A MASS ANALYSIS STUDY OF FORMALDEHYDE FIXATION AND CLEAVAGE OF LACTATE BY PROPIONIBACTERIUM ARABINOSUM*

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Leaver (3) and Leaver and Wood (4) found with resting cells of Propionibacterium arabinosum that formaldehyde-C\textsuperscript{14} was incorporated into each position of propionic and succinic acids during fermentations of glucose, pyruvate, or glycerol. These findings raised the question of whether a total synthesis of the products from formaldehyde had occurred; that is, formation of molecules in which all carbons had been derived from formaldehyde. Another possibility was that formaldehyde-C\textsuperscript{14} had combined with an intermediate produced from the unlabeled substrate, thus yielding a singly labeled compound. The isotope then might be randomized in such a way as to yield three types of singly labeled propionate molecules (C\textsuperscript{14}H\textsubscript{2} CH\textsubscript{3}COOH, CH\textsubscript{3}C\textsuperscript{14}H\textsubscript{2}COOH, and CH\textsubscript{3}CH\textsubscript{2}C\textsuperscript{14}OOH), and therefore the isotope would appear in each position upon degradation. The technique of mass analysis, previously adapted to the study of CO\textsubscript{2} fixation by Wood (5), and employed in the accompanying paper (6) and by Swim and Krampitz (7), has been applied to the above questions. It was known, however, that the carboxyl carbon of propionate is reversibly cleaved during resting cell fermentations (6); therefore any triply labeled propionate would be destroyed even if it were formed initially. Nevertheless, the 2,3-carbons of propionate might be studied to determine whether doubly labeled material was produced at these positions from formaldehyde carbon. It remained possible, however, that propionate or a precursor of propionate might be reversibly cleaved between carbon atoms 2 and 3, and thus doubly labeled material formed from formaldehyde carbon at carbons 2 and 3 would be destroyed also. This possibility was investigated by a procedure similar to that used by Pomerantz (6) in studying cleavage at the propionate carboxyl group.

These mass analysis studies have shown that there is little or no cleavage of lactate between the 2- and 3-carbons during its conversion to pro-

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pionate. The fixation of formaldehyde appears to give rise to a mixture of three types of singly labeled propionate molecules.

**Methods**

*Synthesis of Triply Labeled Lactate*—Triply labeled lactate was synthesized according to the following equations.

\[
\text{BaC}^{13}\text{O}_3 \xrightarrow{\Delta} \text{BaC}^{13}_2 \xrightarrow{\text{H}_2\text{O}} \text{HC}^{13}_2\text{H} \xrightarrow{\text{H}_2\text{O}} \text{C}^{13}_2\text{H}_4 - \text{C}^{13}\text{H}_2
\]

\[ \xrightarrow{1. \text{KCl}} \text{C}^{13}\text{H}_2 - \text{C}^{13}\text{HOH} - \text{C}^{13}\text{OOH} \]

The acetylene was prepared as described by Calvin et al. (8, p. 205), converted to acetaldehyde, and then to lactic acid (9). The lactic acid, after purification on a Celite column (10), was obtained in a yield of 35 per cent from barium carbonate.

*Synthesis of Formaldehyde*—The isotopic formaldehyde was prepared by reduction of CO\(_2\) to methanol with LiAlH\(_4\) (11), followed by catalytic oxidation of the methanol to formaldehyde (12).

*Cultivation of Cells and Fermentation with Resting Cells*—The cells were grown in a medium similar to that previously described (6), except for the fermentations of lactate in which 1.0 per cent sodium lactate and only 0.1 per cent glucose were used. The cells were cultivated successively for 1 day periods in 10 ml., 100 ml., and 1 liter of medium, and then harvested (6). The conditions of the experiments are given in Tables I, III, and V, and the products were separated and purified as described (6).

*Degradation and Isotope Analysis Methods*—Propionate and acetate were degraded by the Schmidt azide method (13) and were oxidized to CO\(_2\) (14) for C\(^{13}\) analysis. Lactate was degraded by the method of Wood et al. (15). C\(^{13}\) analyses and mass analyses of ethylene were performed on a Consolidated Instrument Company mass spectrometer, model No. 101. (See Wood (5) and Pomerantz (6) for the details of corrections for fragmentation and background.)

*Preparation of Ethylene from 2,3-Carbons of Propionate*—The propionate was converted to ethylene by a slight modification of the method of Swim and Krampitz (7).

\[
\text{CH}_3\text{-CH}_2\text{-COONa} \xrightarrow{\text{Na}_2\text{NNa}} \text{CH}_3\text{-CH}_2\text{-NH}_2 \xrightarrow{\text{HCHO}} \text{HCOOH} \]

\[
\text{CH}_3\text{-CH}_2\text{-N(CH}_3)_2 \xrightarrow{\text{CH}_3\text{I}} \text{CH}_3\text{-CH}_2\text{-N}^+\text{(CH}_3)_2\text{I}^- \xrightarrow{\text{OH}^- \Delta} \text{CH}_2=\text{CH}_2
\]

The masses of ethylene involved are 28 (no C\(^{13}\) in molecule), mass 29 (one C\(^{13}\)), and mass 30 (two C\(^{13}\)'s).
Wood has developed (5) Equations 1 and 2 which express the relationship between the labeled types of ethylene and the mass abundances.

\[
\frac{\text{Mass 30}}{\text{Mass 28 \text{ per cent}}} = \frac{100F^2D + 1.09FS + 1.188 \times 10^{-2}(1-D-S)}{(1-F)^2D + 0.989(1-F)S + 0.978(1-D-S)}
\]

\[
\frac{\text{Mass 29 \text{ per cent}}} {\text{Mass 28}} = \frac{200F(1-F)D + [98.9F + 1.09(1-F)]S + 2.16(1-D-S)}{(1-F)^2D + 0.989(1-F)S + 0.978(1-D-S)}
\]

\(F\) is the known fraction of \(\text{C}^{13}\) in the labeled source and \(S\) and \(D\) are the fractions of singly and doubly labeled ethylenes, respectively. The two mass ratios are obtained experimentally\(^1\) and the equations are solved simultaneously for \(S\) and \(D\). \(N\), the fraction of unlabeled ethylene, is obtained by the relationship \(N = 1 - (S + D)\).

**Results**

The object of these mass analysis experiments was to determine whether more than one of the carbons in the same molecule of propionate was derived from formaldehyde. The first problem was to determine whether the \(\text{C}_1\) unit from labeled formaldehyde was diluted by unlabeled substrate. From Equations 1 and 2 it is clear that in order to calculate the amounts of singly and doubly labeled material it is necessary to know \(F\), the fraction of \(\text{C}^{13}\) in the \(\text{C}_1\) compound produced from formaldehyde and subsequently used for the synthesis of propionate. To obtain information on this point two types of experiments were performed. First, labeled formaldehyde was added to fermentations of unlabeled substances, and the residual formaldehyde was recovered. It was found that the tracer in the residual formaldehyde had undergone only a slight dilution. However, it was possible that part of the unlabeled substrate was cleaved to a \(\text{C}_1\) identical with that produced from the labeled formaldehyde. These \(\text{C}_1\) units would mix in the metabolic pools, giving a dilution of the isotope in the \(\text{C}_1\), while the \(\text{C}^{13}\) concentration of the formaldehyde per se remained unchanged.

The second type of experiment involved a study of the cleavage of carbon bonds in the material used as the unlabeled substrate in the formaldehyde experiment. Lactate was chosen initially as the unlabeled substrate because it was possible to synthesize triply labeled lactate and to estimate the production of \(\text{C}_1\) from this source. Previous experiments had indicated that the carboxyl group of lactate apparently did not give rise to a

\(^1\) The correction factors for fragmentation (5) are loss of 1 hydrogen 0.641, and loss of 2 hydrogens 0.623. The equations are slightly different from the ones used earlier because a natural abundance of \(\text{C}^{13}\) of 1.09 per cent was used rather than the 1.00 per cent used by Wood (5).
C₁ similar to that arising from formaldehyde. This was evident because there was very little conversion of C¹⁴ from lactate-1-C¹⁴ to the 2 and 3 positions of propionate (16), whereas formaldehyde was converted to all positions. However, lactate-3-C¹⁴ did give rise to a significant amount of C¹⁴ in all positions of the propionate (16), and on this basis formation of the C₁ from this carbon atom appeared to be a good possibility. Therefore a mass analysis experiment was made to determine how much cleavage occurred between carbon atoms 2 and 3 of lactate during the conversion to propionate. The result of this experiment provided indirect information on the possible cleavage to a C₁.

Lactate Fragmentation—A mixture of triply labeled³ and unlabeled lactate was fermented and the conditions and results of the experiment are given in Tables I and II. Table I gives the data from the oxidation and degradation of the original mixture of triply and unlabeled lactate. From these values the amount of triply labeled and unlabeled material in the original lactate was calculated as shown in Table I. The calculated values of 8.8 per cent triply labeled and 91.2 per cent unlabeled lactate agreed closely with the composition based on the known amounts used in making the mixture.

After fermentation of the lactate mixture, the propionate was isolated, part was degraded to determine the C¹³ content of each position (Table I), and part was converted to ethylene and submitted to mass analysis. The results in Table I indicate that there was little dilution of C¹³ during the conversion of lactate to propionate. This fact assures that the endogenous metabolism was not great. The dilution of the carboxyl carbon was 7.1 per cent. This may have in part arisen by CO₂ fixation since 5 per cent CO₂ was used in the gas phase. The dilution of the 2,3 positions was on the average 2.5 per cent. No correction has been made in the ethylene calculations of Table II for this endogenous dilution since it would be of little over-all significance.

The corrected experimental and the calculated mass abundances for the ethylene are shown in Table II, together with the method for calculating the types of ethylene. The mass abundance values which would have been obtained if the lactate mixture were converted to propionate without cleavage between carbons 2 and 3 are shown in the fourth column of Table II. These values were calculated by substituting F = 0.338, D =

³ Triply labeled lactate was used in the experiment but 2,3 doubly labeled lactate probably would have served as well. It appeared possible that the small amount of the carboxyl carbon of lactate which is converted to the 2,3-carbons of propionate might reach these positions via a symmetrical C₁ intermediate without cleavage. If this occurred, doubly labeled lactate-2,3 C¹³ would give rise to doubly labeled propionate-1,2-C¹³ and carbons 2,3 would be singly labeled but not because cleavage had occurred.
0.088, and \( S = 0.000 \) into Equations 1 and 2. The calculated value for \( \frac{30}{28} \) agrees closely with the observed value (1.09 and 1.06), while the difference between the \( \frac{29}{28} \) values is about 2 per cent (6.32 compared to 6.45). In the fifth and sixth columns of Table II are listed the calculated mass abundances corresponding to 10 and 25 per cent fragmentation of the lactate to a C\(_1\) at carbons 2 and 3. While the \( \frac{29}{28} \) value calculated for 10 per cent fragmentation is close to the value found experimentally,

### Table I

<table>
<thead>
<tr>
<th>Carbon 3</th>
<th>Carbon 2</th>
<th>COOH</th>
<th>CO(_2) from combustion</th>
</tr>
</thead>
<tbody>
<tr>
<td>atom per cent excess C(_{14})</td>
<td>atom per cent excess C(_{13})</td>
<td>atom per cent excess C(_{13})</td>
<td>atom per cent excess C(_{14})</td>
</tr>
<tr>
<td>Initial lactate</td>
<td>2.91</td>
<td>2.81</td>
<td>3.50</td>
</tr>
<tr>
<td>Final propionate</td>
<td>2.81</td>
<td>2.77</td>
<td>3.24</td>
</tr>
</tbody>
</table>

Conditions: cells, 3 per cent wet weight; phosphate buffer, pH 7, 0.15 M; sodium lactate, 0.07 M; total volume, 150 ml.; temperature, 30\(^\circ\)C; gas phase, 95 per cent N\(_2\)-5 per cent CO\(_2\); time, 7.5 hours. The composition of the lactate mixture was calculated as follows: The undiluted triply labeled lactate had an average C\(_{13}\) content of 35.1 atom per cent excess, while the diluted material contained 3.07. The mixture was therefore composed of 8.8 per cent of synthesized triply labeled lactate (3.07/35.1 \times 100), the remainder being unlabeled lactate. The mixture had an excess C\(_{13}\) of 3.50 per cent in the carboxyl carbon. Accordingly the triply labeled lactate is calculated to have 40.0 per cent excess C\(_{13}\) in the carboxyl carbon (3.50 \times 35.1/3.07). This is in agreement with the C\(_{14}\) content of the cyanide used in the synthesis. The average value of the 2- and 3-carbons of the mixture (2.81 + 2.91/2 = 2.86) was used to calculate the C\(_{15}\) content of these positions of the triply labeled lactate (2.86 \times 35.1/3.07 = 32.7 atom per cent excess). The composition of the mixture, therefore, was CH\(_3\)-CHOH—COOH, 8.8 per cent; and CH\(_3\)-CHOH—COOH, 91.2 per cent. The superscripts give atom per cent C\(_{14}\).

The 30/28 values differ by about 7 per cent. Calculations made for 25 per cent fragmentation show a difference of about 19 per cent from the experimental for 30/28. Since the 30/28 value is the most sensitive to changes in composition caused by cleavage of the 2,3-carbon bond of lactate, it is concluded that fragmentation occurred to the extent of 10 per cent or less. This is in agreement with the results of experiments by Leaver et al. (16), in which lactate-3-C\(_{14}\) was incorporated to the extent of about 4 to 9 per cent in the carboxyl carbon of propionate.

**Formaldehyde-C\(_{14}\) Fixation with Lactate As Substrate** — The finding that a maximum of 10 per cent fragmentation could be expected from lactate
during a fermentation made it clear that the formation of a C₃ from the 2,3-carbons of lactate would be small. It was evident also that the 2,3-carbon bond of any doubly labeled propionate (C¹³H₃-C¹³H₂COOH) that might be synthesized from formaldehyde-C¹³ would remain intact during the fermentation. It therefore appeared feasible to undertake a mass

TABLE II
Comparison of Mass Abundance of Ethylene from Carbons 2 and 3 of Propionate (Table I) and Calculated Mass Abundances Based on Fragmentation of Original Lactate Mixture

All the values are expressed in per cent.

<table>
<thead>
<tr>
<th>Masses</th>
<th>Ethylene of propionate</th>
<th>Calculated mass abundance according to lactate fragmentation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Observed spectrometer values</td>
<td>Corrected spectrometer values</td>
</tr>
<tr>
<td>30/28</td>
<td>1.02</td>
<td>1.06</td>
</tr>
<tr>
<td>29/28</td>
<td>6.85</td>
<td>6.45</td>
</tr>
</tbody>
</table>

* The height of the 28 peak was 3.92 x 10⁴ cm.
† See footnote 1 in the text.
‡ The calculations for 10 per cent fragmentation of the 2,3-carbons were made as follows: 10 per cent of the lactate cleaves to make a C₁ unit containing 3.95 per cent C¹³ (0.0875 x 33.8 + 0.9125 x 1.09 = 3.95). This C₁ recombines with C₂ units to make 3.95 1.09 1.09 3.95 33.8 41.1 C₃ units (C—C—COOH, 9.12 per cent and C—C—COOH, 0.88 per cent) which are 33.8 33.8 41.1 1.09 metabolized with the unfragmented C₁ units (C—C—COOH, 7.88 per cent and C—C—COOH, 82.1 per cent) to propionate. The ethylene species involved are CH₁ = 33.8 1.09 1.09, CH₂ = 3.95 33.8 CH₂ (7.88 per cent), CH₃ = CH₂ (82.1 per cent), CH₄ = CH₂ (0.88 per cent), and 3.95 1.09 CH₂ = CH₁ (9.12 per cent). The superscripts give atom per cent C¹³. The contribution of each species to each mass number was calculated, and then the masses totaled and the mass abundances were calculated.
§ The species involved are the same as above, except that the percentages are, respectively, 6.6, 68.4, 2.2, and 22.8.

analysis of the 2,3-carbons of propionate to determine whether there was total synthesis from formaldehyde-C¹³ during a lactate fermentation. The major difficulty encountered was that a really substantial incorporation of formaldehyde was not obtained during lactate fermentations. If the concentration of formaldehyde was increased above 0.007 M, the fermentation of lactate was reduced about 50 per cent.

Tables III and IV give the results of the experiment in which formaldehyde-C¹³ was fermented together with lactate-C¹³. It is evident from Ta-
ble III that most of the added lactate (14 mmoles) was fermented, yielding 10.37 mmoles of propionate and acetate. However, only 13.4 per cent of the C\textsuperscript{18} of the added formaldehyde \((100(3 \times 7.24 + 2 \times 3.13) 0.20/1.12 \times 37.3)\) or the equivalent of 0.15 mmole was recovered in these acids. The C\textsuperscript{18} was found to be equally distributed between the 2- and 3-carbon atoms.

\begin{table}[h]
\centering
\caption{Fixation of Formaldehyde-C\textsuperscript{18} in Products of Fermentation of Lactate}
\begin{tabular}{|c|c|c|c|c|}
\hline
Product & \text{Distribution of C\textsuperscript{18}} & \text{CO\textsubscript{2} of combustion} \\
 & mmoles & \text{atom per cent} & \text{atom per cent} & \text{atom per cent} & \text{atom per cent} \\
 & & \text{access C\textsuperscript{18}} & \text{access C\textsuperscript{18}} & \text{access C\textsuperscript{18}} & \text{access C\textsuperscript{18}} \\
\hline
Propionate & 7.24 & 0.17 & 0.16 & 0.28 & 0.20 \\
Acetate & 3.13 & & & & 0.20 \\
Formate & 0.203 & & & & 31.9 \\
\hline
\end{tabular}
\end{table}

Conditions: formaldehyde, \(5.61 \times 10^{-3} \text{ M} (37.3 \text{ atom per cent excess C}\textsuperscript{18})\); sodium lactate-C\textsuperscript{18}, 0.07 M; potassium phosphate buffer, pH 7, 0.15 M; cells, 5 per cent wet weight; gas phase, 95 per cent N\textsubscript{2}-5 per cent CO\textsubscript{2}; total volume, 200 ml.; time, 17 hours; temperature, 30\textdegree C.

\begin{table}[h]
\centering
\caption{Mass Analysis of Ethylene from Carbons 2 and 3 of Propionate from Lactate Fermentation of Table III and Calculated Values for Doubly and Singly Labeled Molecules}
\begin{tabular}{|c|c|c|c|c|}
\hline
Calculation, No. & \text{C\textsuperscript{18} in labeled source} & \text{Types of ethylene} & \\
 & (P) & \text{D, C\textsuperscript{18}—C\textsuperscript{18}} & \text{S, C\textsuperscript{18}—C} & \text{N, C—C} \\
 & per cent & per cent & per cent & per cent \\
A & 38.4 & 0.03 & 1.5 & 98.5 \\
B & 6.49 & 1.2 & 4.8 & 94.0 \\
\hline
\end{tabular}
\end{table}

The spectrometer values (in per cent) before correction for H fragmentation\textsuperscript{1} were \(30/28 = 0.020\) and \(29/28 = 2.58\); these values after correction were \(30/28 = 0.020\) and \(29/28 = 2.61\). The height of the 28 peak was \(1.59 \times 10^4\) cm.

of the propionate and the C\textsuperscript{18} concentration in these positions was about one-half that of the carboxyl group. This distribution is similar to that observed by Leaver (3). On the basis of the C\textsuperscript{18} concentration of the original formaldehyde, only 0.75 per cent of the carboxyl group originated from the formaldehyde \((100 \times 0.28/37.3)\). Formic acid occurred in substantial amount and it was produced in large part from the formaldehyde, since its C\textsuperscript{18} concentration was 86 per cent of the added formaldehyde. Table IV summarizes the mass analysis data and the calculated values for the doubly and singly labeled molecules of ethylene from carbons 2
and 3 of the propionate, and indicates that only a small number of molecules were actually labeled, and also that there was little or no formation of doubly labeled molecules. Two calculations have been made from the corrected mass values of 30/28 = 0.020 and 29/28 = 2.61 by using Equations 1 and 2. In one calculation the C\textsuperscript{13} concentration of the labeled source (F) was taken as 0.384 (0.373 + 0.0109) and in the other as 0.0649. The former value (Calculation A) is on the assumption that the formaldehyde was converted to a C\textsubscript{1} and utilized in propionate synthesis without dilution by a C\textsubscript{1} from the unlabeled lactate. The value of 0.0649 for F (Calculation B) is on the basis that there was fragmentation of 10 per cent of the lactate which was converted to propionate and acetate (7.24 and 3.13 mmoles, Table III) and the fragmentation gave rise to a C\textsubscript{1} in common with that formed from the formaldehyde-C\textsuperscript{18} during its conversion to propionate and acetate. The per cent C\textsuperscript{13} in the C\textsubscript{1} is thus 6.49 ((0.15 \times 37.3)/(0.1 \times 10.37) + 1.09 = 6.49). The assumption of 10 per cent fragmentation appears to involve the maximal dilution that the formaldehyde-C\textsuperscript{18} might encounter during the conversion to propionate. In fact, there is some reason to believe that the formaldehyde-C\textsuperscript{13} may not have undergone any dilution. In preliminary experiments in which formaldehyde-C\textsuperscript{14} was used, the added formaldehyde had a specific activity of 2.72 \times 10\textsuperscript{5} c.p.m. per mmole and the recovered formaldehyde 2.85 \times 10\textsuperscript{6} c.p.m. per mmole. The residual formaldehyde from the experiment of Table III was lost, but in view of the high C\textsuperscript{13} concentration in the formic acid (31.9 per cent excess) it seems unlikely that there was much dilution of the C\textsuperscript{13} of the formaldehyde. The formic acid probably gives an indication of the intracellular concentration of the C\textsuperscript{13} in the C\textsubscript{1}.

There is some reason to believe that the intracellular formaldehyde partially "equilibrates" with the extracellular formaldehyde, and if formaldehyde was produced from lactate, it would have diluted the tracer in the residual formaldehyde. Leaver (17) fermented glycerol-1,3-C\textsuperscript{14} and unlabeled formaldehyde with washed cells of propionic acid bacteria, and the fermentation was stopped before all the formaldehyde was utilized. The residual formaldehyde was found to have a specific activity equivalent to 65 per cent of the terminal carbons of the glycerol. When formaldehyde-C\textsuperscript{14} was added to the unlabeled glycerol fermentation, the C\textsuperscript{14} was fixed in the products. It is evident that under these conditions formaldehyde which is produced within the cell exchanges with extracellular formaldehyde. There may be, of course, a C\textsubscript{1} intermediate subsequent to formaldehyde, which is not reversibly converted to formaldehyde.

Even with the uncertainties with regard to F, it seems reasonably certain from the data of Table IV that formaldehyde fixation occurred in only a relatively few molecules of propionate. The results of Tables II
and IV complement each other in that the results of Table II indicate that
the conversion of lactate to propionate involves very little cleavage, while
those of Table IV indicate that very little de novo synthesis of carbons 2
or 3 occurs. Because of the small amount of propionate which is syn-
thesized via formaldehyde, the absolute determination of the relative
amounts of doubly labeled and singly labeled species is subject to a large
error. However, as nearly as can be judged from the data at hand, there
appears to be little or no formation of doubly labeled species.

Formaldehyde-$C^{13}$ Fixation with Glucose As Substrate—A much greater
incorporation of formaldehyde-$C^{13}$ occurs in the products of a glucose fer-
mentation than in lactate fermentation. The disadvantage of this fer-
mmentation is that there is no information concerning how much $C_1$ is pro-
duced from glucose. It was not possible to determine the extent of $C_1$
formation by mass analysis of the propionate (in a manner similar to that
with the mixtures of triply labeled lactate and unlabeled lactate (Table II))
because uniformly intramolecularly labeled glucose with a high $C^{13}$ con-
centration was not available. There are some indications, however, that
the amount of formaldehyde produced from glucose is low. Preliminary
experiments with formaldehyde-$C^{14}$ showed that there was little dilution
of $C^{14}$ in the residual formaldehyde during glucose fermentation. Since
there is evidence that the intracellular formaldehyde and extracellular
formaldehyde “equilibrate” (17), it is likely that little free formaldehyde
is formed from glucose in contrast to glycerol (17). For this reason glucose
was purposely selected for study rather than glycerol. It is known, how-
ever, that some formaldehyde is formed from glucose with growing cells.
With dimedon as a trapping agent, Wood and Werkman (18) have isolated
small amounts of formaldimedon.\footnote{In unpublished experiments in these laboratories, Wood and Stjernholm grew
cells of \textit{P. arabinosum} for 7 to 13 days in a yeast extract medium containing dimedon
and 0.08 g glucose. They found from about 0.092 to 0.24 mmole of formaldehyde-
dimedon per liter. It appears that the differences in conditions between the grow-
ing cell experiments and those reported here allow little basis for comparison. It
is perhaps pertinent, however, that even in these experiments only small amounts
of formaldehyde were trapped.}

The results with formaldehyde-$C^{13}$ and glucose fermentation are shown
in Tables V and VI. The total propionate and acetate produced was 5.41
mmoles from the 7.0 mmoles of added glucose (Table V). 0.302 mmole
($\left(3 \times 4.5 \times 0.52\right) + \left(2 \times 0.91 \times 0.56\right) / 26.7$) or 22 per cent of the added
formaldehyde-$C^{13}$ was converted to propionate and acetate. The $C^{13}$ was
equally distributed in the 3-carbons of propionate but there may have been
considerable loss of $C^{13}$ from the carboxyl group because of exchange with
the bicarbonate which was present in substantial amount in this experi-

\footnote{In unpublished experiments in these laboratories, Wood and Stjernholm grew
cells of \textit{P. arabinosum} for 7 to 13 days in a yeast extract medium containing dimedon
and 0.08 g glucose. They found from about 0.092 to 0.24 mmole of formaldehyde-
dimedon per liter. It appears that the differences in conditions between the grow-
ing cell experiments and those reported here allow little basis for comparison. It
is perhaps pertinent, however, that even in these experiments only small amounts
of formaldehyde were trapped.}
MENT. It is to be noted that only a slight dilution of Cl\textsuperscript{13} occurred in the residual formaldehyde (26.0 compared to 26.7).

The mass analysis data and the calculated values for the doubly and singly labeled molecules of ethylene are given in Table VI. Again two

| TABLE V |

**Fixation of Formaldehyde-C\textsuperscript{13} into Products of Fermentation of Glucose**

<table>
<thead>
<tr>
<th>Product</th>
<th>Distribution of C\textsuperscript{13}</th>
<th>CO\textsubscript{2} of combustion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mmoles</td>
<td>CH\textsubscript{3}</td>
</tr>
<tr>
<td>Propionate.......</td>
<td>4.50</td>
<td>0.50</td>
</tr>
<tr>
<td>Acetate...........</td>
<td>0.91</td>
<td>0.91</td>
</tr>
<tr>
<td>Residual formaldehyde*.......</td>
<td>0.145</td>
<td>0.145</td>
</tr>
</tbody>
</table>

Conditions: formaldehyde, 0.007 M (26.7 atom per cent excess C\textsuperscript{13}); glucose, 0.035 M; NaHCO\textsubscript{3}, 0.07 M; cells, 5 per cent wet weight; gas phase, CO\textsubscript{2}; total volume, 200 ml.; time, 8.5 hours; temperature, 30\degree C.

* Analyzed by the method of Nash (19). The dimedon derivative was isolated and oxidized (9) for C\textsuperscript{13} analysis.

| TABLE VI |

**Mass Analysis of Ethylene of Carbon Atoms 2 and 3 of Propionate from Glucose Fermentation of Table V and Calculated Values for Doubly and Singly Labeled Molecules**

<table>
<thead>
<tr>
<th>Calculation, No.</th>
<th>C\textsuperscript{13} in labeled source (F)</th>
<th>Types of ethylene</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>per cent</td>
<td>D, C\textsuperscript{13}--C</td>
</tr>
<tr>
<td>A</td>
<td>27.4</td>
<td>0.07</td>
</tr>
<tr>
<td>B</td>
<td>15.9</td>
<td>0.2</td>
</tr>
</tbody>
</table>

The spectrometer values (in per cent) before correction for H fragmentation\textsuperscript{1} were 30/28 = 0.029 and 29/28 = 3.23; these values after correction were 30/28 = 0.029 and 29/28 = 3.27. The height of the 28 peak was 6.28 \times 10\textsuperscript{8} cm.

Separate calculations have been made, one with F = 0.274 and the other with F = 0.159. The former value (Calculation A) is again based on no dilution of the labeled source by C\textsubscript{13} from unlabeled substrate. The value of F = 0.159 (Calculation B) is based on the assumption that 10 per cent of the C\textsubscript{3} that gave rise to propionate and acetate was cleaved to a C\textsubscript{1} similar to that formed from the formaldehyde-C\textsuperscript{13} (0.302 \times 26.4/0.10 \times 5.41 + 1.09 = 15.9). It is seen in Table VI that most of the propionate appeared
to be formed without involvement of formaldehyde and was unlabeled. There appears to be little or no formation of doubly labeled species from the formaldehyde.

DISCUSSION

Two different types of procedures have been employed in the present studies with the mass spectrometer. In the first procedure an intramolecularly triply labeled substrate was fermented in combination with unlabeled substrate (6). This was done to determine whether or not there was cleavage and resynthesis of the carbon bonds during the conversion of the substrate to the products. If cleavage and resynthesis occurred together with pooling of the cleavage products, then the mass spectrum of the product would differ from the spectrum derived from the corresponding carbons of the substrate. This procedure was important to the present work because it was necessary to know whether the substrate was cleaved to a C1 compound which was used in the synthetic reactions, and whether carbon atoms 2 and 3 of propionate were stable after synthesis. The results showed that there was very little cleavage and resynthesis at carbons 2 and 3 of lactate during the conversion to propionate. This supports the hypothesis that lactate is metabolized largely by a direct reduction to propionate. Furthermore, as noted previously, the C1 that is formed during cleavage and resynthesis of carbon 1 of propionate apparently is not similar to the C1 that is formed from formaldehyde.

The second procedure had the opposite objective, i.e. the estimation of the amount of carbon to carbon synthesis rather than the demonstration of carbon to carbon cleavage. Such synthesis may be demonstrated by mass analysis even under conditions in which it is not possible to show synthesis by the usual balance methods. In this case synthesis is demonstrated by formation of intramolecularly doubly labeled compounds (5, 7). In spite of uncertainties concerning the exact value of the C18 concentration of the labeled source (F), it seems quite certain that there was little doubly labeled material produced because the 30/28 value was very low as compared to the 29/28 value. It therefore appears that the formaldehyde was converted to carbons 2 and 3 of propionate by a process which resulted in singly labeled molecules, and that there was little or no total synthesis of the 2- and 3-carbons of propionate from formaldehyde carbon.

The mechanism by which formaldehyde is fixed in the propionic acid fermentation is unknown but may involve a symmetrical C4 compound. Stjernholm, in unpublished work in these laboratories, has recently shown with P. arabinosum that the C14 of L-glycerol-1-C14 appears not only in the carboxyl carbon but also in carbons 2 and 3 of propionate. This
metabolism differs from that of liver in which L-glycerol-1-C\textsuperscript{14} is metabolized asymmetrically (20) and is converted almost exclusively to the 3,4 positions of the glucose unit of glycogen (21, 22). Rush et al. (23) have found that Aerobacter aerogenes strain 1033 likewise does not handle glycerol asymmetrically. This strain dehydrogenates glycerol to dihydroxyacetone and thus forms a symmetrical C\textsubscript{3} compound. A similar mechanism may account for the randomization of the isotope of L-glycerol-1-C\textsuperscript{14} by propionibacteria and also for the fixation of formaldehyde-C\textsuperscript{13}.

The suggested mechanism for formaldehyde fixation is illustrated in Fig. 1. In this mechanism the C\textsubscript{2} unit from the substrate (glucose or lactate) combines with the C\textsubscript{1} from formaldehyde. The resultant symmetrical C\textsubscript{3} is converted to propionate and at this stage gives two types of propionate, 1- and 3-labeled. Since propionate is known to undergo randomization (6, 24), there would be formation of 2-labeled propionate from 3-labeled propionate. None of the propionate molecules would be doubly or triply labeled.

**SUMMARY**

Formaldehyde is fixed in each position of propionate by propionic acid bacteria (3) during fermentation of lactate or glucose. The question of whether this distribution occurs through a total synthesis of propionate from formaldehyde has been investigated with formaldehyde-C\textsuperscript{13} by the use of mass analysis to determine the types of molecular species of propionate. Only carbons 2 and 3 of propionate were submitted to mass analysis because the carboxyl group of propionate is cleaved reversibly (6).

The calculation of the amount of doubly (C*—C*) and singly (C*—C) labeled molecules which are formed from formaldehyde-C\textsuperscript{13} requires information on the possible dilution of the C\textsuperscript{13} by C\textsubscript{1} which might arise during the accompanying fermentation of the unlabeled substrate. This question was investigated by fermenting a mixture of triply labeled lactate-C\textsuperscript{13} and unlabeled lactate. It was found that there was little differ-
ence between the mass spectrum calculated for carbon atoms 2 and 3 of the lactate mixture and that found for carbons 2 and 3 of the resulting propionate. Thus the mass analysis demonstrated that carbons 2 and 3 remained intact during the conversion to propionate and a C₁ was not involved. It therefore was concluded that there was little formation of C₁ from carbons 2 and 3 of lactate.

The calculations based on the mass spectra of propionates synthesized from formaldehyde-C₁³ and unlabeled lactate or glucose showed that most of the propionate molecules were unlabeled and arose from the unlabeled substrate. Those molecules which did contain fixed formaldehyde-C₁³ appeared to be mostly, if not entirely, singly labeled. A possible mechanism for formation of this mixture of molecules is presented.

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