methane, β-labeled propionic acid would have resulted with all the activity in the methane, and with carboxyl-labeled propionic acid, all the activity would appear in the carbon dioxide.

The results of our experiments, as summarized in Table I, clearly show that both carbon dioxide and methane may be derived from all three carbons. Cultures 1, 2, 3 and 5 were fed β-labeled propionic acid, the conditions and amount of activity ad-

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TABLE I

SUMMARY OF DATA ON RADIOACTIVE PROPIONIC ACID FERMENTATION

Culture no.	Position labeled	μc red.	μc recovered in: CO_2	μc recovered in: CH_4	Activity recovered, %	Activity ratio CO_2:CH
1	Beta	1.760	0.151	0.299	25.5	1:1.98
2	Beta	1.760	.1897	.366	31.5	1:1.93
3	Beta	1.760	.151	.348	28.3	1:2.3
5	Beta	1.056	.212	.572	74.2	1:2.69
7	Alpha	1.118	.241	.600	75.0	1:2.49
8	Alpha	1.118	.291	.493	70.1	1:1.69
9	Carboxyl	1.032	.368	.193	54.6	1:0.526
10	Carboxyl	1.032	.218	.160	36.6	1:0.735
11	Carboxyl	1.032	.459	.193	63.2	1:0.421
12	Carboxyl	1.376	.125	.256		
			.569	.229		
			.694	.485	85.6	1:0.699

ministered varying with the individual cultures. In all cases, it was clearly demonstrated that the methane contained more activity than the carbon dioxide, on the average more than two-thirds of the labeled carbon being recovered in the methane. With culture 5, the incubation period was extended to 16 days, allowing the fermentation to go to completion. Slightly more than 74% of the total activity fed was recovered, and of this activity approximately 73% was found in the methane. Cultures 1, 2 and 3 were incubated for periods of 7, 9 and 8 days, respectively. Subsequent collection and assay of the gases showed that the per cent. activity found in the methane was 66.2 from culture 1, 66.0 from culture 2 and 69.8 from culture 3. The per cent. of the total activity recovered from cultures 1, 2 and 3 was lower than that recovered from culture 5. This may be an illustration of the isotope effect,[12] in that when the tracer reaction was not carried to completion, less than the expected amount of C^{14} had reacted.

Similarly, from the studies on α-labeled propionic acid, the activity was not recovered as anticipated from the original assumption. Culture 7 was incubated at 37° for 12 days and left at room temperature for 5 days before the gas was collected and assayed. Of the activity fed, 75.0% was recovered, and approximately 71% of the activity recovered was found in the methane. Culture 8 was incubated for 8 days at 37° and gave 70% recovery of the total activity fed, of which 62.8% of the activity recovered was present in the methane. As in the studies with β-labeled propionic acid, it appears that the activity recovered and the activity ratio of the carbon dioxide to methane are dependent on the extent of the fermentation period.

The results observed from the fermentation of the carboxyl-labeled propionic acid indicate that methane may be formed by the reduction of carbon dioxide, as is the case in the fermentation of ethyl alcohol.[2] Therefore, the fermentation of unlabeled propionic acid in the presence of labeled $C^{14}O_2$ should result in some labeled methane.

The results of this investigation are shown in Table II. Culture 13, which had been stabilized and maintained on propionic acid without any decrease in gas production, was given an amount of $NaHC^{14}O_3$ containing 1.25 μc. of radioactivity. At the end of 13 days incubation at 37° the gases were collected and assayed for their activity. Of the total activity recovered (62.1%) in the evolved gases, 45.2% was found to be present in the methane. This amount of activity is large enough to assume that the carbon dioxide is reduced to methane.

TABLE II

SUMMARY OF DATA ON FERMENTATION OF NON-RADIOACTIVE PROPIONIC ACID, ETHYL ALCOHOL AND FORMIC ACID IN PRESENCE OF RADIOACTIVE $C^{14}O_2$

Substrate		μc. $NaHC^{14}O_3$ added	μc. recovered in: CO_2	μc. recovered in: CH_4	% activity recovered in: CO_2	% activity recovered in: CH_4	Total % activity recovered
Propionic	(1)	1.25	0.221	0.123	17.6	9.8	27.4
acid[a]	(2)		.181	.168	14.4	13.4	27.8
	(3)		.025	.062	2.0	4.9	6.9
			.427	.353	34.0	28.1	62.1
Ethyl alcohol[b]		1.25	0.706	0.275	56.5	22.0	78.5
Formic	(1)	1.25	0.328	0.104	26.4	8.3	34.7
acid[c]	(2)		.310	.120	24.7	9.6	34.3
			.628	.224	51.1	17.9	70.0

[a] Culture 13 incubated at 37° for 4 days and portion (1) assayed—incubated 6 days longer and portion (2) assayed—incubated 4 days longer and portion (3) assayed—the culture was not incubated to completion and was not acidified upon the final removal of gas. [b] Culture 15 incubated for 8 days at 37°. [c] Culture 14 incubated at 37° for 4 days and portion (1) assayed—incubated 8 days longer to completion and acidified, portion (2) then assayed.

Since this is so and a reduction of carbon dioxide to methane also occurs in the fermentation of ethyl alcohol, it was thought that the latter compound may be an intermediate in the breakdown of propionic acid. When ethyl alcohol was fed to a stabilized propionic acid culture, there was no decrease in the rate of gas production. In accordance with the simultaneous adaptation theory,[13] this would indicate that ethyl alcohol could be an intermediate in the fermentation of propionic acid. This culture, after stabilization to ethyl alcohol, was given $NaHC^{14}O_3$ containing 1.25 μc. of radioactivity and of the 78.5% activity recovered, 22% was present in the methane. The culture was allowed to incubate for 8 days at 37°. The results of the fermentation are presented in Table II.

If the assumption is made that ethyl alcohol is a product of the hydrolysis of propionic acid. it necessarily follows that formic acid is also an intermediate. Formic acid has been shown to yield carbon dioxide and methane,[14] and, hence, is an example of methane arising from a carboxyl group. Formic acid was fed to a stabilized propionic acid culture and, as was the case for ethyl alcohol, no change in the rate of gas production was observed. Again, in accordance with the simultaneous adaptation theory, this suggests that formic acid could be an intermediate in the fermentation of propionic acid. Table II shows the results obtained when formic acid was fermented

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(14) D. Tarvin and A. M. Buswell, THIS JOURNAL, **56**, 1751 (1934).

in the presence of $NaHC^{14}O_3$ containing 1.25 μc. of radioactivity. Of the total activity (69.0%), recovered, 26.2% was present in the methane.

It is evident that the results obtained from the fermentation of α-, β- and carboxyl-labeled propionic acid must, therefore, be interpreted keeping in mind the fact that carbon dioxide is reduced to methane. The extent of this reduction depends upon the duration of the fermentation.

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY AND PURDUE RESEARCH FOUNDATION, PURDUE UNIVERSITY]

The Dipole Moments of Some Fluorine-Containing Organic Compounds.[1] I

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A heterodyne beat frequency method was employed for the determination of dipole moments of fifteen fluorine-containing organic compounds by the dilute solution technique. Hedestrand's equation was used to calculate the polarization of the solute at infinite dilution.

The purpose of this work was the determination of the dipole moments of certain fluorine-containing compounds. A solution technique permitting the use of Hedestrand's equation was employed.[2]

Experimental

Materials.—Barrett thiophene-free benzene was purified by recommended procedures. After the final distillation it was stored over sodium.[3] The refractive index and density were checked and were found to agree very well with the values listed by Rossini.[4]

Paragon cyclohexane was rectified and a fraction boiling within a 0.1° boiling range was collected, refluxed and stored over sodium.[3] The density at 25° was 0.7713 as compared with 0.77389 reported by Rossini.[4]

The physical constants and sources of the other materials employed are given in Table II.

Apparatus.—The dielectric constant measuring cell had a cylindrical plate assembly consisting of three concentric nickel cylinders.[5] Its air capacity was about 188 mmf., its volume 30 ml. The cell and a solution mixing chamber attached to it were encased in a metal can extending to the bottom of the mixing chamber which was external to this can. Water from a temperature-controlled bath (25 ± 0.02°) was circulated through the can by means of a rotary pump.

A heterodyne beat frequency oscillator was employed for the measurements. The stable oscillator was frequency controlled at 100 kc. with a crystal. The variable oscillator was a modified Hartley circuit. Its frequency was determined by the cell and a variable capacitor in parallel. The output from a mixer stage and amplifier was compared with the 60-cycle, 110-volt a.c., current from the Public Service Co. lines. A Lissajou pattern obtained on an oscilloscope facilitated the tuning of the variable oscillator to exactly 99,940 cycles per second. A comparison capacitor (General Radio 722-D) could be switched into the cell circuit parallel to the variable capacitor in place of the measuring cell. This capacitor was used to correct for oscillator drift. If the comparison capacitor was set at some fixed value and the oscillator did not drift, then a given setting of the variable (measuring) capacitor should be found to always produce the same frequency in the heterodyne beat frequency oscillator output. If the oscillator drifted, the setting of the variable capacitor would also vary and this correction could be noted. The variable capacitor was a General Radio 722-M capacitor calibrated in capacitance removed up to 1,000 mmf. A vernier scale permitted the setting to be read to the nearest 0.02 mmf.

A Sprengel–Ostwald pycnometer was used for determining densities of liquid. A Pulfrich refractometer was used for determining refractive indices.

Procedure.—With dry air in the cell two readings of the variable capacitor were recorded, one with the measuring cell in the circuit and the other with the comparison capacitor in the circuit. The solvent to be employed (benzene or cyclohexane) was transferred from the storage bottle to the mixing chamber by means of a 30-ml. hypodermic syringe. The solvent was forced into the measuring cell up to a marked level above the top of the plates. After a sufficient time interval to permit temperature equilibrum to be established, two more variable capacitor readings were taken as previously described.

A small quantity of the sample being investigated (0.2 g.) was added to the mixing chamber and mixed with the solvent by forcing the liquid back and forth between the cell and the mixing chamber. When the sample was considered sufficiently dispersed, another set of variable capacitor readings were taken. This was continued until the readings for five or six different concentrations of the solution were obtained.

Since the mole fraction of the solute was never more than 0.02, the change in density with mole fraction was assumed linear. The change in dielectric constant with mole fraction (linear in dilute solution) was determined by the method of least squares. This datum and the change in density with mole fraction were substituted in the Hedestrand equation

$$P_{2\infty} = P_1\frac{M_2}{M_1} - \frac{P_1\Delta d}{d_1 f_2} + \frac{3P_1}{(D_1 - 1)(D_1 + 2)}\,\frac{\Delta D}{f_2}$$

$P_{2\infty}$ = polarization of solute at infinite dilution
P_1 = polarization of solvent
d_1 = density of solvent
M_2 = molecular weight of solute
M_1 = molecular weight of solvent
Δd = density of solution minus density of solvent
f_2 = mole fraction of solute
D_1 = dielectric constant of solvent
ΔD = dielectric constant of solution minus dielectric constant of solvent

The dipole moment of the solute was calculated from the equation

$$\mu = 0.01281\ [(P_{2\infty} - P_e)T]^{1/2} \times 10^{-18}\ \text{e.s.u.}$$

The electronic polarization of the solute, P_e, and the polarization of the solvent, P_1, were considered equal to the molar refractivity. These were calculated with the Lorentz–Lorenz equation except in the case of the perfluorocyclohexanes. For these the refractive indices were too low to be determined with the instruments available, hence, the molar refractivities were calculated from atomic refractivities.

Results

The results of the determination of the dipole moments of seventeen compounds are listed in

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(1) Abstracted from the Doctoral Thesis of Paul E. Brown, whose present address is Westinghouse Electric Corporation, Pittsburgh, Pa.

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