STUDIES ON THE METHANE FERMENTATION

XII. The Pathway of Hydrogen in the Acetate Fermentation[1]

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Most of the examined methane fermentations are consistent with the general oxidation-reduction scheme represented by the equation

$$4AH_2 + CO_2 \rightarrow CH_4 + 4A + 2H_2O.$$

In these fermentations, the radioisotope of C^{14}-enriched carbon dioxide can be recovered more or less quantitatively in the methane produced, and conversely labeled hydrogen donor does not generate labeled methane except by prior generation of carbon dioxide (Stadtman and Barker, 1951*a*).

In the fermentation of acetate, however, Buswell and Sollo (1948), and Stadtman and Barker (1949) found that the methyl carbon of the substrate substitutes for carbon dioxide in a discrete labeling pattern:

$$\overset{*}{CH_3}\overset{\circ}{C}OOH \rightarrow \overset{*}{CH_4} + \overset{\circ}{CO_2}$$

the superscripts denoting separate C^{14} labelings.

In order to determine whether the hydrogens attached to the methyl carbon are removed in the course of this exceptional carbon transfer, we reinvestigated the fermentation with deuterium as a tracer. We have found that the substrate hydrogen, like the methyl carbon, is quantitatively transferred to methane.

EXPERIMENTAL METHODS

Enrichment cultures of acetate-fermenting, methane-producing bacteria were obtained from mud collected from the eastern shore of San Francisco Bay, using the elective procedure and the medium described by Stadtman and Barker (1951*a*). In the first tracer experiment a small amount of washed sediment from a secondary enrichment was used as the inoculum after gas evolution had ceased. The results were sufficiently clear-cut to justify the use of unwashed inocula from primary and secondary enrichments in later experiments. Substrate contamination then became evident, but could be corrected for.

An all-glass apparatus was used for the fermentations and for the collection of gas. Small tubes were filled almost to capacity with 15 ml of freshly steamed medium containing 1.6 millimoles of normal or deuterierated acetate prepared by the method of Wilson (1935). The exchangeable hydrogen had been removed by neutralizing a concentrated solution of the acid with $BaCO_3$, followed by precipitation of the salt in acetone and displacement of the cation with "dowex 50" (Na) or by precipitation with Na_2SO_4.

After inoculation, the tubes were evacuated and incubated at 37 C for several days. The first few milliliters of methane formed were transferred over Hg layered with alkaline pyrogallol and collected over anhydrous $Mg(ClO_4)_2$ or dried through a dry ice-isopropanol cooled trap.

Deuterierated acetate was assayed by the combustion and reduction technique of Graff and Rittenberg (1952). Samples were determined as the Ba salt and combusted repeatedly until the enrichment values showed no consistent deviation. For convenience we examined the *m/e* 4/3 ratios, rather than the usual 3/2 ratios, for substrates containing high deuterium enrichment.

The deuterium enrichment of the methane was calculated from a direct scan of the nine possible spectral peaks (*m/e* 12 to 20) of the uncombusted gas with a Consolidated-Nier isotope-ratio mass spectrometer. In comparison with the combustion procedure, direct scanning is superior because of its simplicity, its applicability to small gas samples and to wide ranges of deuterium enrichment, and its consistent reproducibility. In ad-

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dition, it is possible to determine whether the enrichment is indiscriminately randomized, or whether it is excluded from any of the substituent sites in the molecule. To determine the existence and abundance of the five possible species of deuterierated methane (CH_4 to CD_4), we referred to the spectra of Dibeler and Mohler (1950). Minor corrections were computed from the deviations between our spectra and theirs for normal methane. These were applied to their spectra for the deuterierated methanes, taking into account the ion abundances computed by them for all possible dissociations.

Corrections were also made for background peaks, for naturally occurring C^{13}, assumed to be 1.1 per cent of the C^{12}, for the dissociation peaks of any extraneously introduced N_2, O_2, A, or CO_2, and for the differences in sensitivity with respect to the isotopic methanes. Water peaks could not be corrected for with certainty, but they could not have given rise to serious errors.

The total D enrichment in the gas could be found directly by tallying the individual isotopic contributions of each methane species. Determining their relative abundance involves evaluating the heaviest methane from its pure parent peak, subtracting its contribution of dissociated peaks from the residual, and calculating the successively lighter methanes in a like manner. In samples containing more than 2 isotopic methanes, the considerable accumulation of corrections increases the uncertainty of the lower values. Therefore, for complex distributions, when warranting the assumption of random distribution of the isotope in the enriched substituent sites, the total D enrichment was calculated from the mole fraction (N) of the heaviest methane alone. This was considered the ultimate term of the expansion $(a + b)^x$, x being the number of enriched substituent sites (i. e., the number of D atoms in the heaviest methane observed), and a and b the total atom fractions of H and D respectively in those sites. The term b would thus be

$$\sqrt[x]{N CD_xH_{(4-x)}} \text{ or } \sqrt[x]{\frac{v_o}{V_o} \cdot \frac{V_r}{v_r}} \text{ atom per cent D;}$$

v and V refer to the voltages of the uppermost peak and the total peaks, respectively; and the subscripts o and r refer to sample values, and the corrected literature values of the heaviest methane species in the sample, respectively.

The original assumption of random labeling in the enriched substituent sites could be verified by comparing the observed spectrum with a theoretical one. The latter was tallied from the corrected literature values for the observed concentration of the heaviest methane, and the corresponding concentrations of the successively lighter methanes that should be equilibrated with it. These would correspond to the terms containing successively lower powers of b in the above expansion.

Procedures for C^{14} assays are described elsewhere (Stadtman and Barker, 1949).

RESULTS

Fermentation of deuterierated acetate and unlabeled water. As indicated in Experiment I, table 1, the virtual absence of CD_4 (m/e 20) and the abundance of CD_3H (m/e 19) show that the isotope is restricted to 3 substituent sites in the methane. The close agreement of the experimental and theoretical values justifies the assumption of random distribution of the isotope in those positions, and hence the validity of the enrichment calculation. The enrichment of 73.5 atom per cent excess D in the "methyl" moiety of methane is in close agreement with the value of 75 atom per cent excess D in the original acetate sample. The deuterium appears to be transferred quantitatively and without isotope effect in accordance with the equation:

$$CD_3CO_2H \rightarrow CD_3H + CO_2.$$

Fermentation of normal acetate in deuterium-labeled water. Experiment II of table 1 shows that the one remaining hydrogen of methane is derived from water as would be expected from the previous experiment:

$$CH_3CO_2D \rightarrow CH_3D + CO_2.$$

The deuterium transfer is accompanied by an appreciable isotope selection, the deuterium enrichment in the single position of methane amounting to 53 per cent of the water enrichment.

The theoretical and experimental magnitudes of hydrogen isotopic selection are discussed by Eidinoff (1953). It will suffice to mention that the fractionation encountered in the present fermentation is large, but not maximal in comparison with the fractionations that can be reached in some rate-limiting biological systems.

Fermentation of unequilibrated CD_3CO_2H in un-

TABLE 1

Mass spectra and D enrichments of deuterierated substrate and product in the acetate fermentation

	Expt. I $CD_3CO_2Na + H_2O$		Expt. II $CH_3CO_2Na + D_2O$	Expt. III Unequilibrated $CD_3CO_2Na + H_2O$		
	Mass spectrum of methane					
	Observed	Theoretical	Observed	Observed	Theoretical	
					Non-randomized[f]	Randomized[g]
	%	%	%	%	%	%
D enrichment in labeled substrate	75 atom per cent excess D		4.80 atom per per cent excess D	7.5 atom per cent excess D[e]		
m/e—20	0.00	0.00	0.00	0.00	0.00	0.00
19	19.5	19.5[b]	0.00	1.41	1.95	0.0195
18	28.3	26.3	0.07[c]	2.25	2.83	0.736
17	28.7	30.4	1.21	2.91	3.32	9.70
16	14.6	14.7	48.4	45.9	45.2	45.88
15	4.86	4.70	41.0	38.6	37.5	34.65
14	2.25	2.45	5.67	5.45	5.39	5.42
13	0.86	0.92	2.70	2.54	2.53	2.57
12	0.89	0.96	0.99	0.97	1.00	1.03
D enrichment in labeled substituent sites of methane	73.5 atom per cent excess D as CD_3H[a]	73.5 atom per cent excess D as CD_3H[b]	2.55 atom per cent excess D as CDH_3[d]	7.35 atom per cent excess D as CD_3H[e]		

[a] Calculated from the abundance of the *m/e* 19 peak, assuming random distribution of D in 3 substituent sites.

[b] Value set to equal the observed value. The deuteromethane enrichments that would follow, assuming random distribution in 3 substituent sites would be: CD_3H, 38.8 mole per cent; CD_2H_2, 43.0 mole per cent; CDH_3, 15.5 mole per cent; and CH_4, 1.86 mole per cent. Corresponding peak values were derived from the deuteromethane spectra of Dibeler and Mohler (1950).

[c] value of uncertain origin, probably due to water.

[d] Tally of the contributions of CDH_3 and CH_4.

[e] Derived from the corresponding acetate or methane values of Experiment I, correcting for a 10-fold carrier dilution.

[f] Tally of the contribution of $\frac{1}{10}$ of each of the spectral values of Experiment I, and $\frac{9}{10}$ of each of the spectral values of normal methane.

[g] Computed from the deuteromethane spectra of Dibeler and Mohler (1950) assuming complete randomization of H containing 7.35 atom per cent excess D enriched in 3 substituent sites.

labeled water. The quantitative deuterium transfer in Experiment I and the confinement of label to the appropriate methane species in Experiments I and II indicate an intact methyl transfer. However, the alternate possibility exists that one or more deuterium atoms are transferred to an acceptor that does not exchange with water, and add back in discrete steps to the carbon skeleton of methane. If unaccompanied by isotopic fractionation this would give the observed results and yet involve a different pathway. The several direct hydrogen transfers demonstrated for isolated pyridine nucleotide dehydrogenases (Vennesland and Westheimer, 1954) provide illustrations of this possibility. However, transfer of hydrogen or deuterium by such a mechanism would redistribute the label among the different species of methane. Since the deuterium in the acetate sample of Experiment I was probably randomly distributed to begin with, this redistribution would not be apparent.

We therefore examined the fermentation of acetate deuterierated in a non-random manner. A sample of the $(CD_3CO_2)_2Ba$ used in Experiment I was diluted tenfold with carrier acetate. Adapting the mathematical expansion of the

previous section to the randomization of D and H as substituents for the three valences of a methyl group, the concentration of each deuterierated acetate species (CH_3CO_2H to CD_3CO_2H) is a cubic function of the total D enrichment. Since carrier dilution lowered all of these values directly, the enrichment in the acetate sample is non-randomized. If this distribution were preserved in the methane produced by an intact methyl transfer, the spectrum of the gas should show an identical 10-fold lowering of each isotopic peak, as represented in the theoretical non-randomized spectrum in table 1, Experiment III. Alternatively, the activation of all substituents would completely randomize the isotope in the three positions and yield the theoretical randomized spectrum in table 1, Experiment III. The same spectrum would obtain for two substituent activations if they occurred separately or were unlinked. For a single activation, the partially randomized spectrum would not diverge as much from the non-randomized one, but its distinctive pattern would be obvious; its *m/e* 19 peak would be one-tenth that of the non-randomized one, assuming no isotope effect. The spectrum actually observed (table 1, Experiment III) agrees closely with the non-randomized one. The comparatively small, more or less similar deviations at the highest peaks are probably caused by some remaining unlabeled acetate introduced from the inoculum which would uniformly decrease all of these peaks from their expected values. Isotope equilibration on the other hand would lower the peaks exponentially. The non-equilibration permits a simple calculation of unlabeled contamination, which would be that value which lowers the *m/e* 19 abundance below the theoretical value, or the observed value for an uncontaminated sample.

Confirmatory analyses. We attempted to verify the direct scan assay of methane for deuterium with the more conventional combustion procedure, and with C^{14} labeling as used by Stadtman and Barker (1949). The use of acetate-2-C^{14} was necessary to show that the reaction catalyzed by our enrichment cultures of acetate-fermenting methane bacteria was the same as that previously observed.

A sample of the $(CD_3CO_2)_2Ba$ was supplemented with a small amount of $C^{14}H_3CO_2Na$ that did not introduce appreciable H to the total. Approximately 4 ml of methane were collected from the fermentation of the derived $C^{14}D_3CO_2Na$ and a small aliquot was assayed by direct spectral inspection. The total deuterium enrichment, taking into account the hydrogen contribution from all 4 methane substituents, and substrate contamination amounting to 44.1 per cent normal methane was estimated as 30.6 atom per cent excess D. The remaining gas was divided into 3 aliquots which were combusted separately and bracketed between determinations of a D_2O standard containing 30.6 atom per cent excess D. The highest value reached for the gas samples was 29.7 atom per cent excess D. This value is somewhat uncertain because of the difficulties inherent in adapting the combustion procedure to small gas samples containing high deuterium enrichments. Nevertheless the result is sufficiently reliable to support the authenticity of the deuterierated methane peaks and the validity of the direct scanning procedure.

Small subsequent gas samples from the fermentation were then analyzed alternatively for C^{14} activity and for deuterium content by direct scanning. The average deuterium determination showed a substrate contamination of 38.7 per cent CH_4, and hence a CD_3H yield of 61.3 per cent. In comparison to the substrate C^{14} activity of 246,000 ct/min/meq, the average of three determinations of methane is 154,000 ct/min/meq, equivalent to a C^{14} yield of 62.4 per cent, which is in good agreement with the deuterium determination. The carbon and hydrogens of the acetate and methyl group are thus both quantitatively transferred to methane. This experiment parallels the labeling experiment of Stadtman and Barker (1949), and indicates the identity of the metabolic reactions occurring in the two sets of experiments.

DISCUSSION

Our finding of intact methyl transfer from acetate to methane identifies the reduction stage of intermediate *X*, presumed by Stadtman and Barker (1949, 1951*b*) to be a common methane precursor in both the carbon dioxide utilizing and the non-utilizing types of methane fermentations. Evidently the ultimate intermediate step in the methane fermentation is a reductive demethylation, since the methyl moiety of acetate remains reduced and attached by only one valence throughout its fermentative transformations.

For comparison of the acetate fermentation with a carbon dioxide utilizing fermentation, we performed a deuterium labeling experiment with *Methanobacterium omelianskii*, which oxidizes alcohol to acetate with the concomitant reduction

of carbon dioxide to methane. The results will be presented in detail in another paper. Here it will suffice to mention that the fermentation of unlabeled ethanol in water enriched with D_2O yields methane enriched in all positions (CD_4) with an over-all isotope yield of about 80 per cent of the water enrichment. The lower deuterium content of the methane as compared with the water can be attributed to an isotope selection effect, since some fractionation of this magnitude undoubtedly would be expected from stepwise hydrogen transfers. The abundant entry of the label of water into all valences of methane in this fermentation emphasizes the distinctness of substrate hydrogen incorporation in the acetate fermentation.

The superficial simplicity of the labeling pattern does not imply that the fermentation proceeds by a simple decarboxylation as proposed by Buswell and Sollo (1948). The arguments against this idea are discussed by Stadtman and Barker (1949). It is theoretically possible for acetate or its products to undergo separate oxidations and reductions without labilization of the hydrogen atoms attached to the methyl group. One possibility would be an oxidative decarboxylation of acetate yielding a methylated derivative and a reduced acceptor, followed ultimately by a compensatory oxidation-reduction between the products.

Amplifying the general scheme of Stadtman and Barker (1949) for the critically examined methane fermentations, the acetate fermentation may be expressed:

$$\begin{array}{ccc} CH_3COOH + HX & \xrightarrow{-2H} & CH_3X + CO_2 \\ & & \downarrow{\scriptstyle +2H} \\ & & CH_4 + HX \end{array}$$

in which acetate substitutes for the more common 1-carbon source, carbon dioxide, as the precursor of the penultimate intermediate in the methane fermentation.

The present findings provide a new example of methyl group transfer. It remains to be seen how closely this process is related to the more general examples of transmethylation and of 1-carbon transfer. In mammalian systems, acetate can be incorporated into 1-carbon donors, but evidently by an indirect route, such as the one suggested by Nyc and Zabin (1955), which would preclude any extensive intact transfer of H labeling.

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SUMMARY

Experiments with deuterium-labeled acetate show that, in the methane fermentation of acetate, the methyl group of the substrate is transferred intact into methane.

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