A method has been developed for the positional $^{13}$C isotope analysis of pyruvate and acetate by stepwise quantitative degradation. On its base, the kinetic isotope effects on the pyruvate dehydrogenase reaction (enzymes from Escherichia coli and Saccharomyces cerevisiae) for both of the carbon atoms involved in the bond scission (double isotope effect determination) and on C-3 of pyruvate have been determined. The experimental $k_1/k_3$ values with the enzyme from *E. coli* on C-1 and C-2 of pyruvate are $1.0093 \pm 0.0007$ and $1.0213 \pm 0.0017$, respectively, and, with the enzyme from *S. cerevisiae*, the values are $1.0238 \pm 0.0013$ and $1.0254 \pm 0.0018$, respectively. A secondary isotope effect of $1.0031 \pm 0.0009$ on C-3 (CH$_3$-group) was found with both enzymes.

The size of the isotope on C-1 indicates that decarboxylation is more rate-determining with the yeast enzyme than with the enzyme from *E. coli*, although it is not the entirely rate-limiting step in the overall reaction sequence. Assuming appropriate values for the intrinsic isotope effect on the decarboxylation step ($k_d$) and the equilibrium isotope effect on the reversible substrate binding ($k_1$, $k_3$), one can calculate values for the partitioning factor $R$ ($k_1/k_3$; *E. coli* enzyme 4.67, *S. cerevisiae* enzyme 1.14) and the intrinsic isotope effects related to the carboxyl-C ($k_1/k_1'$ = 1.019; $k_3/k_3'$ = 1.033).

The isotope fractionation at C-2 of pyruvate gives strong evidence that the well known relative carbon-13 depletion in lipids from biological material is mainly caused by the isotope effect on the pyruvate dehydrogenase reaction. In addition, our results indicate an alternating $^{13}$C abundance in fatty acids, that has already been verified in some cases.

**The abbreviations used are:** TPP, thiamin pyrophosphate; Lip$_{SH}$, lipoic acid.

The standard is carbon dioxide from PeeDee Belemnite isotopic standard limestone (4).

C$_4$ plants are distinctly different ($-26 \pm 3\%$ and $-12 \pm 3\%$ respectively). However, within both of these plant groups, the lipids are depleted in carbon-13 relative to the carbohydrates by 4–6% (5–7). Therefore, one has to postulate that, in the course of the metabolic pathway from carbohydrates to lipids, an irreversible step at a branching point of metabolism must occur, that is accompanied by a carbon isotope effect.

Abelson and Hoering (8) presented indirect evidence that glycolysis proceeds without isotope discrimination. Monson and Hayes (9) indirectly deduced a kinetic carbon isotope effect of 1.023 for the pyruvate dehydrogenase reaction (enzyme from *Escherichia coli*); however, the isotope effect of this important reaction has not yet been determined experimentally.

The formation of acetyl-CoA from pyruvate (oxidative decarboxylation)

\[
\begin{align*}
H_2C-CO-COO^+ & + HSCoA + NAD^+ \\
\rightarrow H_2C-CO-SCoA + CO_2 + NADH
\end{align*}
\]

is a (multistep) reaction sequence, catalyzed by a multienzyme complex of three different catalytic subunits, the relative number and geometric arrangement of which depend on its origin. The different enzymes are pyruvate dehydrogenase (E1 (EC 1.2.4.1), coenzyme thiamin pyrophosphate = TPP$^+$), dihydrolipoamide acetyltransferase (E2 (EC 2.3.1.12), coenzyme lipoic acid = Lip$_{SH}$), and dihydrolipoamide dehydrogenase (E3 (EC 1.8.1.4), coenzyme flavin adenine dinucleotide = FAD). The overall process is a sequence of five reactions (10):

\[
\begin{align*}
H_2C-CO-COOH + TPP-E1 & \\
\rightarrow (H_2C-CHOH-TPP)-E1 + CO_2 \\
(H_2C-CHOH-TPP)-E1 + (LipSH)_2-E2 & \\
\Rightarrow (H_2C-CO-SLipSH)-E2 + TPP-E1 \\
(H_2C-CO-SLipSH)-E2 + HS-CoA & \\
\Rightarrow (Lip(SH)_2)-E2 + H_2C-CO-S-CoA \\
(Lip(SH)_2)-E2 + FAD-E3 & \Rightarrow (LipS)-E2 + FAD-H_2-E3 \\
FAD-H_2-E3 + NAD^+ & \Rightarrow FAD-E3 + NADH + H^+
\end{align*}
\]

Reaction 2 corresponds to the pyruvate decarboxylase reaction (11–14) for which kinetic isotope effects have already been determined. There is also evidence from various kinetic investigations (15–18) that for the oxidative decarboxylation the reactions catalyzed by the E1 subunit comprise the rate-limiting step. The proper step itself, however, has not yet been precisely identified. In addition, the relative position of the different parts of the reaction may also be influenced by the structure and symmetry of the multienzyme complexes.

$^{1}$ The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

$^{2}$ The abbreviations used are: TPP, thiamin pyrophosphate; Lip$_{SH}$, lipoic acid.

8159
In the present study, the kinetic isotope effect on the pyruvate dehydrogenase reaction is investigated with multienzyme complexes of yeast and prokaryote (E. coli), which are known to be different in their tetramer structure. In order to get more information on the relative velocities of the different steps, the double isotope effect method (12, 19) is applied in which the isotope effects on both atoms separated in the course of the reaction are determined. This demands a positional isotope analysis of the substrate pyruvate and the product acetate. Hence, sophisticated quantitative and isotope effect free degradation methods had to be developed.

**MATERIALS AND METHODS**

**Chemicals and Enzymes**—Chemicals (highest purity available) were from E. Merck, Darmstadt, FRG and Fluka AG, Buchs, Switzerland. Biochemicals were purchased from Boehringer Mannheim GmbH, Mannheim, FRG, and Sigma, and was phosphotransacetylase (EC 2.3.1.8) from Clostridium kluyveri. Pyruvate dehydrogenase from Saccharomyces cerevisiae was a gift from Dr. G.-B. Kreuze (Boehringer Mannheim). The enzyme, a suspension in 0.1 M potassium phosphate buffer (pH 7.6) (for determination of the activity of the enzyme, in which the formation of NADH was coupled with the phosphotransacetylase reaction. The incubation method of Kresze and Ronft (20) was used for the spectrophotometric determination of the activity of the enzyme, in which the formation of NADH was measured. One unit of enzyme is defined as the amount of CO2 formed (spectrophotometric measurement of NADH concentration). The incubation was stopped by the addition of 0.4 ml of 10 N H2SO4. While the solution was kept at -126°C (freezing methylenechlorane), the CO2 was isolated on a high vacuum line by freezing in liquid nitrogen (-196°C). Repeated freeze/thaw cycles gave quantitative yields. The fraction of reaction was determined manometrically from the amount of CO2. The gas was then used for isotope ratio analysis. From the media for the CO2 isolation, the reaction was stopped by the addition of 0.4 ml of 10 N H2SO4. While the solution was kept at -126°C (freezing methylenechlorane), the CO2 was isolated on a high vacuum line by freezing in liquid nitrogen (-196°C). Repeated freeze/thaw cycles gave quantitative yields. The fraction of reaction was determined manometrically from the amount of CO2. The gas was then used for isotope ratio analysis. From the media for the CO2 isolation, the reaction was stopped by the addition of 0.4 ml of 10 N NaOH. The mixture was heated to 70°C for 20 min in order to hydrolyze acetyl-CoA and acetate. Then, the solution was concentrated to about 2 ml under reduced pressure, and 50 ml of concentrated HCl and 0.5 ml of 10 N H2SO4 were added. The solution in the closed flask was heated to 600°C and then brought to -2°C. After the excess pyruvic acid had formed a semicarbazone, the acetate acid could be isolated by lyophilization. The cold trap (-196°C) contained 0.1 nmol of freshly prepared NaOH solution. The sodium acetate solution obtained was brought to dryness in an evacuated desiccator. The salt was either directly combusted for a total mean isotope determination or submitted to pyrolytic degradation for positional isotope analysis. The δ13C value of acetate isolated by this procedure in control experiments did not differ by more than ±0.4‰ from that of the starting material.

**Degradation of Pyruvate and Acetate for Positional Isotope Analysis**—The first step of the degradation of pyruvate was an oxidation with H2O2, yielding C-1 as CO2 and C-2 and -3 as acetic acid. In the second step, the pyrolysis of sodium acetate, CH3CO2Na, was performed with the CH3 group and Na13CO3 from the carboxyl group.

The decarboxylation of sodium pyruvate for the isotope analysis in position 1 was performed in a three-compartment vessel: 1 ml of 85 mM sodium pyruvate was put into the main compartment, the side arms contained 0.2 ml of 30% H2O2 and 0.5 ml of 10 N H2SO4, respectively. The solutions were degassed by repeated freezing and thawing under vacuum. After addition of the H2O2 to the substrate, the mixture was kept at 22°C for 30 min. Then, the H2SO4 was added and the CO2 formed was isolated as described above. Completeness of the reaction was controlled by manometric CO2 determination. The gas was used for mass spectrometric isotope analysis. The isolation of the acetic acid by lyophilization was performed as described for the enzyme-reacted samples (see above).

For the isotope analysis of acetate (corresponding to C-2 and -3 of pyruvate), 3-10 mg of the sodium salt (isolated from an enzymatic or H2O2-degradation of pyruvate) and a 3-4-fold excess of dry CO2-free NaH were heated for 10-15 min to 480°C in an evacuated reaction tube (Fig. 1) which was directly connected to a combustion line (Heraeus Micro U elemental analyzer). The CH3 group (C-2 of acetate corresponding to C-3 of pyruvate) was formed, then washed with O2/helium (1:2) through the combustion unit, and the CO2 formed was introduced into the mass spectrometer. The reaction tube was evacuated, and 1 ml of 10 N H2SO4 was added through the gas inlet. The CO2 (carbonate from C-1 of acetate corresponding to C-2 of pyruvate) was isolated for mass spectrometry.

The total mean δ13C value of pyruvate or acetate was obtained by the combustion of 4-6 mg of the salts in the presence of 50 mg of NaHPO4 and subsequent mass spectrometric analysis of the CO2 formed.

**Isootope Ratio Measurement and Isotope Effect Calculation**—The isotope analysis of the CO2 samples (0.04-0.2 mmol) of different origins was performed on an isotope ratio mass spectrometer (VG Micromass 903) relative to a laboratory standard. Corrections for δ13C and the correlation to the Pee Dee Belemnite isotopic standard were automatically computed to give δ13C values from which the isotope ratios P1/P2 were obtained. On their base and the turnover of the reaction, the isotope effects were calculated by means of equation (7):

$$k_{34}/k_{35} = \log(1 + f)/\log(1 + f(P_1/P_2)/(P_3/P_4))$$

with $f = $ fraction of reaction; $P_1/P_2 = [13C]/[12C]$ in the product after partial reaction; $P_3/P_4 = [13C]/[13C]$ in the product after total turnover which is identical with that in the substrate at the same carbon position.

The isotope effects at C-1 and C-3 of pyruvate were directly obtained from the δ13C values of the CO2 and the CH3H, respectively. For the calculation of the isotope effects at C-2 of pyruvate, the δ13C value of the acetate obtained was used, which was corrected for the δ13C value of C-3 (mean value for CH3 after partial turnover).

**RESULTS**

**Positional Isotope Analysis of Pyruvate**—The double isotope effect method implies the measurement of the isotope ratios of both carbon atoms involved in a bond scission before and
after partial reaction. This demands a positional isotope analysis of substrate and products, hence a quantitative and isotope effect-free degradation of pyruvate and acetate in the case of the pyruvate dehydrogenase reaction.

Methods according to the following equations were developed:

\[
\begin{align*}
H_3C-CO-COOH + H_2O \rightarrow H_3C-COOH + CO_2 + H_2O \quad (8) \\
H_3C-COOH + NaOH \rightarrow CH_4 + Na_2CO_3 \quad (9) \\
CH_4 + 2 O_2 \rightarrow CO_2 + 2 H_2O \quad (10)
\end{align*}
\]

The oxidative degradation of pyruvate by H$_2$O$_2$ was first described by Hollemann (22). As the reaction proceeds with a carbon isotope fractionation, experimental conditions had to be developed to assure quantitative turnover. Systematic research on the influence of pH value and reaction time on the yield led to the conditions described under “Materials and Methods.”

Among several methods tested for the degradation of acetic acid (Kolbe electrolysis, Schmidt degradation, pyrolysis), the most promising seemed to be the pyrolysis of sodium acetate in the presence of an excess of NaOH. However, as the yield of CH$_4$ by the method of Bromley et al. (23) was unsatisfactory, a modification was developed in which a stoichiometric yield of pure methane (mass spectrometric control) was attained. In a simple device (Fig. 1), the isotope analysis for both carbon atoms of acetate from the same sample was possible.

As shown in Table I, the arithmetic mean of the $\delta$ values for the individual carbon atoms fits very well with the mean value of the whole molecule obtained by combustion. The same is true with the results for the acetate resulting from the degradation, while the corresponding values for a standard acetate sample differ by 1.3%. Obviously, in the latter case, the larger difference between the $\delta$ values of the different carbon atoms implied a greater error. Nevertheless, one can conclude that the whole procedure can be performed without isotope fractionation and thus can be used for positional isotope analysis of pyruvate and analytic control of the isotope effect of the enzymatic oxidative decarboxylation of pyruvate.

**Kinetic Carbon Isotope Effects on the Pyruvate Dehydrogenase Reaction**—The kinetic isotope effects of the pyruvate dehydrogenase reaction catalyzed by multienzyme complexes from yeast (eukaryote) and from E. coli (prokaryote) were investigated. As the reactions catalyzed by the subunits E1 and E2 are inhibited by the product acetyl-CoA (24, 25), all incubations were performed in the presence of phosphotransacetylase. The isotope effects on both carbon atoms of the bond split in the course of the reaction and on C-3 were determined by positional isotope analysis of the substrate before (isotope ratio $P_{\text{C}}/P_{\text{C}}$) and the products after a given turnover (isotope ratio $P_{\text{C}}/P_{\text{C}}$). The turnover rate (between 4 and 14%) was determined from the amount of CO$_2$ or NADH formed.

With both multienzyme complexes, the pyruvate dehydrogenase reaction implies a small isotope fractionation on the CH$_3$-group (1.0031 ± 0.0009; Table I). As C-3 is not directly involved in the bond-scission during the reaction, only a secondary isotope effect of this size can be expected. A similar effect was found by DeNiro and Epstein (12) in the CH$_3$-group of pyruvate for the pyruvate decarboxylase reaction ($h_{\text{H}}/h_{\text{H}} = 1.0001$).

The isotope effect at C-2 (CO-group), determined by combustion of the acetate formed under regard of the isotope fractionation at the CH$_3$-group, is 1.0213 ± 0.0017 for the enzyme of E. coli and 1.0254 ± 0.0016 for the enzyme from S. cerevisiae (Table II). These values are in the range of a primary kinetic carbon isotope effect of an enzyme-catalyzed reaction and indicate that bond-making or bond-breaking at this atom is partially rate-limiting with both enzymes.

The kinetic isotope effect at C-1 (COOH-group) is distinctly different for the enzymes from the two sources (Table II). While with the enzyme from S. cerevisiae it is quite similar (1.0238 ± 0.0013) to that at the adjacent C-2, it is distinctly smaller (1.0093 ± 0.0007) in the case of the E. coli enzyme. This indicates a different relative velocities of various steps with the two enzymes. The result is obviously a demonstration for the value of the double isotope effect method, by which more detailed and precise conclusions on the reaction mechanism are possible in comparison to the classical method, as is shown below.

**DISCUSSION**

**Interpretation of the Results Regarding Reaction Mechanism and Different Enzyme Source**—Experiments by Danson et al. (15) with E. coli-pyruvate dehydrogenase and by Caté et al. (16) with the bovine kidney enzyme have given evidence that one of the steps catalyzed by the E1-subunit should be the slowest in the overall reaction sequence. Using rapid mixing quench techniques, Akiyama and Hammes (17, 18) deduced that the decarboxylation of the TPP-pyruvate adduct could be rate-limiting.

The E1-catalyzed first part of the pyruvate dehydrogenase reaction comprises two steps leading to enzyme-substrate complexes with covalent binding between thiamin pyrophosphate and pyruvate or acetaldehyde. Nucleophilic attack on the carbonyl carbon of pyruvate is facilitated by thiamin $^{3}$E. Melzer and H.-L. Schmidt, unpublished results.
pyrophosphate reacting in the carbanionic form at C-2 of the thiazolium moiety. As this ionization occurs in a fast pre-equilibrium step without interaction of pyruvate, it will not be included in the following rate constant discussion. The first pyruvate-related step is reversible (covalent binding), while the second, comprising the bond-splitting between C-1 and -2 of pyruvate and CO₂ elimination, is irreversible.

The determination of the kinetic isotope effect on an enzyme-catalyzed reaction provides an independent means for the identification of the rate-limiting step prior to and including the first irreversible step. More detailed information is to be expected from the double isotope effect determination, as in the first (reversible) step only C-2 of pyruvate is involved, while in the second step both C-1 and -2 are involved (C-C bond fission). In equation 11, kr is the rate constant for the substrate binding, k₁ for the decomposition of the intermediate into the reactants, and k₃ the rate constant for the decarboxylation. The mathematical expression for the correlation between an experimentally determined isotope effect k₁₂/k₁₃ (exp) and the rate constants of the corresponding reaction sequence is given in equation 12 (k indicates the rate constant for the heavy isotope, R is partitioning factor or commitment; Ref. 2):}

\[
k_{12}/k_{13}(\text{exp}) = k_{1}/k_{1}' = k_{1}/k_{1}' + R \quad \text{k}_{2}/k_{2}' = k_{2}/k_{2}' + R
\]

Therefore, one can deduce from an experimentally determined isotope effect (e.g. k₁₂/k₁₃ in Table II) the real isotope effect on an individual step (intrinsic isotope effect), when the rate constants of the other steps are known.

For the reactions in question, this is not the case, but certain assumptions can be made. An isotope effect on C-1 should predominantly be caused by the second step, because in the first step this atom could only participate with a secondary isotope effect. Hence, the first step should practically not be "isotope-sensitive" with regard to C-1, and k₁/k₁' and k₂/k₂' should be near unity. In this case, equation 12 simplifies to the form:

\[
k_{12}/k_{13}(\text{exp}) = k_{2}/k_{2}' + R/1 + R
\]

Intrinsic isotope effects for rate-determining decarboxylations in chemical reactions are in the range of 1.03–1.07 (21). Jordan et al. (13) found a value of 1.051 (pH 5.0; 45.6 °C) for the isotope effect of the decarboxylation of 2-(1-carboxy-3-hydroxyethyl)-3,4-dimethylthiazolium chloride, which is a model for the covalent TPP-pyruvate adduct in the enzyme. If one takes this value as a realistic intrinsic isotope effect for the corresponding enzyme-catalyzed decarboxylation, one can calculate values for the partitioning factor R. For the E. coli enzyme, R = k₂/k₁ turns out to be 4.67, for the S. cerevisiae enzyme 1.14. Thus, in the latter case, decarboxylation and decomposition of the enzyme-substrate complex occur at almost the same rate, and substrate binding (k₁) as well as decarboxylation (k₂) are partially rate-limiting steps. With the pyruvate dehydrogenase from E. coli, however, the TPP-pyruvate complex undergoes decarboxylation (k₂) nearly 5 times faster than decomposition to substrate and enzyme (k₃). We therefore conclude that with this enzyme, substrate binding is more rate-determining than decarboxylation.

For the discussion of the isotope at C-2, equation 12 can be applied. The experimental isotope effects and the R value (see above) are known for both enzymes. In order to obtain a solution for the two intrinsic isotope effects k₁/k₁' and k₂/k₂', two further assumptions have to be made. First, the intrinsic isotope effect should be the same with both enzymes as the mechanism of the reaction (TPP-pyruvate adduct) is identi-
Carbon Isotope Effects on Pyruvate Dehydrogenase Reaction

IMPORTANT OF THE ISOPOE EFFECTS ON THE CARBON-13 CONTENT OF LIPIDS—Pyruvate is a key intermediate in both aerobic and anaerobic metabolism. It is the starting substrate for glucoseogenesis, the biosynthesis of several amino acids, and the formation of acetaldehyde or acetyl-CoA. The acetyl group of acetyl-CoA may either be degraded in the citric acid cycle or serve as the carbon source in the biosynthesis of lipids. As the pyruvate dehydrogenase reaction is one of several reactions at a branching point, its isotope effects should be manifested in the carbon-13 content of the products synthesized from acetyl-CoA.

As the isotope effect on the pyruvate dehydrogenase reaction results in a depletion of carbon-13 in the C-1 of the acetyl group, all lipids should be depleted in the heavy carbon isotope. Theoretically, from our results, the overall depletion of 4.5% for the acetyl subset of acetyl-CoA should be expected, as strong polarization at the site of reaction in both the substrate and the coenzyme facilitate C-C bond-making. The intrinsic isotope effect on the decarboxylation step (1.033) is somewhat smaller at C-2 than the assumed one at C-1 (1.051). This is an expression for the different changes in bonding in the neighborhood of the bond involved in the scission. A similar difference for the isotope effects on adjacent carbon atoms was found by Loudon et al. (27) for the decarboxylation of malonic acid (k_{obs}/k_0 = 1.017 and 1.049, respectively; 150 °C).

The relative rate constant and isotope effect differences found with the enzymes from different origins may probably be due to structural differences influencing polarization of substrate and coenzyme and providing different microenvironments. Finally, the known differences in the quaternary structure of the two multi-enzyme complexes can influence substrate diffusion rates within the complex and thus change the relative reaction rates.

The carbon isotope effects on the pyruvate decarboxylation reaction (enzyme from yeast) as reported in the literature range between 1.004 and 1.009 for C-1, and between 1.015 and 1.018 for C-2 (11–14). Comparing these values to our results on the pyruvate dehydrogenase reaction, certain differences can be seen, especially for the two enzymes from yeast. As mentioned above, in spite of the same reaction mechanism (for the first two steps), enzymatic catalysis can cause different expression of the presumable same intrinsic isotope effect.

Acknowledgments—We wish to thank Dr. G.-B. Kresse (Boehringer Mannheim, Tutzing) and Dr. H. Bisswanger, University of Tübingen, for generously providing the pyruvate dehydrogenase preparations. We are grateful to Prof. M. H. O’Leary, Madison, Wisconsin, for helpful discussions and review of the manuscript.

REFERENCES

Carbon Isotope Effects on Pyruvate Dehydrogenase Reaction