Mechanism of Carbon Isotope Fractionation Associated with Lipid Synthesis

Abstract. *The low carbon-13/carbon-12 ratio of lipids is shown to result from isotopic fractionation during the oxidation of pyruvate to acetyl coenzyme A. In vitro analysis of the kinetic isotope effects of this reaction indicates that there will be a large, temperature-dependent difference in the carbon-13/carbon-12 ratio between the methyl and carbonyl carbon atoms of acetyl coenzyme A and between those carbon atoms of lipid components which derive from them.*

There are differences in the carbon isotopic composition of the major biochemical components of an organism. The lipid fraction has a lower $\delta^{13}C$ value (*1*) than the other major components (protein and carbohydrate fractions) and the total organism (*2–4*). The basis for the lower $\delta^{13}C$ value of lipids has not been experimentally determined (*5*).

Microorganisms grown in minimal media (inorganic salts, trace quantities of vitamins, and a single source of carbon) provide a simple system for identifying the biochemical steps during which the ^{13}C depletion of lipids occurs. Abelson and Hoering (*3*) showed that both *Escherichia coli* and *Chlorella* grown on glucose have lipid fractions which are depleted in $\delta^{13}C$ by 7.0 per mil relative to the glucose carbon. They also presented indirect evidence which indicates that the glucose used in these experiments was isotopically homogeneous (that is, its individual carbon atoms had the same isotopic composition). The ^{13}C depletion of the lipid fraction must therefore be due to isotope effects during the metabolism of glucose (*6*).

The metabolic pathway involved in lipid synthesis from glucose in these organisms is shown in simplified form in Fig. 1. Glucose is metabolized via the Embden-Meyerhof pathway to pyruvate. Pyruvate is then decarboxylated and oxidized by the pyruvate dehydrogenase complex to acetyl coenzyme A (CoA). The acetyl group of acetyl CoA serves as the main source of carbon for lipid biosynthesis. The steps in this metabolic sequence responsible for the ^{13}C depletion of lipids can be identified by growing *E. coli* on carbon sources which enter as intermediates after glucose but before lipid synthesis. Growth on a carbon source which enters before such steps will result in a lipid fraction depleted in ^{13}C relative to the carbon source. Conversely, the ^{13}C depletion of the lipid fraction relative to the carbon source will be eliminated if the carbon source enters after the steps causing the isotopic fractionation.

The results of experiments in which *E. coli* was grown in minimal media with glucose, pyruvate, or acetate as the sole carbon source are presented in Table 1. Cells grown on glucose or pyruvate have lipid fractions which are depleted in $\delta^{13}C$ by 6.5 and 8.4 per mil relative to their respective carbon sources, while growth on acetate produces a lipid fraction which is depleted by only 1.0 per mil relative to the carbon source.

Abelson and Hoering (*3*) presented indirect evidence which indicates that there is little or no isotopic fractionation during the metabolism of glucose to pyruvate. Their observation that, in *Chlorella* grown on glucose as the sole carbon source, alanine has a $\delta^{13}C$ value only 0.5 per mil more positive than that of glucose supports this idea since alanine derives its carbon atoms directly from pyruvate through a transamination reaction (*7*). This conclusion is also consistent with the results cited above, which indicate that growth on glucose and growth on pyruvate give ^{13}C depletions in the lipid fraction of similar magnitude. Growth on acetate, which is metabolized directly to acetyl CoA (*8*) before lipid synthesis, results in a much smaller isotopic fractionation. Therefore, most of the ^{13}C depletion of the lipid fraction must occur during the oxidation of pyruvate to acetyl CoA.

Table 1. Effect of the carbon source on the carbon isotopic composition of the *E. coli* lipid fraction. *Escherichia coli* K12, strain AB1621, was inoculated into 1 liter of medium, which contained only inorganic salts (*13*), 1 mg of thiamine, and either glucose (28 m*M*), sodium pyruvate (56 m*M*), or sodium acetate (84 m*M*). The cultures were maintained at 25°C with vigorous aeration. Cells were harvested in log phase at 2×10^8 cells per milliliter; at this concentration, less than 2 percent of the available carbon was assimilated into cell matter. Lipid extraction (*14*) and sample combustion to CO_2 (*15*) were done by standard techniques. The uncertainty due to sample preparation and measurement is ± 0.2 per mil. Two cultures were grown and processed separately for each carbon source.

Carbon source	$\delta^{13}C$ (per mil)	
	Carbon source	Lipid
Glucose	−9.5	−15.7
		−16.3
Sodium pyruvate	−20.5	−28.9
		−28.9
Sodium acetate	−20.1	−21.5
		−20.7

Pyruvate is oxidized to acetyl CoA by the pyruvate dehydrogenase complex. After the initial step of the pyruvate dehydrogenase complex reaction

$$\underset{\text{(enzyme)}}{E} + \underset{\text{(pyruvate)}}{CH_3COCOOH} \rightarrow \underset{\text{(hydroxyethyl-E)}}{CH_3CHOH\text{-}E} + CO_2 \quad (1)$$

the enzyme-bound hydroxyethyl group will be completely converted to acetyl CoA, since the intermediates of the reaction do not dissociate from the complex (*9*). Therefore, the ^{13}C depletion of the lipid fraction must result from kinetic isotope effects during this initial step producing hydroxyethyl groups depleted in ^{13}C relative to the pyruvate. One method of determining the kinetic isotope effects of a reaction is based on the comparison of $\delta^{13}C$ values of the products of the reaction run to only a few percent of completion (so as not to change the isotopic composition of the reactant pool) with the $\delta^{13}C$ values of the reactants, which can be determined by running the reaction to completion. The enzyme pyruvate decarboxylase from yeast was selected for the study of the isotope effects in question. It catalyzes reaction 1, but the CH_3CHOH-E complex then breaks down to E and CH_3CHO (acetaldehyde) (*10*). This allows precise measurement of the kinetic isotope effects of the enzymatic reaction, since both carbon dioxide and acetaldehyde can be isolated by vacuum techniques, permitting their $\delta^{13}C$ values to be determined by simple and direct methods.

The results of kinetic isotope effect studies with yeast pyruvate decarboxylase are presented in Table 2. The acetaldehyde formed in the 1 percent reaction at 25°C (experiment C) has a $\delta^{13}C$ value of −28.5 per mil, which is 7.9 per mil lower than that of the methyl and carbonyl carbon atoms of pyruvate (experiment A) from which it is derived, which have a $\delta^{13}C$ value of −20.6 per mil. Analysis of each of the three carbon atoms of pyruvate (experiment E) and of the products of the 1 percent reaction at 25°C (experiment F) indicates that the $\delta^{13}C$ depletion of acetaldehyde, which was found to be 7.5 per mil by this method, is confined largely to the carbonyl carbon atom. However, the $\delta^{13}C$ depletion of 1 per mil in the methyl carbon atom of acetaldehyde is statistically significant (by Student's *t*-test at the $P = .05$ level). The isotopic fractionation in acetaldehyde is concentrated in the carbonyl carbon atom because the formation of a carbonyl addition complex between the enzyme

and pyruvate (*10*) is the main rate-determining step in the enzymatic reaction mechanism (*11*). The small ^{13}C depletion in the methyl carbon atom of acetaldehyde is a secondary isotope effect, since this atom undergoes no change in bonding during the enzymatic reaction (*10*). The isotope effect on the CO_2 indicates that the decarboxylation step in the enzymatic reaction mechanism also must be partially rate-determining (*11*). Temperature effect studies indicate that the magnitude of the $\delta^{13}C$ depletion of acetaldehyde relative to the methyl and carbonyl carbon atoms of pyruvate increases by 3.7 per mil over the range 15° to 35°C (experiments B, C, and D). Since the secondary isotope effect on the methyl carbon atom will not vary significantly with temperature, the temperature effect must manifest itself primarily in the carbonyl carbon atom. Therefore, the observed difference in $\delta^{13}C$ values between the methyl and carbonyl carbon atoms of the acetaldehyde must increase by approximately 0.4 per mil per 1°C increase in temperature.

The depletion and distribution of ^{13}C in the lipid fraction of microorganisms grown on isotopically homogeneous glucose are accounted for by the following model. There is little or no isotopic fractionation when glucose is converted to pyruvate via the Embden-Meyerhof pathway. Oxidation of pyruvate to acetyl CoA by the pyruvate dehydrogenase complex results in a decrease in ^{13}C content of the acetyl group relative to the pyruvate, the magnitude of the ^{13}C depletion depending on the temperature-dependent kinetic isotope fractionation of the initial step of the pyruvate dehydrogenase complex reaction and on the partitioning of pyruvate between this and other reactions. The ^{13}C depletion of acetyl CoA is concentrated primarily in the carbonyl carbon atom, with the methyl group retaining the $\delta^{13}C$ value of the glucose. Incorporation of ^{13}C-depleted acetyl groups into lipid components results in the ^{13}C depletion of the lipid fraction. The difference in $\delta^{13}C$ values between the methyl and carbonyl carbon atoms of acetyl CoA is preserved in the carbon atoms of lipid components which derive from them; the difference between the $\delta^{13}C$ values of the glucose and the lipid fraction is half as large as that between the methyl and carbonyl carbon atoms of acetyl CoA.

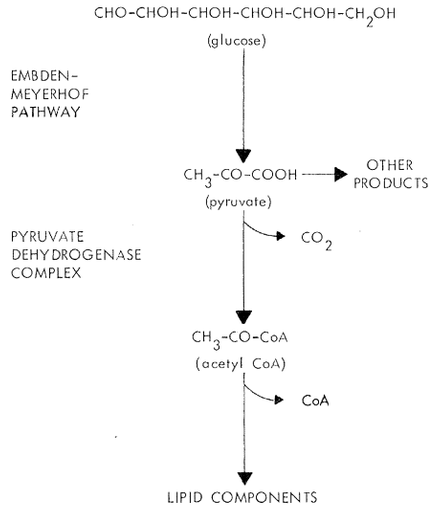

Fig. 1. Simplified version of lipid synthesis in microorganisms growing on glucose. The indicated intermediates are all capable of undergoing other reactions.

It is important to note that if all the pyruvate were oxidized to acetyl CoA, the acetyl group would have the same isotopic composition as the pyruvate. As smaller proportions of the total pyruvate pool pass through the pyruvate dehydrogenase complex, the magnitude of the ^{13}C depletion of the acetyl group relative to pyruvate increases toward a maximum value, which is reached if only a few percent of the available pyruvate is converted to acetyl CoA. Other reactions involving pyruvate as a reactant modulate the fraction of pyruvate which is oxidized to acetyl CoA. The observation that the magnitude of the ^{13}C depletion of the lipid fraction has a negative correlation with the amount of lipid for several plant species (*2*) is consistent with this feature of the model.

The model can be used to explain the observation (*3*) that in *Chlorella* and in *E. coli* grown on glucose, the $\delta^{13}C$ value of the carboxyl carbon atom of leucine is 9 to 17 per mil lower than the $\delta^{13}C$ values of the carboxyl carbon atoms of the eight other amino acids which were analyzed. The carboxyl carbon atom of leucine is derived from the carbonyl carbon atom of acetyl CoA, while the carboxyl carbon atoms of the eight other amino acids are derived from intermediates of either the Embden-Meyerhof pathway or the tricarboxylic acid cycle (*7*). Relative to the glucose carbon source, the carboxyl carbon atom of leucine has a $\delta^{13}C$ value of -14.0 per mil for *Chlorella* and -18.5 per mil for *E. coli*, while the $\delta^{13}C$ values for the lipid fractions of both organisms are -7.0 per mil (*3*). The low $\delta^{13}C$ value of the leucine carboxyl carbon atom is consistent with the proposal that the difference between the $\delta^{13}C$ values of the glucose carbon source and the lipid fraction will be half as large as that between the methyl and carbonyl carbon atoms of acetyl CoA.

The basic design of lipid synthesis is the same in all organisms. Kinetic isotope effects during the pyruvate dehydrogenase reaction account for the ^{13}C depletion of the lipid fraction observed in organisms as they exist in nature. Species-specific differences in the magnitude of the ^{13}C depletion could arise from different kinetic isotope effects during the specific pyruvate dehydrogenase reactions and from different flow rates of pyruvate to its other metabolic reactions. The ^{13}C depletion would be limited primarily to the carbonyl carbon atom of acetyl CoA. Differences in the $\delta^{13}C$ values of individual carbon atoms of pyruvate would be an additional factor influ-

Table 2. Kinetic isotope effects of pyruvate decarboxylase. The $\delta^{13}C$ values of the different carbon atoms of pyruvate were determined by running the reaction to completion (experiments A and E). The $\delta^{13}C$ values of the different carbon atoms of the products of the enzymatic reaction run to approximately 1 percent of completion were then determined at several temperatures (experiments B, C, D, and F). The number of replicates for each experiment is indicated by *N*. Sodium pyruvate ($\delta^{13}C = -21.0$ per mil) was incubated with yeast pyruvate decarboxylase in degassed 100 m*M* citrate buffer at *p*H 6.0. The reaction was stopped by the addition of 100 percent phosphoric acid. The CO_2 and acetaldehyde were separated from the reaction mixture by distillation through a Dry Ice trap and from each other by condensation of the acetaldehyde with frozen *n*-pentane. For experiments A to D, the acetaldehyde was combusted at 850°C directly to CO_2. For experiments E and F, the acetaldehyde was pyrolyzed in the presence of H_2S to yield quantitatively the carbonyl and methyl carbon atoms as carbon monoxide and methane (*16*). These were separated from each other and converted to CO_2 by combustion in the presence of a CuO catalyst at 450°C (carbon monoxide is oxidized to CO_2; methane does not react) and at 850°C (methane is oxidized to CO_2) (*17*). Details of these procedures will be presented elsewhere (*11*).

Experiment	Experimental conditions	$\delta^{13}C$ (per mil)		
		Acetaldehyde		Carbon dioxide
A	100 percent reaction ($N = 3$)	-20.6 ± 0.4		-22.5 ± 0.3
B	15°C, 0.5 percent reaction ($N = 4$)	-26.5 ± 0.2		-28.1 ± 0.5
C	25°C, 1.4 percent reaction ($N = 4$)	-28.5 ± 0.2		-28.8 ± 0.1
D	35°C, 0.8 percent reaction ($N = 4$)	-30.2 ± 0.7		-30.2 ± 0.5
		Methyl	Carbonyl	
E	100 percent reaction ($N = 9$)	-20.1 ± 0.6	-19.9 ± 0.3	-22.3 ± 0.2
F	25°C, 1.9 percent reaction ($N = 5$)	-21.1 ± 0.6	-34.0 ± 0.4	-28.8 ± 0.1

encing the intramolecular isotopic distribution in lipid components. The pyruvate internal isotopic arrangement is in turn a function of the isotopic distribution in its metabolic precursors. In plants, the cyclic nature of the Calvin cycle produces random incorporation of ^{14}C into photosynthetic carbohydrate in the long run (*12*), which implies that there will be no large isotopic differences in the carbon atoms of the hexoses which are the basic starting materials for metabolism. In animals, intramolecular isotopic distribution in carbohydrates would be a function of the relative contribution of de novo synthesis and assimilation of precursors from the diet; the further an animal is from plant carbon in the food chain, the more complex the factors determining the intramolecular isotopic arrangement of its pyruvate.

Techniques that allow for precise comparison of the isotopic composition of carbon atoms of lipid components which are known to derive from the methyl and carbonyl carbon atoms of acetyl CoA are not known at present. Their development would permit the direct demonstration of temperature-dependent intramolecular carbon isotopic heterogeneity in extant lipid components and would provide a method which might yield information as to the source and early thermal history of fossil lipids.

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References and Notes

1. Carbon isotopic composition is determined by converting the sample to CO_2 and comparing the $^{13}C^{12}C$ ratio of the sample with that of a standard by mass spectrometry. The results are reported in the notation

$$\delta^{13}C \text{ (per mil)} = \left[\frac{(^{13}C/^{12}C)_{sample}}{(^{13}C/^{12}C)_{standard}} - 1\right] \times 10^3$$

For the work reported here, the standard is the Pee Dee belemnite (PDB) carbonate.
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5. Two hypotheses have been advanced, one by Park and Epstein (*2*) and one by A. A. Ivlev, M. Ya. Koreleva, A. G. Kaloshin [*Dokl. Akad. Nauk SSSR* **217**, 224 (1974)]. The results of this study are not consistent with the main features of either hypothesis.
6. Most of the carbon atoms of the lipid fraction derive from carbon atoms 1, 2, 5, and 6 of the glucose molecule. If these atoms were sufficiently depleted in ^{13}C relative to carbon atoms 3 and 4 of glucose, the ^{13}C depletion of the lipid fraction could be explained without recourse to isotopic effects during metabolism.
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A Bactericidal Effect for Human Lactoferrin

Abstract. Streptococcus mutans *and* Vibrio cholerae, *but not* Escherichia coli, *were killed by incubation with purified human apolactoferrin. Concentrations of lactoferrin below that necessary for total inhibition resulted in a marked reduction in viable colony-forming units. This bactericidal effect was contingent upon the metal-chelating properties of the lactoferrin molecule.*

Lactoferrin (LF), an iron-binding protein synthesized by neutrophils (*1*) and glandular epithelial cells (*2*), has been detected in most of the major secretions that bathe the human mucosal surfaces (*3*). Because it retards the growth of bacteria (*2, 4, 5*) and fungi (*6*) in vitro, LF has been suggested as a possible contributor to defense against local mucosal infections (*3*). The antimicrobial action of LF and its serum counterpart, transferrin (TF), has been attributed to the ability of these compounds to chelate iron, an action that makes this essential nutrient inaccessible to an invading microorganism (*7*). The ready reversibility of this antimicrobial effect, when an excess of iron is supplied to the nutritionally deprived organisms, suggests a simple bacteriostatic effect for LF. Our study, however, provides evidence that LF is capable of a direct bactericidal action on certain bacteria.

Purified LF was obtained as follows: (i) globulins were separated from a pool of human colostrum by ammonium sulfate precipitation, (ii) passed over CM (carboxymethyl) Sephadex C-50, (iii) subjected to ion exchange chromatography on A-25 (diethylaminoethyl) DEAE Sephadex, and (iv) filtered on Sephadex G-200 (*8*). The purity of the LF was assessed by immunoelectrophoresis against antiserum to normal human serum and antiserum to normal human colostrum and by disk-gel electrophoresis (*8*). Apo LF (iron-free) and saturated LF were prepared by dialysis against either 0.1*M* citric acid (*p*H 2.3) or saturated ferrous ammonium sulfate, respectively, then each was dialyzed against double-distilled water (*8*).

Three types of pathogens associated with exocrine secretions and mucosal surfaces were selected for this study: *Streptococcus mutans*, an etiologic agent of dental caries in humans and animals; *Vibrio cholerae*, the causative agent of Asiatic cholera; and *Escherichia coli*, an enteropathogen isolated from an infant diarrhea. *Streptococcus mutans* AHT (Bratthall serotype a) was grown under an atmosphere of 5 percent CO_2 in N_2 at 37°C for 18 hours in a partially defined (PD) medium containing 120 μM iron as $FeCl_3$. *Vibrio cholerae* was grown under conditions of constant shaking in 1.5 percent peptone broth at *p*H 8.0 and 37°C for 6 hours. *Escherichia coli* 0126 B16, also subjected to constant shaking, was cultured in PD-glucose medium at 37°C for 6 hours. Each culture was washed three times in sterile saline and resuspended to a concentration of approximately 2.0×10^9 colony-forming units (CFU) per milliliter.

In sterile cuvettes, 100 μl (2×10^8 CFU) of the various bacterial suspensions were incubated with 200 μl of various concentrations of LF (1.0 to 2000 μg) for 1 hour at 37°C. After incubation, 2.7 ml of the appropriate medium was added to the reaction mixture, and growth at 37°C was monitored spectrophotometrically at 660 nm (Fig. 1). The growth of *S. mutans* was totally inhibited by prior incubation with 50 or more micrograms of LF per 300 μl ($\geq 2\ \mu M$) of reaction mixture (Fig. 1A). Furthermore, with concentrations less than 50 μg, there was a dose-dependent delay in the onset of detectable growth. Similarly, *V. cholerae* was totally inhibited by LF concentrations of or in excess of 100 μg and, at lesser concentrations, showed a delay in the onset of exponential growth proportional to the LF concentrations (Fig. 1B). In contrast, the growth of *E. coli* was not inhibited by concentrations of apo LF as high as 2000 μg per reaction mixture (80 μM). Saturated LF, in concentrations of 2000 μg, did not alter the