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Carbon Isotopic Fractionations Associated with Acetic Acid Production by *Acetobacter suboxydans*

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Abstract—*Acetobacter suboxydans*, strain 8.3, was grown using ethanol, made by the hydration of ethylene, as the sole energy source. After the microorganism had oxidized some of the ethanol to acetic acid, the unassimilated alcohol and produced acetic acid were isolated from the supernatant. Carbon isotopic analyses of these compounds and the starting ethanol show: (1) the methyl carbon of the starting alcohol is enriched in carbon-13 by 4.6 ‰ relative to the hydroxyl carbon, (2) the starting alcohol contains 2‰ less carbon-13 than the unassimilated ethanol and (3) the carboxyl carbon of the acetic acid excreted by the *A. suboxydans* is enriched by 3.8 ‰ in carbon-13 relative to the methyl carbon. These results support earlier findings which indicate that organisms preferentially utilize compounds enriched in carbon-12 and tend to concentrate carbon-12 in the reduced carbons relative to the oxidized carbons of metabolic products.

Introduction

THE REACTION rates and equilibrium constants are changed when one carbon isotope is substituted for another in reacting molecules.[1] These effects give rise to intermolecular and intramolecular isotopic fractionations. Park and Epstein[2] have measured the intermolecular variations of the stable carbon isotopes in tomato plants, and Abelson and Hoering[3] have shown that the carboxyl carbons are enriched in carbon-13 relative to the other carbons in amino acids of biological origin. We have previously reported marked differences in the intramolecular isotopic compositions of acetic acid isolated from apple cider vinegar and of acetic acid commercially synthesized from ethylene.[4]

Theoretical considerations and analytical results clearly indicate that intramolecular isotopic measurements can be used to define metabolic processes, and to distinguish biological reactions and products from nonbiological or abiotic reactions and products. Our specific interest in acetic acid stems from the fact that acetyl-CoA, a derivative of acetic acid, is the fundamental precursor of biological lipids which include biogeochemically important compounds. Extensive investigations have established the biosynthetic pathways of lipids and the presence in sediments and ancient rocks of compounds structurally equivalent to constituents of biological lipids.[5–10] The potential importance of intramolecular isotopic analyses in studies of metabolic processes in extant and pre-existant organisms has led us to investigate the fractionation of carbon isotopes that occurs during the production of acetic acid in a biologically, chemically and isotopically defined system.

Experimental

A pure culture of *Acetobacter suboxydans*, strain 8.3, was grown on a 2% by volume solution containing 0.0343 mol of synthetic ethanol (Commercial Solvents Corp.) in distilled water. The ethanol was the sole carbon energy source of the microorganism. The culture was maintained at its optimum growth temperature, 30 °C, for 72 h. The supernatant was recovered from the harvested cells by centrifugation and decantation. The excreted acetic acid (0.0242 mol) and unassimilated ethanol (0.0101 mol) were isolated from the supernatant by preparative gas-liquid chromatography.

A separate 5 ml sample of the starting ethanol was oxidized to acetic acid by adding the alcohol to a solution composed of 19 g $Na_2Cr_2O_7$ and 10 ml of concentrated H_2SO_4 in 120 ml of distilled water and stirring the mixture for 30 min at 20 °C. The reaction mixture was transferred to a 500 ml separatory funnel and extracted with four successive 50 ml portions of diethyl ether. The extracts were combined and the ether was removed from the acetic acid by evaporation. The acetic acid was purified by distillation. The yield of acetic acid after purification was approximately 90%.

Carbon isotopic analyses were separately obtained for the acetic acid samples isolated from the *A. suboxydans* growth medium and the $Na_2Cr_2O_7$ oxidation products by the method previously described for the acetic acid samples from vinegar.[4] This method involves measurements of the carbon isotopic ratios in carbon dioxide samples produced by the combustion of (a) acetic acid and (b) methane derived from the methyl carbon of the acetic acid by pyrolysis of sodium acetate.[11] The combustion and pyrolysis are carried out in the apparatus shown in Fig. 1. The CO_2 produced by combustion is collected in a trap immersed in liquid nitrogen. The trap is then transferred to a modified McLeod gauge[12] (Fig. 2), where the carbon dioxide is distilled, measured and retrapped prior to analysis in a computer controlled ion counting mass spectrometer.[13]

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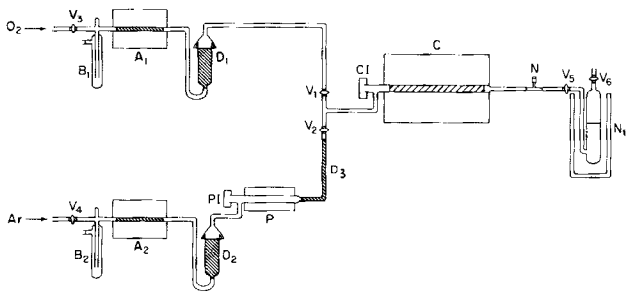

FIG. 1. Pyrolysis and combustion apparatus. A_1, A_2 = carrier gas purification furnaces (770 °C); B_1, B_2 = pyrex fluometers; D_1, D_2, D_3 = ascarite traps; V_1, V_2, V_3, V_4, V_5, V_6 = valves; C = combustion oven (775 °C); P = pyrolysis oven (375 °C); CI = sample inlet for combustion; PI = sample inlet for pyrolysis; N = needle valve; N_1 = liquid nitrogen trap.

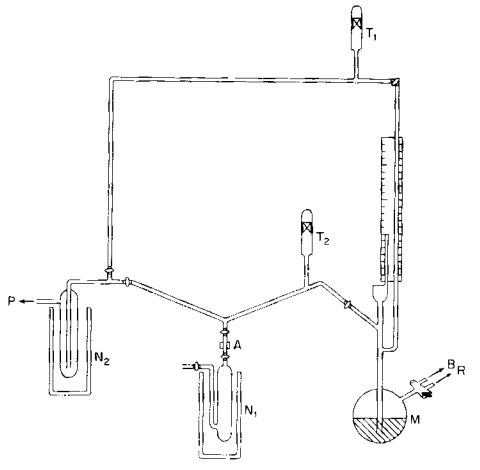

FIG. 2. Modified McLeon gauge. T_1, T_2 = thermocouple pressure gauge; N_1 = sample contained in liquid nitrogen trap; N_2 = liquid nitrogen trap; M = mercury flask; P = to high vacuum; R = to mechanical pump; B = to air; A = adaptor.

Results and discussion

Our results are expressed in Table 1 both in the conventional $\delta^{13}C_{PDB}$ scale[12] and in terms of % ^{13}C. The latter values are calculated by reference to the measurement[13] of the absolute carbon-13 abundance in the PDB standard and involve a series of interlaboratory comparisons leading to our standards. These indirect determinations increase the possible error of our absolute carbon-13 measurements to ±0.01 %, but the difference between carbon-13 abundances in the carboxyl and methyl carbon positions is established within the confidence intervals of approximately ±0.002 % as reported in Table 1. These results show that the methyl carbon is enriched in carbon-13 by 4.6‰ relative to the hydroxyl carbon of the starting ethanol. This enrichment is apparently a consequence of the isotopically selective protonation reaction occurring in the first step of the synthesis of ethanol from ethylene.[4] The carboxyl carbon in the acetic acid excreted by the *A. suboxydans* is derived from the hydroxyl carbon of the starting ethanol. As shown in Table 1, the carboxyl carbon has a 3.8‰ greater $\delta^{13}C$

TABLE 1. Carbon isotopic distributions of 'feed' ethanol and acetic acid[a] from *Acetobacter suboxydans*

CH_3CH_2OH	CH_3–	CH_3COOH	–COOH[b]	Difference
δ_Σ(‰)	δ_M(‰)[c] ^{13}C(%)	δ_Σ(‰)	δ_c(‰) ^{13}C(%)	$\delta_c - \delta_M$(‰)
Inorganic ethanol	Acetic acid synthesized from inorganic ethanol			
−28.9±1	−24.8±1	−27±1		
−28.0±1	−25.6±1	−27.8±1		
−28.3±1	−26.1±1	−28.5±1		
	−26.1±1			
	−25.1±1			
	Averages			
−28.4±.6	−25.5±.4 1.083	−27.8±.6	−30.1±1.2 1.077	−4.6±1.4
Unreacted acetic acid excreted in the supernatant				
ethanol				
−26.9±1	−33.3±1	−32.0±1		
−26.1±1	−35.1±1	−33.1±1		
−26.9±1	−34.7±1	−33.2±1		
	−35.4±1			
	Averages			
−26.6±.6	−34.6±.5 1.073	−32.7±.6	−30.8±1.3 1.077	3.8±1.6

[a] Error limits noted are 95 % confidence limits.
[b] $\delta_c = 2(\delta_\Sigma - \delta_M)$.
[c] Measured on CO_2 produced by combustion of CH_4 formed by pyrolysis of CH_3COONa.

than the methyl carbon in the excreted acid. Thus, a reversal in the carbon-13 enrichment pattern takes place when the *A. suboxydans* oxidizes the ethanol to acetic acid, and the overall increase in the carbon-13 content of the carboxyl carbon of the acid, measured relative to the methyl carbon of the ethanol, is 8.4‰. Additionally, the δC value of the starting ethanol is 2‰ less than that of the unassimilated ethanol. The increase in carbon-13 in the unassimilated ethanol suggests the preferential uptake of carbon-12 enriched ethanol across the bacterial cell membrane.

Acetobacter suboxydans, strain 8.3, are well suited for an isotopic study of this type because the culture is essentially a closed system. It does not oxidize the acetic acid it excretes[15] and manometric studies on the oxidation of ethanol by resting cells of *A. suboxydans* show a quantitative production of acetic acid without CO_2 evolution.[16] These observations are confirmed by the results of DeLey and Stouthamer[17] and our gas-liquid chromatographic analyses. The only compounds found by us in the bacterial supernatant were acetic acid and the unassimilated ethanol.

If our culture was a closed system, as previous studies indicate,[15–17] the following equation may be used to evaluate data reported in Table 1:

$$xA = yB + zC + wD \qquad (1)$$

where A = mol of ethanol initially fed to the bacterium; B = mol of unreacted ethanol in the bacterial supernatant; C = mol of acetic acid in the supernatant; D = increase in bacterial mass; x, y, z and w are their mean $\delta^{13}C$ values, respectively.

Since the increase in mass of *A. suboxydans* grown solely on ethanol as an energy source is small (less than 30 mg), wD may be ignored in the solution of Eqn (1). The following molar and $\delta^{13}C$ values have been measured for the variables in Eqn (1): $A = 0.0343$ mol, $B = 0.0242$ mol, $C = 0.0101$ mol, $x = -28.4‰$, $y = -26.6‰$ and $z = -32.7‰$. Substituting these values and solving Eqn (1) gives

$$(-28.4)(0.0343) = (-26.6)(0.0242) + (0.0101)(-32.7)$$

$$\text{or } 0.974 = 0.974.$$

The identity of this solution indicates that the determined isotopic values are as recorded in Table 1.

In conclusion, the controlled experiment in which *A. suboxydans*, strain 8.3, was grown on ethanol of a determined isotopic composition provides evidence in support of our previous interpretation of the intramolecular carbon isotopic analyses of acetic acid. Because carbon-13 is probably enriched in the carboxyl carbon of the acetyl group in acetyl-CoA, as it is in the carboxyl carbon of biologically produced acetic acid, the individual carbon atoms in lipids that are biosynthesized from acetyl-CoA should retain much of the 'isotopic order' it displays. Such an 'isotopic order' could be useful in defining metabolic pathways and distinguishing biological remnants from abiotically or randomly produce carbon compounds in ancient rocks.

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