I. CARBON ISOTOPE DISTRIBUTION IN FOOD CHAINS

II. MECHANISM OF CARBON ISOTOPE FRACTIONATION ASSOCIATED WITH LIPID SYNTHESIS

Thesis by

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In Partial Fulfillment of the Requirements

For the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1977

(Submitted March 7, 1977)

ACKNOWLEDGEMENTS

I am grateful to Sam Epstein for providing the intellectual atmosphere and experimental facilities which allowed a wide-ranging study such as this to be pursued; I would also like to thank him for his more recent efforts to insure that none of my untimely semi-colons or parentheses would reach the public eye.

I thank James Bonner for his encouragement with regard to the subject matter of the second half of this thesis.

Tom Hoering was most generous in sharing his ideas and techniques with me during my short visit to his laboratory. The Carnegie Institution of Washington provided partial support during my stay there.

The following people either provided or helped me in culturing organisms raised on defined diets: Betty Aalseth, Carl Johnson, Ilga Lielausis, Gail Lyon, and Ben Swart, all of Caltech; H. H. Crowell, Oregon State University; R. E. Pfadt, University of Wyoming; J. E. Baker, Stored-Product Insects Research and Development Laboratory, Savannah, Georgia; J. Baker, San Joaquin Valley Agricultural Research and Extension Center, Parlier, California; J. A. Onsager, Rangeland Insect Laboratory, Bozeman, Montana; and J. G. Riemann, Metabolism and Radiation Research Laboratory, Fargo, North Dakota.

I thank Steve Lambert for introducing me to the daily operations of the stable isotope laboratory and Joop Goris for running almost all of my samples on the mass spectrometer.

Bill Klein and Russell Potter provided helpful discussions.

The initial drafts of this thesis were typed by Sue Yamada, while Janet Boike did the final version. The illustrations were drawn by Jan Scott.

I reserve my deepest gratitude for my wife, Mary Beth, not only for supporting us during my graduate career, but for her patience while waiting for this study to be completed.

I was funded by a National Institutes of Health Fellowship during the earlier part of my tenure as a graduate student, and more recently by National Science Foundation grant DES71-00558 A03.

ABSTRACT

The distribution of carbon isotopes in food chains was investigated by analyzing animals grown in the laboratory on diets of constant isotopic composition. The isotopic composition of an animal reflects the isotopic composition of its diet, but the animal is generally enriched by about 1 ‰ in $\delta^{13}C$ relative to the diet. An isotopic mass balance exists for an animal-diet system. In three of four cases analyzed, the ^{13}C enrichment of the body relative to the diet is balanced by a ^{13}C depletion of the respired CO_2.

The carbon isotope distribution among different suborganismic components of animals was also analyzed. The relationships among the $^{13}C/^{12}C$ ratios of the major biochemical fractions, such as lipid, carbohydrate, and protein, appear to be preserved in going from one trophic level to the next, but the actual isotopic composition of a fraction in an animal and its diet can differ considerably. The relationship between the carbon isotopic composition of a tissue in a mouse and the carbon isotopic composition of the diet is affected both by the type of tissue analyzed and by the diet. The $^{13}C/^{12}C$ ratios of the biochemical components collagen, chitin, and the insoluble organic fraction of shells, which are commonly preserved in fossil material, also show a correlation with the isotopic composition of the diet. However, the magnitude of the difference between the $^{13}C/^{12}C$ ratios of the component and the diet depends on the animal, the diet, and the biochemical nature of the component.

These results suggest that it will be possible to perform dietary

analysis based on the relationship between the $^{13}C/^{12}C$ ratios of animals and their diets. The limits of accuracy of this method, as established by the experiments reported here, will restrict its application to situations in which the diet is derived from sources with large differences in their $\delta^{13}C$ values, such as terrestrial vs. aquatic organisms and C_3 vs. C_4 plants. This method should be applicable in fossil as well as modern situations.

The second part of this study involved the elucidation of the basis of the low $^{13}C/^{12}C$ ratio of the lipid fraction relative to the carbon isotopic composition of the whole organism and the other major biochemical fractions. Experiments in which E. coli was grown on carbon sources which enter at different steps of the metabolic sequence leading to lipid formation indicate that the carbon isotope fractionation resulting in the ^{13}C depletion of the lipid fraction occurs during the oxidative decarboxylation of pyruvate to acetyl CoA by the pyruvate dehydrogenase complex.

The isotopic fractionations occurring during this step were analyzed indirectly in a series of in vitro experiments with pyruvate decarboxylase. These experments indicate that:

1) An isotopic fractionation of the expected direction and magnitude occurs during the pyruvate dehydrogenase step.

2) The ^{13}C depletion of the acetyl CoA formed in the reaction is concentrated primarily in the carbonyl carbon atom, with the methyl carbon atom retaining the $^{13}C/^{12}C$ ratio of pyruvate.

3) The difference in the $^{13}C/^{12}C$ ratios of the methyl and carbonyl carbon atoms of acetyl CoA is twice as large as the

^{13}C depletion of the lipid fraction.

4) The difference in the $^{13}C/^{12}C$ ratios of the methyl and carbonyl carbon atoms of acetyl CoA is temperature-dependent.

5) There will be a large, temperature-dependent difference in the carbon isotopic composition of those carbon atoms of lipid components which derive from the methyl and carbonyl carbon atoms of acetyl CoA.

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I. CARBON ISOTOPE DISTRIBUTION IN FOOD CHAINS

I. INTRODUCTION AND REVIEW OF THE LITERATURE

1.1. Introduction

The introduction of a high resolution mass spectrometer which permitted the detection of small variations in the abundance of the stable isotopes of the light elements (McKinney et al., 1950) led to the widespread use of this technique in the area of geochemistry. The stable isotopes of carbon have been studied most thoroughly, partly because of the ease with which samples can be prepared for isotopic analysis and also because of the importance of this element in many geochemical processes. Since so much of the carbon cycle involves carbon in organic matter, these studies have included determinations of carbon isotopic abundance in biological matter. Figure 1-1 presents a summary of the research in this area, emphasizing the influence of biological processes on carbon isotope distribution in nature. The figure and the following discussion are divided into three sections: primordial carbon, carbon dioxide cycle, and organic matter.

1.2. Definition of $\delta^{13}C$.

The carbon isotope ratios are presented as $\delta^{13}C$ values where

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

The $\delta^{13}C$ values discussed in this work are presented relative to the PDB standard, which is CO_2 prepared from the calcium carbonate of the Cretaceous belemnite Belemnitella americana from the Peedee formation

Figure 1-1. Range of $\delta^{13}C$ values of some major reservoirs of carbon in nature. The superscripts refer to the following references:

[1](Bender et al., 1973)

[2](Degens, 1969)

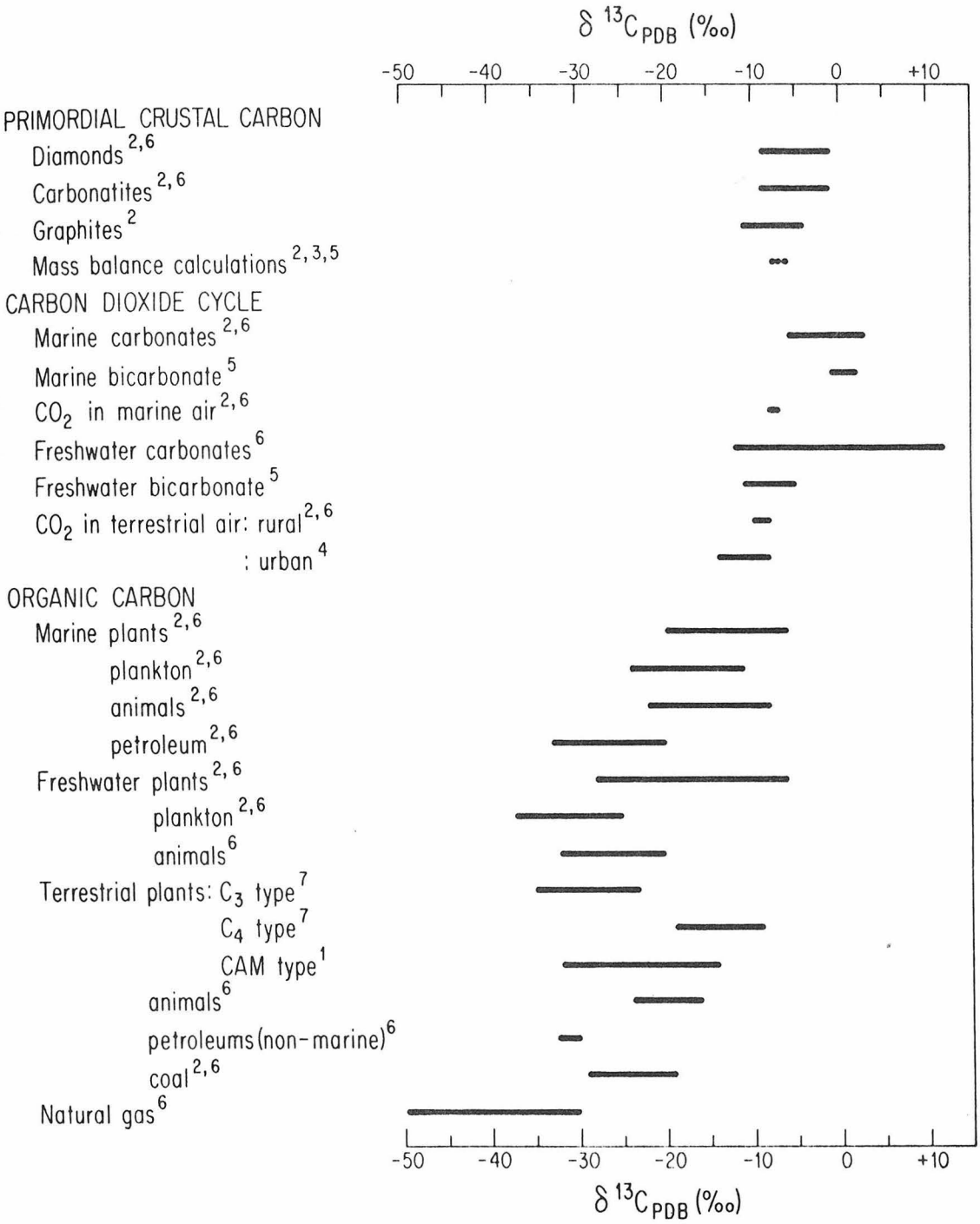
[3](Epstein, 1969)

[4](Epstein, 1977)

[5](Hoefs, 1973)

[6](Schwarz, 1969)

[7](Smith and Epstein, 1970).


δ 13C PDB (‰)
-50
-40
-30
-20
-10
0
+10
PRIMORDIAL CRUSTAL CARBON
Diamonds 2,6
Carbonatites 2,6
Graphites 2
Mass balance calculations 2,3,5
CARBON DIOXIDE CYCLE
Marine carbonates 2,6
Marine bicarbonate 5
CO2 in marine air 2,6
Freshwater carbonates 6
Freshwater bicarbonate 5
CO2 in terrestrial air: rural 2,6
: urban 4
ORGANIC CARBON
Marine plants 2,6
plankton 2,6
animals 2,6
petroleum 2,6
Freshwater plants 2,6
plankton 2,6
animals 6
Terrestrial plants: C3 type 7
C4 type 7
CAM type 1
animals 6
petroleums (non-marine) 6
coal 2,6
Natural gas 6
-50
-40
-30
-20
-10
0
+10
δ 13C PDB (‰)

of South Carolina. A $\delta^{13}C$ value of -10.0‰ indicates that the sample carbon has a $^{13}C/^{12}C$ ratio which is 1.00% less than that of the PDB standard carbon.

1.3. Distribution of carbon isotopes in nature.

1.3.1. Primordial carbon. The $\delta^{13}C$ value of the original crustal carbon reservoir can be estimated by analyzing the carbon contained in minerals which derive from deep-seated magmas which presumably do not contain any recycled carbon. The $\delta^{13}C$ values of suitable samples of diamonds, carbonatites (magmatic carbonates), and igneous graphites fall in a range of (-1 to -11)‰. This method is complicated by the many ways of changing the $\delta^{13}C$ value of the small carbon reservoirs from which the mineral samples were formed, by either addition or subtraction of carbon as CH_4, CO or CO_2. A second, more reliable, method for determining this value involves the estimation of the size and $\delta^{13}C$ values of all the present-day crustal carbon reservoirs. A mass balance calculation using these estimates provides a $\delta^{13}C$ value for total crustal carbon, which will equal that of primordial crustal carbon assuming that there has been no significant loss of carbon from the crust since its formation. Crustal carbon is contained almost entirely in carbonates (73% of the total carbon) and in the organic matter of shales and other sedimentary deposits (>26% of the total)(Rubey, 1951), so that the $\delta^{13}C$ values of these two reservoirs are the major factors in determining the $\delta^{13}C$ value of crustal carbon. Three such calculations have yielded values of -6‰, -7‰, and -7.5‰ (Degens, 1969; Epstein, 1969; Hoefs, 1973), indicating that the $\delta^{13}C$ value of primordial crustal

carbon was in the neighborhood of -7‰.

1.3.2. Carbon dioxide cycle. The primary factor which determines the isotopic composition of all organic matter is the isotopic fractionation associated with carbon fixation during photosynthesis. It is therefore necessary to understand the factors which can influence the isotopic composition of the components of the carbon dioxide cycle which serve as the source of carbon for photosynthesis.

The overriding feature influencing the isotopic composition of the components of the carbon dioxide cycle is the isotopic equilibrium between the species CO_2(g.) - HCO_3^-(aq.) - $CaCO_3$(s.). In an equilibrium system, the $\delta^{13}C$ value of the CO_2(g.) will be 10.2‰ less than that of the $CaCO_3$(s.) and 8.4‰ less than that of HCO_3^-(aq.) at 20°C. The temperature variations for these equilibrium fractionations are small, being -0.063‰ per 1°C for $CaCO_3$(s.) - C)$_2$(g.) and -0.109‰ per 1°C for HCO_3^- (aq.) - CO_2(g.) (Emrich et al., 1970).

This equilibrium fractionation largely explains the distribution of carbon isotopes in the marine carbon dioxide cycle, in which CO_2(g.) is depleted in $\delta^{13}C$ by 6-10 ‰ relative to the HCO_3^-(aq.) and the limestones (calcium carbonates), although there is a considerable spread in the $\delta^{13}C$ values of the limestones. The non-marine system (freshwater lakes and rivers plus terrestrial air) is more complicated due to several factors. Rural area air contains CO_2 whose $\delta^{13}C$ value is not very different from that of marine air, but the CO_2 of urban air may be slightly depleted in ^{13}C due to the addition of ^{13}C-depleted CO_2 from the burning of fossil fuels (Epstein, 1977). The HCO_3^-(aq.) of

freshwater systems in equilibrium with CO_2 in rural air would be expected to have a distribution similar to that of HCO_3^-(aq.) in marine water, but the contribution of CO_2 from decaying organic matter and from respiration of organisms, with both processes producing CO_2 whose $\delta^{13}C$ values are more negative than that of atmospheric CO_2, tends to reduce the $\delta^{13}C$ values of freshwater HCO_3^- (aq.). It should be noted that high rates of photosynthesis possibly could increase the $\delta^{13}C$ of the HCO_3^- (aq.) of freshwater by preferentially fixing ^{12}C. Freshwater limestones come from a variety of environments, such as caves, springs, and lakes. The different amounts of CO_2 derived from decaying organic matter and from biological activity in these environments explains the wide range and low $\delta^{13}C$ values of these limestones.

In summary, then, the $\delta^{13}C$ values of the carbon dioxide cycle components which serve as the carbon sources for photosynthesis, CO_2 (g.) and HCO_3^- (aq.), vary depending on the environment from which they are isolated; this variation must be considered in interpreting the $\delta^{13}C$ values of organic matter which originates in these environments.

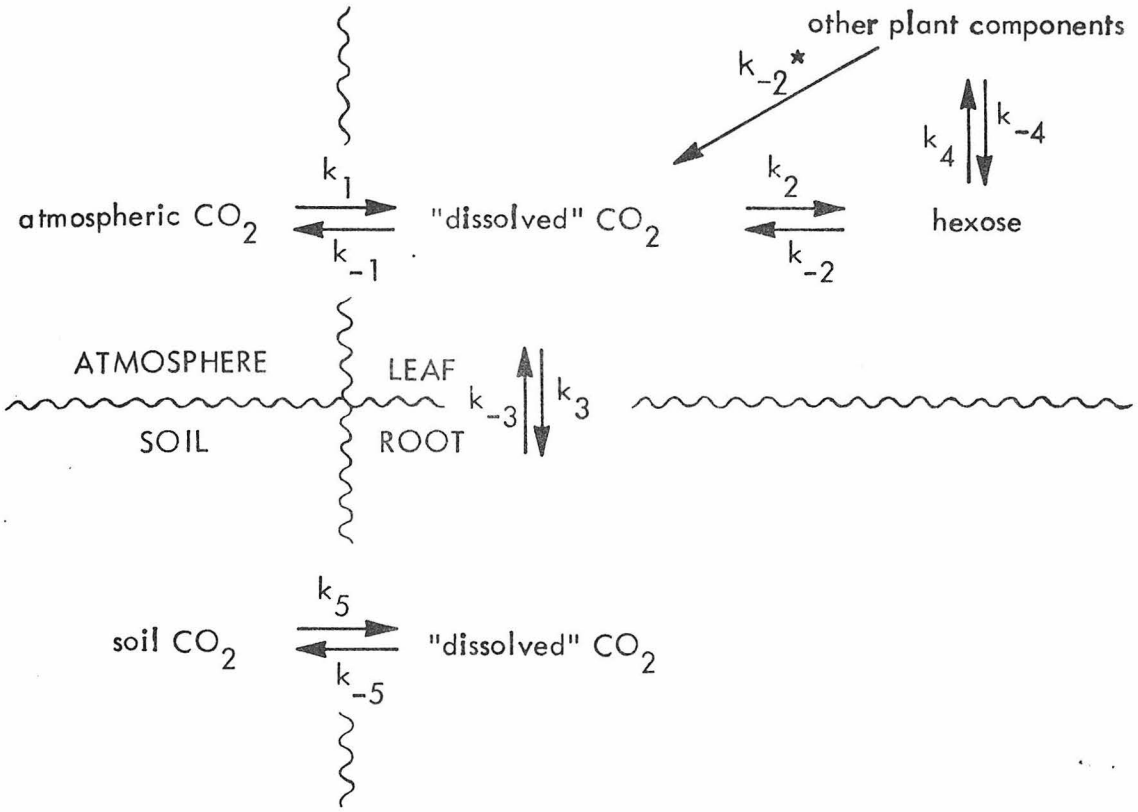
1.3.3. Organic matter.

1.3.3.1. Plants. Figure 1-1 indicates that plants taken from either marine, freshwater, or terrestrial environments have lower $\delta^{13}C$ values than the inorganic CO_2 (g.) or HCO_3^- (aq.) which serves as the carbon source for those plants. Park and Epstein (1960) have offered a model which can be used to explain the characteristic $\delta^{13}C$ values of plants taken from different environments and of plants which have different biosynthetic pathways for fixing CO_2. Their model is shown in

Figure 1-2. Step k_1 is the diffusion of atmospheric CO_2 into the leaf; the maximum fractionation of this step was estimated to be -14 ‰ by analogy with the fractionation accompanying the absorption of CO_2 by a $Ba(OH)_2$ solution. Step k_2 is the fixation of CO_2 by the enzyme ribulose 1,5-diphosphate carboxylase, which was shown to have a maximum fractionation of -17 ‰ (for the enzyme from tomato plants). The k_3 step, which involves translocation of "dissolved" CO_2 in the leaf to the roots, where it is lost from the plants by the k_5 step, prevents unreacted "dissolved" CO_2 from being fixed by the enzyme. Complete conversion of "dissolved" CO_2 to hexose would result in no isotopic fractionation in the k_2 step. The model predicts that, starting with the atmospheric CO_2 whose $\delta^{13}C = -7‰$, the maximum fractionations in k_1 and k_2 would lead to values of -21 ‰ for "dissolved" CO_2 and -38 ‰ for the whole plant carbon (assuming no overall isotope effect during incorporation of hexose carbon atoms into the other plant biochemical components). More positive values for both pools could be obtained if k_{-1} or k_{-2} were sufficiently fast or if k_3 were sufficiently slow. Although details of the model have been challenged (Whelan *et al.*, 1973) and it has been suggested that the $\delta^{13}C$ values of plants are determined by the photosynthesis/photorespiration ratios in plants (Lowdon and Dyck, 1974), the model can be used to explain most of the variations of $\delta^{13}C$ values observed in various types of plants.

Aquatic plants, both marine and freshwater, are enriched in ^{13}C relative to those terrestrial plants which fix CO_2 via the ribulose 1,5-diphosphate carboxylase reaction (C_3-type). Park and Epstein

Figure 1-2. Park and Epstein (1960) model for carbon isotope fractionation during photosynthesis.



k_1	absorption of CO_2 into leaf
k_{-1}	desorption of CO_2 from leaf
k_2	photosynthetic fixation of CO_2
k_{-2}, k_{-2}^*	production of CO_2 by respiration
k_3	translocation of CO_2 by vascular system
k_4	synthesis of other plant components
k_5	desorption of CO_2 from root

(1960) pointed out that aquatic plants would not have a fractionation during the k_1 step if communication between aquatic HCO_3^- (aq.) and "dissolved" CO_2 in the plant were rapid. Since the HCO_3^- (aq.) has a $\delta^{13}C$ value more positive than CO_2 (g.) for both marine and freshwater systems, and since the isotopic fractionation of the k_1 step would be absent in aquatic plants, aquatic plants have more positive $\delta^{13}C$ values than those terrestrial plants which fix carbon by the C_3 pathway.

The difference in $\delta^{13}C$ values between C_3 plants, which fix CO_2 with ribulose 1,5-diphosphate (RuDP) carboxylase, and C_4 plants, which fix CO_2 (initially, at least) with phosphoenolpyruvate (PEP) carboxylase, has been ascribed to differences in the fractionation of carbon isotopes during CO_2 fixation by the two enzymes. PEP carboxylase from sorghum was shown to have a maximum fractionation of only -3 ‰, compared with a fractionation of -34 ‰ for RuDP carboxylase from the same plant (Whelan et al., 1973). Although the CO_2 initially fixed by PEP carboxylase probably is released and subsequently refixed by RuDP carboxylase (Black, 1973), if the refixation is complete the $\delta^{13}C$ values of C_4 plants will be determined solely by the fractionation of the PEP carboxylase step (Whelan et al., 1973). The smaller fractionation of this step relative to the CO_2-fixing step in C_3 plants explains the higher $^{13}C/^{12}C$ ratios of C_4 plants relative to C_3 plants.

The $\delta^{13}C$ values of crassulacean acid metabolism (CAM) plants overlap the values of C_3- and C_4-type plants. These plants are capable of either C_3- or C_4-type metabolism depending on environmental conditions.

It has been shown that conditions presumably favorable to CO_2 fixation by the C_4 pathway (high light intensity, high temperature) result in CAM plants with C_4-like $\delta^{13}C$ values, while those conditions favoring CO_2 fixation by the C_3 pathway (low light intensity, low temperature) result in CAM plants with C_3-like $\delta^{13}C$ values (Bender et al., 1973).

1.3.3.2. Animals. The $\delta^{13}C$ values of animals from marine, freshwater, and terrestrial environments fall within the range of plant $\delta^{13}C$ values from the particular environment. This observation has been taken to mean that no large fractionation occurs during the passage of carbon to various trophic levels. The magnitude and basis for changes in the isotopic composition of carbon as it passes through food chains is the subject matter of this study, and will be commented on below.

1.3.3.3. Fossil organic matter. The $\delta^{13}C$ values of fossil organic matter are determined by the $\delta^{13}C$ values of the biogenic carbon which serves as the starting material for these fractions and the isotopic fractionation associated with the steps leading to their formation. The highly aromatic structure of coal has been interpreted as evidence that coal derives primarily from the aromatic plant fraction lignin. The similarity between the $\delta^{13}C$ values of coals and extant terrestrial plants and their lignin fractions has been taken to indicate that there is no fractionation of carbon isotopes during coalification (Craig, 1953; Degens, 1969). By contrast, however, the $\delta^{13}C$ values of petroleums, both marine and non-marine, are more negative than the $\delta^{13}C$ values of the whole organisms from which they derive their carbon.

This difference has been ascribed to the preferential incorporation of the lipid fraction of organisms into petroleums (Silverman and Epstein, 1958), since the lipid fraction of an organism has a more negative $\delta^{13}C$ value than the whole organism (see Section 5.1). The very low $\delta^{13}C$ values of natural gases can be accounted for by both isotopic fractionation during the production of methane either by thermal cracking of sedimentary organic matter (Silverman, 1967) or by bacterial fermentation (Rosenfield and Silverman, 1959) and by direct equilibrium isotopic exchange with CO_2 (Bottinga, 1969).

1.4. Previous work relating to factors influencing the carbon isotope composition of animals.

It has generally been assumed that there are no large isotopic fractionations associated with the incorporation of diet carbon into an animal's body. This assumption was supported by the observation, noted above, that the $\delta^{13}C$ values of animals from marine, freshwater, and terrestrial environments fall within the range of $\delta^{13}C$ values of plants from the respective environment. Additionally, the observation that the $\delta^{13}C$ values of plankton samples were not affected by the amounts of zooplankton present in the sample (the zooplankton presumably having fed on phytoplankton present in the sample) was interpreted as support for this assumption (Degens et al., 1968)

Two studies have been published in which the authors have directly examined the question of isotope effects during incorporation of carbon into animals. Smith and Epstein (1970), based on a comparison of $^{13}C/^{12}C$ ratios of fourteen macroscopic plants and six animals occurring

together in a salt marsh, conclude that "isotopically these animals nicely reflected their presumptive diets". However, the authors did not indicate what the presumptive diets were. Among the animals analyzed, there were a filter feeder, a detritus feeder, a grazer-scraper, and three predators (Hyman, 1967; Ricketts et al., 1968), so that the presumptive diet of none of the animals was actually analyzed. Since the isotopic composition of the actual diets might differ significantly from the isotopic composition of the plants which were analyzed (e.g. due to fractionation of carbon isotopes during detritus formation or during passage of carbon through trophic levels), the conclusion of this study does not appear to have been substantiated. In a more rigorously controlled field study (Minson et al., 1975), cows were grazed in pastures which contained predominantly one or a few plant species of either the C_3 or C_4 type. The $\delta^{13}C$ values of the pasture plants and of the cows' milk and hair are shown in Table 1-1. The large difference in $^{13}C/^{12}C$ characteristic of plants possessing the different photosynthetic pathways was reflected in the isotopic composition of milk and hair of cows from the respective pastures, although there was significant variability in the relationship of the $^{13}C/^{12}C$ ratios of the diets and the animal components which appeared to be dependent both on the diet and on the component analyzed.

Several factors which cannot be controlled in field studies limit the validity of the conclusions which derive from them. Seasonal variation in the isotopic composition of maple tree leaves and grasses (up to 6 ‰ and 5 ‰ respectively) has been demonstrated (Lowdon and

Table 1-1. $\delta^{13}C$ values of pasture plants and the milk and hair of cows grazed on them. (Minson et al., 1975)

PHOTOSYNTHETIC PATHWAY OF PASTURE PLANTS	$\delta^{13}C$ (‰)		
	Plants	Milk	Hair
C_4	-14.0	-15.5	-12.1
C_4	-12.4	-15.0	-15.1
C_3	-25.4	-22.5	-22.3
C_3	-28.9	-26.0	-26.2

Dyck, 1974); variations of similar magnitude probably exist in other plants, since the basis for the variation was ascribed to changes in the $^{13}C/^{12}C$ ratio of atmospheric CO_2. Unless recently synthesized animal components were analyzed, or an integrated value for the isotopic composition of the diet is used, determination of the relationship between plant and animal isotopic composition will be in error. Secondly, the assumption that herbivores will consume the available plants at random is implicit in the design of natural experiments; the existence of selectivity in the choice of food plants, coupled with differences in their isotopic compositions, will cause error associated with this assumption. Minson et al. (1975) recognized such a possibility (e.g. that a cow might graze on a few C_4 plants in a predominantly C_3 pasture) and used it as a possible explanation for the differences between the isotopic composition of the pasture plants and the cows' components. The possibility that herbivores may be selective in avoiding C_4 species in a mixed population of potential food plants (Caswell et al., 1973) would lead to large errors if random feeding is assumed, due to the large difference of $^{13}C/^{12}C$ ratios for C_3 and C_4 plants. A third consideration with regard to natural experiments relates to the observation that the isotopic composition of the major biochemical components of plants varies systematically, with the series protein-carbohydrate-lipid showing a progressive decrease in ^{13}C content (see Section 5.1). The proportions of these components which are assimilated into animal carbon will be a function of both their availability and the animal's metabolic requirements, so that changes in the physiological states of both the diet organism and the consumer will be

factors in field studies. Controlled laboratory experiments, where animals are reared on a single diet of constant isotopic composition, avoid most of the complexities of interpreting observations based on naturally occurring diet-animal relationships, and allow for a more precise determination of the isotopic relationship between consumed and incorporated carbon. These findings can then be applied to the interpretation of the isotopic relationship between animals and their diets as they exist in the field.

1.5. Overview of work presented in this study.

This study was initiated with the long-term objective of understanding the basis for the carbon isotope distribution in animals at the organismic and suborganismic levels. Because there has been no systematic analysis of these distributions, let alone of the factors responsible for them, much of the work presented here is of a survey nature. It is hoped that the findings based on these studies will serve as the foundation for a rigorous application of stable isotope methodology to problems of animal physiology and ecology.

The first specific goal of this study was to define the relationship between the $\delta^{13}C$ value of an animal and the $\delta^{13}C$ value of its diet. The results of experiments in which a number of animals were raised on diets of constant $\delta^{13}C$ value are presented in Section 3.2. Some of the causes for the differences observed between the $\delta^{13}C$ values of animals and their diets were analyzed and are discussed in Section 3.3 and Section 3.4.

A second objective of this study was to determine if the dietary

history of an animal can be reconstructed from the $\delta^{13}C$ value of the animal's carbon. This approach involves two steps. First, the $\delta^{13}C$ value of the diet is estimated from the $\delta^{13}C$ value of the animal. Second, the relative contribution of diet sources of known isotopic composition is calculated from the diet $\delta^{13}C$ value. In practice, it may not always be possible or desirable to measure the $\delta^{13}C$ value of a whole animal in order to determine the $\delta^{13}C$ value of the diet. Therefore, the relationships between the $\delta^{13}C$ values of the diet and several types of suborganismic components of animals were determined. The isotopic relationships are discussed for some of the major biochemical fractions (lipid, glycogen, and protein) in Section 3.5, for different tissues from mice in Section 3.6, and for several organic components which are often preserved as fossil material in Section 3.7. Finally, a discussion of some of the practical considerations involved in the application of the method of dietary analysis based on the determination of the $\delta^{13}C$ value of animal carbon is presented in Section 3.8.

2. ANALYTICAL TECHNIQUES

2.1. Sampling methods.

2.1.1. Preparation of diets and growth of animals. All animals in this study were raised from birth on a single diet of constant $\delta^{13}C$ value. The diets, which are listed in Table 2-1, were prepared by the following methods. The algae were grown under fluorescent lighting in a medium containing only inorganic salts through which tank CO_2 and compressed air were bubbled (Swart, 1977). Bacteria were cultured in liquid minimal media with glucose as the carbon source (Johnson, 1977). Corn and wheat seedlings were grown in vermiculite soaked in an inorganic nutrient solution (Yih and Clark, 1965) with CO_2 in the laboratory air serving as the carbon source. The light source was a bank of fluorescent lights supplemented with sunlight from a nearby window. Grape leaves, milkweed seeds, Romaine lettuce leaves, and wheat seeds were obtained from plants grown in the field. Horsemeat was obtained from Breeder's Choice Pet Foods, Irwindale, California, while the pork was provided by the Clougherty Meat Company, Vernon, California. Mouse diets were purchased from commercial suppliers: JAX 911A from the Jackson Laboratory, Bar Harbor, Maine; Purina Rat Chow from the Ralston Purina Company, St. Louis, Missouri; and Wayne Lab-Blox F6 from Allied Mills, Inc., Chicago, Illinois.

The techniques used in raising the animals are outlined in Table 2-1.

2.1.2. Sample collection. The diets were sampled by one of three methods. In those cases in which the animal and its diet were supplied

Table 2-1. Animals, diets, diet sampling methods, and animal culture techniques.

ANIMAL	DIET-DIET SAMPLING METHOD*	ANIMAL CULTURE TECHNIQUE
Artemia salina (brine shrimp)	Algae - a2 (*Dunaliella* sp.)	Newly hatched shrimp were added to an aerated culture of algae. The shrimp were later separated from the algae by serial washings.
Caenorhabditis elegans (nematode)	Bacteria - a2 (*Escherichia coli*)	A liquid suspension of bacteria was innoculated with a small quantity of worms; the worms were later separated from the bacteria by centrifugation (Johnson, 1977).
Calliphora vicina (fly)	Horsemeat - a2 (*Equus caballus*) or Pork - a2 (*Sus scrofa*)	Eggs were placed directly on the meat, which was then covered with sawdust. After 13 days, pupae were transferred to an empty container. Adults were collected within 12 hours of emergence from the pupal cases.
Desmia funeralis (moth)	Grape leaves - a1 (*Vitis vinifera*)	The animals have been maintained in the laboratory for several generations on the specified diet; they were given grape leaves and water *ad libitum* (Aliniazee and Stafford, 1973).
Helix aspersa (snail)	Romaine lettuce leaves - b (*Lactuca sativa*)	The animals, starting with newly hatched snails, were provided with Romaine lettuce leaves and water *ad libitum*. In some cases, the diet was supplemented with $CaCO_3$ or $Ca_3(PO_4)_2$.

*See Section 2.1.2.

ANIMAL	DIET-DIET SAMPLING METHOD*	ANIMAL CULTURE TECHNIQUE
Melanoplus sanguinipes (grasshopper)	Corn seedlings - b (*Zea mays*) or Wheat seedlings - b (*Triticum aestivum*)	The animals, starting with newly hatched nymphs, were provided with the specified seedlings and water *ad libitum*.
Mus musculus strain AQR (mouse)	Purina Rat Chow mixture - b	Three-week-old female mice were obtained from the animal room of C.I.T., where they had been maintained on the specified diet for a number of generations. They were given the specified diet and water *ad libitum*.
Mus musculus strain BALB/c (mouse)	Wayne Lab-Blox F6 mixture-b	Thirteen-week-old female mice were obtained from the L. C. Strong Company, San Diego, California, where they had been maintained on the specified diet for a number of generations. They were provided with the specified diet and water *ad libitum*.
Mus musculus, strain BALB/cJ (mouse)	JAX 911A mixture - b	Five-week-old female mice were obtained from The Jackson Laboratory, Bar Harbor, Maine, where they had been maintained on the specified diet for a number of generations. They were provided with the specified diet and water *ad libitum*.

*See Section 2.1.2.

ANIMAL	DIET-DIET SAMPLING METHOD*	ANIMAL CULTURE TECHNIQUE
Musca domestica (fly)	Horsemeat - a2 (Equus caballus) or Pork - a2 (Sus scrofa)	Eggs were placed directly on the meat which was then covered with sawdust. After 10 days, pupae were transferred to an empty container. Adults were collected within 12 hours of emergence from the pupal cases.
Oncopeltus fasciatus (milkweed bug)	Milkweed seeds - a1 (Asclepias syriaca)	The animals have been maintained for many generations in the laboratory on the specified diet; they were given milkweed seeds and water ad libitum (LaChance and Riemann, 1973).
Sitophilus granarius (weevil) and Sitophilus oryzae (weevil)	Wheat seeds - a1 (Triticum aestivum)	The animals have been maintained for several generations in the laboratory on the specified diet; they were provided with wheat seeds and water ad libitum (Lum and Baker, 1973).

*See Section 2.1.2.

to us, a sample of the diet was taken immediately upon receipt (sampling technique a1, Table 2-1). In these cases, the diet was drawn from a large homogeneous reservoir so that variation in the $\delta^{13}C$ value of the diet was assumed to be small over the lifetime of the animal. In those cases in which the animal was given its entire lifetime supply of food at one time, a sample of the diet was taken only when the diet was first introduced to the animal (sampling technique a2). If several batches of food were required during the lifetime of the animal, at least one sample from each batch was taken (sampling technique b). The samples of the diets were stored at -20°C.

The presence of food in the gut of some animals, which represents contamination if the $\delta^{13}C$ value of the whole animal is to be determined, necessitated the adoption of several variations in the sampling technique for the animals. All insects, except for Calliphora and Musca, were held in cages with water but no food for 24 hours before collection to permit them to empty their guts by the normal processes of excretion. This treatment was not considered to be necessary for Calliphora and Musca, since the food, which was available only to larval stages, presumably would not be present in the gut of the adults which were analyzed. Artemia and Caenorhabditis were maintained in culture without food for four hours in order to purge their digestive tracts. The gut and its contents were removed from Helix specimens by dissection. Contamination by gut contents was not a consideration with mice, since organs and tissues exclusive of the alimentary canal, rather than the whole organism, were analyzed. The contribution of gut contents to the

whole animal body carbon was probably small in most cases. For example, the isotopic composition of Oncopeltus specimens which had been starved, -26.7 ± 0.5 ‰ (n=6), did not differ from that of specimens which had not been starved, -26.6 ± 0.2 ‰ (n=4). All animal specimens with the exception of mice were killed by immersion in liquid nitrogen and were stored in their entirety at -20°C. Mice were killed by cervical dislocation, dissected, and tissues and organs were stored at -20°C.

2.2. Isolation of biochemical components from specimens.

All chemicals used in these procedures were of reagent grade. Triply distilled water was used in making solutions.

The specimens from which lipid, total or soluble protein, or glycogen were to be isolated were first lyophilized, then ground to a powder with a mortar and pestle. Special procedures were required to prepare the specimens from which collagen, chitin, or the insoluble organic fraction of shells were to be isolated. These procedures are described when the isolation technique for each of these components is discussed below.

2.2.1. Lipids. The method of Bligh and Dyer (1959) was used to extract lipids. 50-100 mg. of sample was ground in 2 ml. of a homogenous mixture of methanol: chloroform: water (2.0: 1.0: 0.8). The solution was centrifuged at 1800 g., and the supernatant held in reserve. The pellet was extracted twice more with 2 ml. of the methanol:chloroform: water mixture and once with 2 ml. of methanol. The supernatants were combined, and chloroform and 0.18 M KCl were added to bring the final methanol:chloroform:water +0.18 M KCl ratio to 2.0:2.0:1.8. The

solution was mixed and allowed to separate overnight. The denser chloroform phase, which contained the total lipid fraction, was isolated with a separatory funnel. The chloroform was evaporated and the residual lipid redissolved in 1 ml. of chloroform. An aliquot of this solution was transferred to a pre-weighed, pre-combusted porcelain boat, the chloroform was evaporated, and the sample weighed before combustion. Experiments in which this extraction procedure was applied to a blank sample gave no detectable CO_2 following combustion (<0.2 μ moles).

2.2.2. Total protein. The method of Marroquin and Farber (1965) was used to prepare the total protein fraction. After the lipids had been extracted from a 50-100 mg. sample by the procedure detailed above, the residual pellet was ground in 2 ml. of ice-cold 10% (w/w) TCA (trichloroacetic acid). After 50 minutes, the mixture was centrifuged at 1800 g. and the supernatant solution poured off. The extraction procedure was repeated four times. Next, 2 ml. of 10% (w/w) TCA was added to the pellet, and the mixture was put in a 90°C water bath for 30 minutes. The resulting suspension was filtered through a carbon-free glass-fiber filter (Reeve Angel Co.), and the residue was washed with absolute ethanol and diethyl ether. After the filter was lyophilized, it could be cut into strips and combusted, or the residue could be scraped off. Combustion of a filter from an experiment in which the procedure was applied to a blank sample gave no detectable CO_2 (<0.2 μ moles).

2.2.3. Soluble protein. A modification of the procedure to isolate

the total protein fraction (Section 2.2.2.) was used to prepare the soluble protein fraction. A sample of 0.3-1.0 g. was sonicated in 40 ml. water and then centrifuged at 2000 g. The supernatant was isolated and solid TCA was added to it to bring the final concentration of TCA to 10% (w/w). The solution was cooled to 4°C and centrifuged at 1800 g. The supernatant was discarded. 5 ml. of 10% TCA was added to the pellet, and the mixture was put in a 90°C water bath for 30 minutes. The mixture was filtered through a carbon-free glass fiber filter, and the residue washed with absolute ethanol and diethyl ether. After lyophilization, the residue was scraped from the filter and combusted.

2.2.4. Glycogen. The methods of Stetten et al. (1956) and Jacobson et al. (1972) were used for glycogen preparation. The sample was taken through the soluble protein isolation procedure. Glycogen was isolated from the 2000 g., 10% (w/w) TCA supernatant by precipitation either by addition of ethanol to a final concentration of 67% (v/v) or by centrifugation at 20000 g. The glycogen was dissolved in water and reprecipitated with ethanol in the first case, while in the second case it was dissolved in water, washed with n-butanol in a separatory funnel, and reprecipitated by centrifugation at 20000 g.

2.2.5. Collagen Mouse bones were prepared for the collagen extraction procedure by the following steps. The flesh was removed from the bones during a prolonged exposure to dermestid beetle larva. Superficial debris was removed by sonicating the stripped bones in distilled water. The bones were then lyophilized and any remaining debris was removed with forceps under a dissecting microscope. The cleaned bones were

crushed in a rock piston. Collagen was isolated by the method of Longin (1971). 50-150 mg. of crushed bone was mixed with 50 ml. of 1 M HCl at room temperature; after 20 minutes, the mixture was filtered through a carbon-free glass-fiber filter. The filter was transferred to a 100 ml. beaker, 50 ml. of 10^{-3} M HCl was added, and the beaker covered with aluminum foil. The solution was heated at 90-95°C for 9-10 hours and then filtered through another carbon-free glass-fiber filter. The filtrate was reduced to a small volume by evaporation and then lyophilized. The residue is collagen.

2.2.6. Chitin. The method of Tsao and Richards (1952) was used to isolate chitin from insects. A whole insect was refluxed in 40 ml. of 10% (w/w) NaOH for three days, with the solution being changed daily. The insoluble residue was rehydrated by taking it through a series of ethanol-water mixtures of decreasing ethanol content, soaked in 0.4% (v/v) HCl for ten minutes to remove carbonates, and washed in water. This residue is chitin.

2.2.7. Shell insoluble organic fraction. The method of Weiner et al. (1976) was used to isolate the insoluble organic fraction of Helix shells. The snail shell was broken; the flesh and superficial debris were removed by scrubbing in water. For decalcification purposes, the shell was broken to smaller pieces and these were dialyzed against 10% (w/v) EDTA, buffered to pH 7.0 with phosphate and poisoned with a small amount of sodium azide. After decalcification, the insoluble organic fraction was recovered by centrifugation at 3170 g. and washed extensively with water.

2.3. Mass spectrometer analysis.

All carbon was converted to CO_2 by one of several methods outlined below prior to isotopic analysis by mass spectrometry. The CO_2 samples were analyzed in a 60° sector, double-collecting mass spectrometer, with the modifications of McKinney et al. (1950) on the original design of Nier (1947), in which the $^{13}C/^{12}C$ ratio of a sample CO_2 is compared with the $^{13}C/^{12}C$ ratio of a standard CO_2. The results are expressed as a $\delta^{13}C$ value:

$$\delta^{13}C \text{ (in ‰)} = \left[\frac{R_{sample}}{R_{standard}} - 1\right] \cdot 10^3$$

where R is the ratio of mass 45 ($^{13}C^{16}O^{16}O$) to mass 44 ($^{12}C^{16}O^{16}O$). Corrections for machine background, mixing of sample and standard gases in the inlet system, and $^{12}C^{17}O^{16}O$ contribution to the mass 45 peak have been made (Craig, 1957; Deines, 1970). All results are reported as $\delta^{13}C_{PDB}$ values, where the standard gas is CO_2 prepared from the calcium carbonate of the Cretaceous belemnite Belmnitella americana from the Peedee formation of South Carolina. The precision of mass spectrometer analysis was better than 0.1 ‰.

2.4. Carbon dioxide sample purification and yield determination.

The CO_2 samples from the combustion of organic matter were generally contaminated with oxides of nitrogen and sulfur. The respired CO_2 samples also contained unidentified contaminants which interferred with the isotopic analysis. All CO_2 samples, except those prepared from carbonates, were exposed first to Cu at 450°C to remove the oxides of nitrogen, then to MnO_2 at 450°C to remove oxides of sulfur.

As shown below, these procedures did not alter the isotopic composition of the CO_2.

Run number	$\delta^{13}C$ (of pure CO_2 before purification)	$\delta^{13}C$ (after purification)
MJDR-567	-21.3	-21.4
MJDR-589	-20.8	-20.8
MJDR-438	-20.5	-20.6

The volume of the CO_2 was measured after purification in a mercury manometer. The yield of CO_2 determined in this manner was accurate to within 1 μ mole.

2.5. Preparation of samples for mass spectrometer analysis.

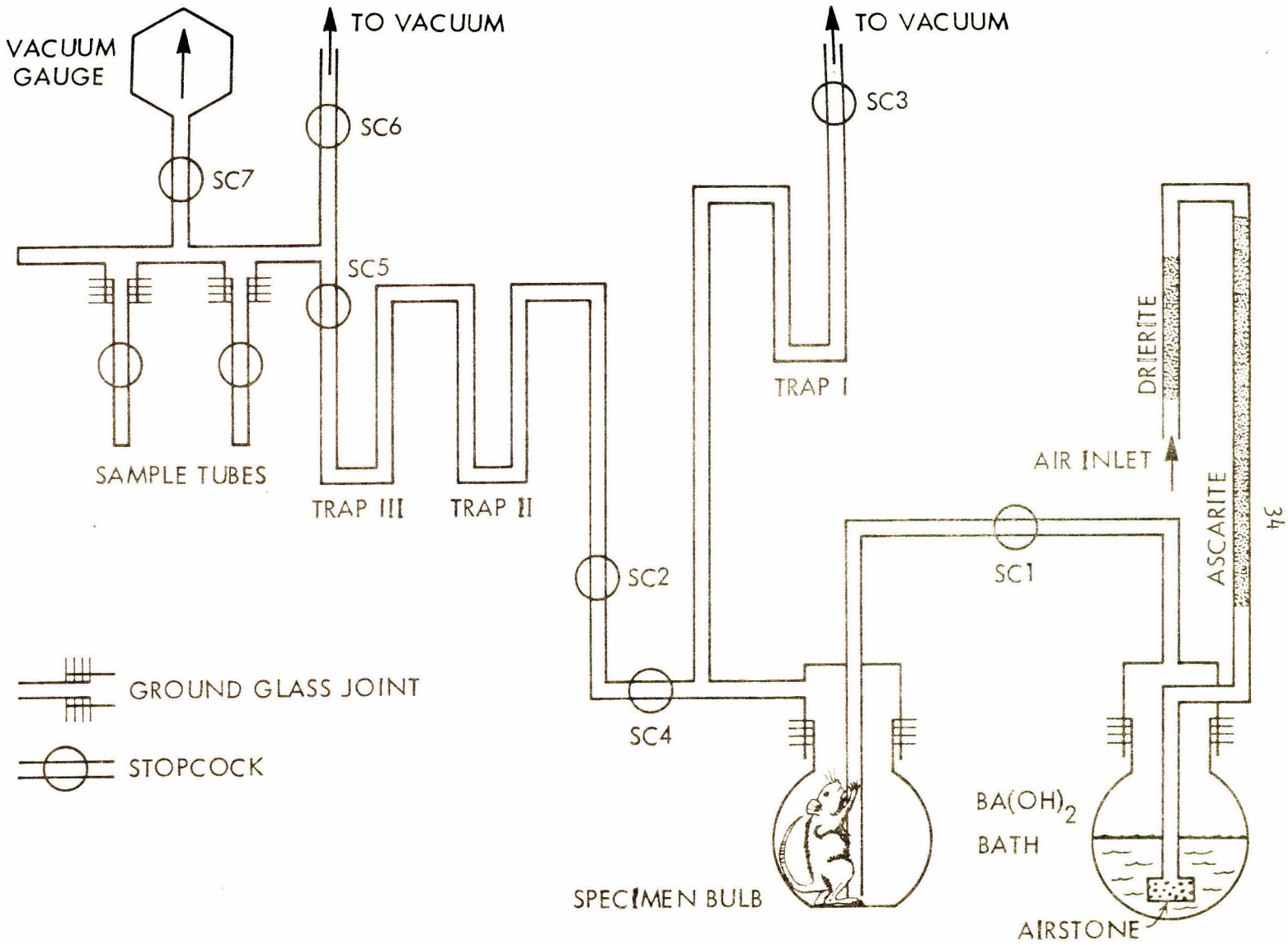
The following abbreviations are used in this section. Dry ice-M17 is a slurry of powdered dry ice and M17 (an organic non-polar solvent); H_2O but not CO_2 will condense out at the temperature of the slurry, -80°C. LN_2 is liquid nitrogen (-180°C), which is used to condense CO_2. CRV is a carbonate reaction vessel, which is identical in design to an enzyme reaction vessel (Figure 6-1), except that it doesn't have the large sidearm.

2.5.1. Preparation of CO_2 from carbonates. CO_2 was prepared from the calcium carbonate of snail shells, mouse bones, and a commercial $CaCO_3$ sample by the method of McCrea (1950). The shell and bone samples were ground to a powder and pretreated with a 50% aqueous solution of commercial Chlorox to destroy organic matter which might interfere with the isotopic analysis of the carbonate (Lowenstam and Epstein, 1957). The sample was then washed extensively with water and lyophilized. The carbonate sample was put in the main well of a carbonate reaction

vessel (CRV), 3 ml. of 100% H_3PO_4 was put in the sidearm, and the vessel was evacuated. The stopcock was closed and the CRV was transferred to a 25°C water bath. After 3 hours, the phosphoric acid was tipped into the main well. The products of the reaction, CO_2 and H_2O, were condensed in a trap immersed in LN_2. The trap was then warmed to dry ice temperature, and the sublimed CO_2 condensed to a manometer with LN_2, where its volume was measured prior to isotopic analysis. The reproducibility of replicate analyses of carbonates was ± 0.1 ‰.

2.5.2. Collection of respired CO_2. Respired CO_2 was collected from mice by the following procedure. (The modifications of the procedure necessary for the collection of required CO_2 from snails and insects are discussed below.) A mouse was placed in a one liter bulb which was attached to the line shown in Figure 2-1 through an airtight greased ground glass joint. The line between the air inlet and SC3, which included the specimen bulb, was at atmospheric pressure, while the line beyond SC4 was held under vacuum. Trap I was immersed in LN_2. The condensation of O_2, H_2O and CO_2 in the trap caused the pressure in the line to drop, but air flowed through the air inlet to balance this pressure drop, so that the animal was not exposed to any significant pressure trauma. The air entering the specimen bulb had been scrubbed of CO_2 by passage over Ascarite (which is NaOH absorbed onto asbestos) and by bubbling through a $Ba(OH)_2$ bath. After ten minutes of flushing the specimen container with CO_2-free air, SC2 was closed, so that any CO_2 that was introduced when the specimen bulb was attached to the line was isolated in Trap I. Trap III was then immersed in LN_2 and Trap II

Figure 2-1. High vacuum line for the collection of respiratory CO_2.


VACUUM GAUGE
TO VACUUM
TO VACUUM
SC7
SC6
SC5
SC3
SAMPLE TUBES
TRAP III
TRAP II
TRAP I
DRIERITE
AIR INLET
ASCARITE
SC2
SC1
SC4
GROUND GLASS JOINT
STOPCOCK
SPECIMEN BULB
BA(OH)2
BATH
AIRSTONE

in dry ice-M17. SC5 was closed and SC4 was opened, permitting any CO_2 respired by the animal to be condensed in Trap III. The specimen container was again flushed for ten minutes, after which SC4 was closed in order to isolate the traps from the specimen bulb. The non-condensible gases and liquid oxygen contained in Trap III were pumped away. Trap III was then warmed to dry ice temperature and the sublimed CO_2 condensed into a sample tube with LN_2 for subsequent purification and analysis.

For snails and insects, the following modifications in the procedure were necessary in order to collect a reasonably large CO_2 sample. 1-6 specimens, depending on the size of the animals, were placed in either the one liter specimen bulb or in a 150 ml. bulb. After the initial ten minute flushing period, SC1, SC2 and SC4 were closed, isolating the specimen bulb. After 12-24 hours, Trap III was immersed in LN_2 and SC1 and SC4 were opened. The specimen container was then flushed with CO_2-free air for ten minutes. The rest of the procedure outlined for mice was followed.

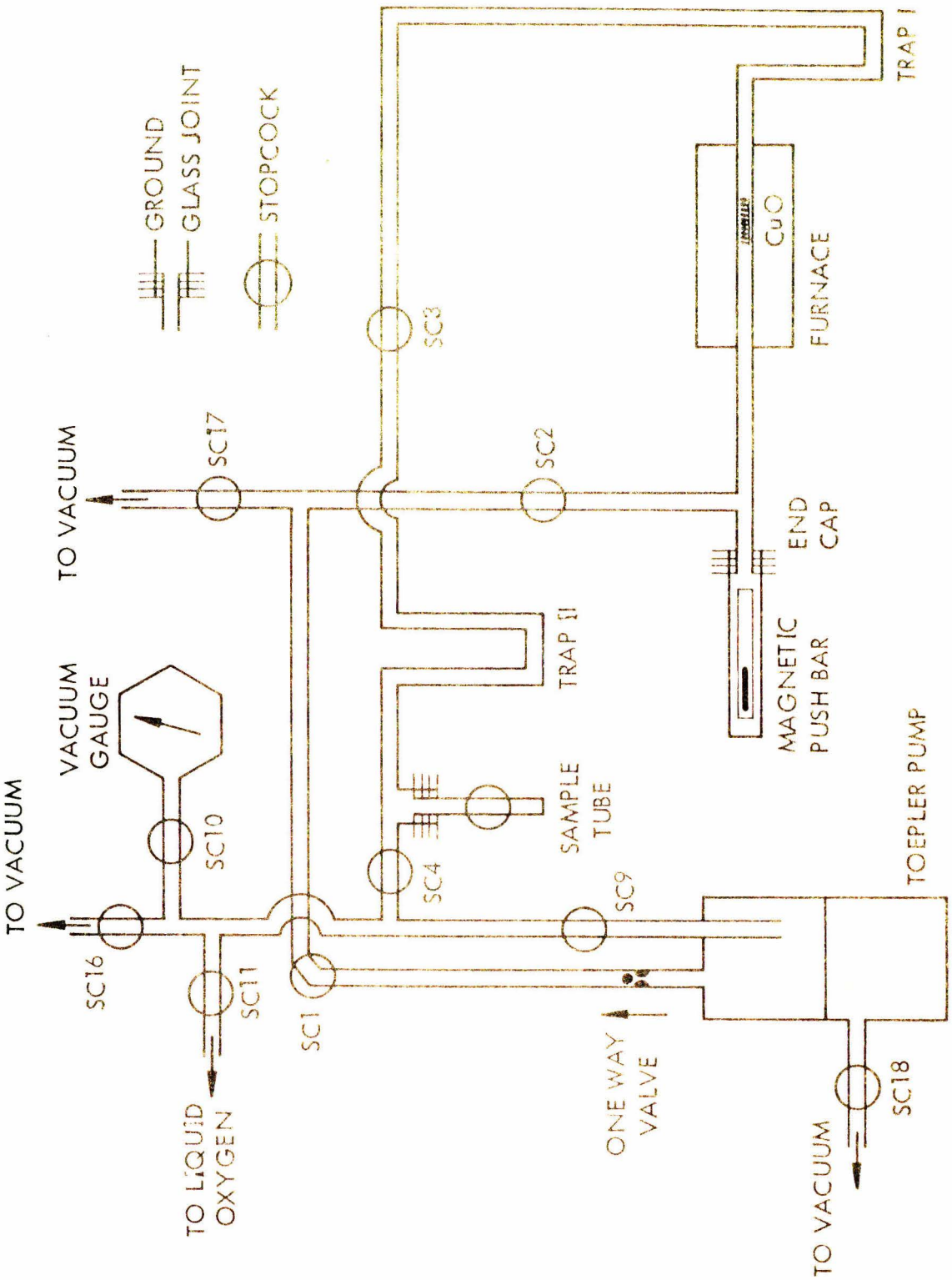
Two control experiments were done. Runs with an empty specimen bulb gave no detectable CO_2 (<0.2 μ moles). Since all respired CO_2 samples were larger than 100 μ moles, this indicates that background contribution of CO_2 was negligible. Control experiments in which a vial containing CO_2 of known isotopic composition was broken in an otherwise empty specimen bulb and the released CO_2 then collected by the standard procedure showed that the error due to the collection procedure was less than 0.3 ‰. The results of this second set of control runs are shown below.

Run number	$\delta^{13}C$ (‰), before collection	$\delta^{13}C$ (‰) after collection
MJDR-41	-22.2	-22.5
MJDR-42	-22.2	-22.4
MJDR-43	-30.0	-29.9

2.5.3. Combustion of organic samples to CO_2. Organic samples were converted to CO_2 by combusting the sample in a stream of free oxygen at 850°C in the line shown in Figure 2-2. All samples were lyophilized prior to combustion. The combustion furnace on the line consisted of two elements whose temperatures could be controlled separately. The section close to the end cap was kept at 550°C, while the other section was maintained at 850°C. There was a plug of CuO in the part of the line at 850°C. 1-20 mg. of sample was placed in a pre-combusted porcelain boat, which was then put in a cool part of the line near the end cap. The line was heated with an annealing flame and evacuated. The stopcocks leading to the vacuum were closed. Trap I was immersed in dry ice-M17 and Trap II was immersed in LN_2. An excess of O_2 was admitted from a reservoir of liquid oxygen through SC11, which was then closed. The automatic Toepler pump was activated, the porcelain boat was pushed into the furnace by the action of a quartz rod containing a magnet, and the temperature of the cooler part of the furnace was raised to 850°C. After twenty minutes, the line was evacuated through SC16, which was then closed. Trap II was warmed to dry ice temperature and the sublimed CO_2 condensed into sample tube I with LN_2. The CO_2 was purified and its volume determined prior to isotopic analysis.

For lipid samples, in order to insure complete combustion, it was

Figure 2-2. High vacuum line for the combustion of organic samples to CO_2.


GROUND
GLASS JOINT
STOPCOCK
TO VACUUM
VACUUM GAUGE
TO VACUUM
SC17
SC3
SC2
TRAP I
CuO
FURNACE
SC16
SC10
SC11
SC1
SC4
SAMPLE TUBE
TRAP II
END CAP
MAGNETIC PUSH BAR
SC9
TOEPLER PUMP
TO LIQUID OXYGEN
ONE WAY VALVE
SC18
TO VACUUM

necessary to circulate the oxygen and the combustion products for twenty minutes without LN_2 or dry ice-MI7 on either trap. Trap II was then immersed in LN_2 to condense out the combustion products, from which CO_2 was isolated as described above.

The completeness of the combustion, which insures no isotopic fractionation, was demonstrated by the following series of combustions of tripalmitin.

Run number	Yield	$\delta^{13}C$
MJD-249	99.6%	-22.2 ‰
MJD-268	90.2%	-22.2 ‰
MJD-269	96.8%	-22.2 ‰
MJD-270	100.1%	-22.2 ‰
MJD-281	98.7%	-22.3 ‰

The precision of the combustion method was determined by the replicate analyses of two standards which were combusted periodically during the course of this study, as indicated below.

Standard	Number of Analyses	$\delta^{13}C$
Grape leaf	20	-29.5 ‰ ± 0.2 ‰
Hemoglobin	9	-13.2 ‰ ± 0.3 ‰

3. RESULTS AND DISCUSSION

3.1. Isotopic composition of the diets.

The $\delta^{13}C$ values for the diets used in this study are indicated in Table 3-1. Standard deviations are reported only for those cases in which the diet was sampled more than once (sampling method b, Section 2.1.2.). Duplicate analyses of the diet in cases in which the diet was sampled only once (sampling methods a1 and a2) differed by 0.1 ‰ or less. In most cases the animals were cultured over one continuous period, so that a single sample or set of samples was taken. However, in the cases of Helix raised on Romaine lettuce leaves, the animals were cultured during two different periods, so two sets of samples were taken. The cause for the small difference in $\delta^{13}C$ values of the two diets used for the two growth periods is not known.

The $\delta^{13}C$ values for corn and wheat seedlings used in this study are considerably more negative than values reported in the literature. Smith and Epstein (1970) report values of -14.0 ‰ for corn and -23.7 ‰ for wheat grown in nature, while our laboratory-grown specimens had values of -22.6 ‰ and -40.2 ‰ respectively. Note also that our wheat seeds, which were grown in nature, had a $\delta^{13}C$ of -25.0 ‰. The source of CO_2 for plants grown in our laboratory was a mixture of Los Angeles atmospheric CO_2 and human respired CO_2. The $\delta^{13}C$ value of this CO_2 would be more negative than the average $\delta^{13}C$ value of -7 ‰ for rural atmosphere CO_2 (Keeling, 1958), because Los Angeles urban CO_2 ranges from -7 ‰ to -12 ‰ due to the contribution of ^{13}C-depleted CO_2 from the burning of fossil fuels (Epstein, 1977), while human respired CO_2 has even more negative $\delta^{13}C$ values (Broecker and Olson, 1961; La Croix

Table 3-1. $\delta^{13}C$ values of the diets.

DIET	$\delta^{13}C$ (‰)
Algae	-20.4
Bacteria	-24.4
Corn seedlings	-22.6 ± 0.6 (n = 3)
Grape leaves	-29.5
Horsemeat	-23.9
JAX 911A	-22.3 ± 0.5 (n = 10)
Milkweed seeds	-27.1
Pork	-13.5
Purina Rat Chow	-18.3 ± 0.6 (n = 4)
Romaine lettuce leaves #1	-27.6 ± 0.1 (n = 2)
Romaine lettuce leaves #2	-26.6 ± 0.3 (n = 3)
Wayne Lab-Blox F6	-19.3 ± 0.4 (n = 4)
Wheat seed	-25.0
Wheat seedling	-40.2 ± 0.8 (n = 3)

et al., 1973). The low $\delta^{13}C$ values of the seedlings grown in our laboratory must reflect the isotopic composition of their CO_2 source.

The difference in the $\delta^{13}C$ values of the pork and horsemeat has a similar explanation. The horsemeat was taken from an animal whose primary source of carbon must have been C_3 plants, since its $\delta^{13}C$ value, -23.9 ‰, falls in their range. The pork was obtained from a pig which was fed large amounts of corn (John, 1977), and its $\delta^{13}C$ value, -13.5 ‰, reflects the isotopic composition of its diet.

3.2. Relationship between the $\delta^{13}C$ values of whole animals and their diets.

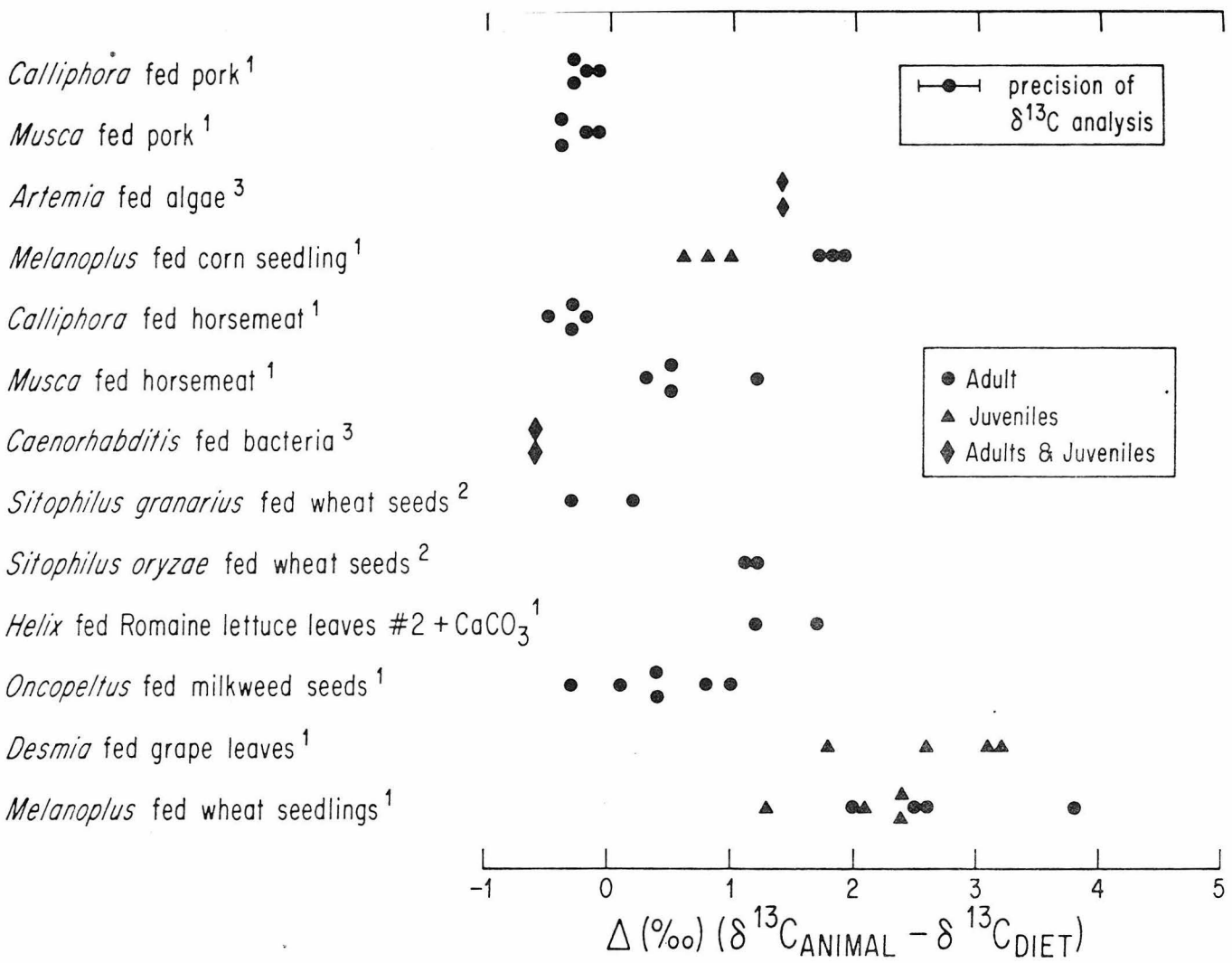
The relationship between the $\delta^{13}C$ value of a whole animal and the $\delta^{13}C$ value of its diet is shown in Figure 3-1, in which the $\Delta_{ANIMAL-DIET}$ values ($\delta^{13}C_{ANIMAL} - \delta^{13}C_{DIET}$) are plotted for all animals studied except mice. A positive $\Delta_{ANIMAL-DIET}$ value indicates that an animal's carbon is enriched in ^{13}C relative to its diet.

The following conclusions can be made from the data of Figure 3-1.

1) The animal carbon is generally enriched in ^{13}C relative to the diet carbon. The average $\Delta_{ANIMAL-DIET}$ value is +0.8 ± 1.0 ‰, with average values for the different species of animals ranging from -0.6 ‰ for Caenorhabditis raised on bacteria to +2.7 ‰ for Desmia raised on grape leaves and Melanoplus adults raised on wheat seedlings.

2) There can be considerable range in the $\delta^{13}C$ values of several individuals of an animal species fed the same diet. The range of $\Delta_{ANIMAL-DIET}$ values varies from a low of 0.2 ‰ for Calliphora raised on pork (n=4) to a high of 1.8 ‰ for Melanoplus adults raised on wheat seedlings (n=4).

Figure 3-1. Relationship between the $\delta^{13}C$ values of whole animals and the $\delta^{13}C$ values of their diets. The data are presented as $\Delta_{ANIMAL-DIET}$ values ($\delta^{13}C_{ANIMAL} - \delta^{13}C_{DIET}$). The superscripts indicate that each point represents the analysis of: [1]a single animal; [2]five animals; or [3]many animals.


Calliphora fed pork [1]
Musca fed pork [1]
Artemia fed algae [3]
Melanoplus fed corn seedling [1]
Calliphora fed horsemeat [1]
Musca fed horsemeat [1]
Caenorhabditis fed bacteria [3]
Sitophilus granarius fed wheat seeds [2]
Sitophilus oryzae fed wheat seeds [2]
Helix fed Romaine lettuce leaves #2 + $CaCO_3$ [1]
Oncopeltus fed milkweed seeds [1]
Desmia fed grape leaves [1]
Melanoplus fed wheat seedlings [1]
precision of $\delta^{13}C$ analysis
Adult
Juveniles
Adults & Juveniles
−1
0
1
2
3
4
5
Δ (‰) ($\delta^{13}C_{ANIMAL} - \delta^{13}C_{DIET}$)

3) The effect of the species of animal on the $\Delta_{ANIMAL-DIET}$ relationship is small. Different animal species feeding on the same diet, Calliphora and Musca raised on horsemeat, Calliphora and Musca raised on pork, and Sitophilus granarius and S. oryzae raised on wheat seeds, do not show large differences when their $\Delta_{ANIMAL-DIET}$ values are compared. The largest difference in the $\Delta_{ANIMAL-DIET}$ values for different animal species fed the same diet is ~1 ‰.

4) The effect of diet on the $\Delta_{ANIMAL-DIET}$ relationship is also small. The same animal species feeding on different diets, Calliphora raised on horsemeat and pork, Musca raised on horsemeat and pork, and Melanoplus raised on corn and wheat seedlings, do not have large differences in their $\Delta_{ANIMAL-DIET}$ values. The largest difference in the $\Delta_{ANIMAL-DIET}$ values for the same animal raised on different diets is on the order of 1 ‰.

3.3. Carbon isotope mass balance for animals and their diets.

The isotopic composition of carbon which enters an animal as its diet (input) must equal the isotopic composition of the carbon which is used by or lost from the animal (output). The output consists of incorporation of carbon into the body and loss of carbon from the body via either excretion or respiration. The overall carbon isotopic budget must represent a mass balance:

$$\delta^{13}C_i \cdot n_i = \delta^{13}C_j \cdot n_j$$

where i and j refer to input and output respectively and n is the number of moles of carbon in a component.

The isotopic mass balance expression indicates that a ^{13}C

enrichment relative to the diet in one output component must be balanced by a ^{13}C depletion of at least one other output component. This suggests that the ^{13}C enrichment of the whole animal relative to its diet must be balanced by a ^{13}C depletion, relative to the diet, of either the respired CO_2 or the excreted carbon. Melanoplus adults raised on wheat seedlings had the largest average $\Delta_{ANIMAL-DIET}$ values of the animals analyzed. The expected ^{13}C depletion, relative to the diet, of some output component other than incorporated carbon should be larger and hence more easily detected in Melanoplus raised on wheat seedlings than in the other animals of the study. Therefore, the $\delta^{13}C$ values of the input and output components for Melanoplus adults raised on wheat seedlings were determined.

The results of these determinations are shown in Figure 3-2a. Relative to the diet, the respired CO_2 has a lower $\delta^{13}C$ value, while the feces and the whole body carbon have more positive $\delta^{13}C$ values. It should be noted that in insects the nitrogenous waste products which contain carbon are incorporated into the feces (Wigglesworth, 1972), rather than excreted separately, so that the feces in this case represents all the output carbon lost through excretion.

The isotopic mass balance expression for Melanoplus raised on wheat seedlings is solved in Table 3-2. The amounts of carbon in the various components were approximated using the data of Smith (1959) for the amount of input (weight of wheat seedlings eaten) and output (weight of feces produced and increase in body weight) over a five-day period for adults of the related grasshopper, Melanoplus bilituratus. The amount of carbon lost as respired CO_2, which Smith did

Figure 3.2. $\delta^{13}C$ values of input and output components for:

a) *Melanoplus* raised on wheat seedlings

b) *Melanoplus* raised on corn seedlings

c) *Oncopeltus* raised on milkweed seeds

d) *Helix* raised on Romaine lettuce leaves #2 plus $CaCO_3$.

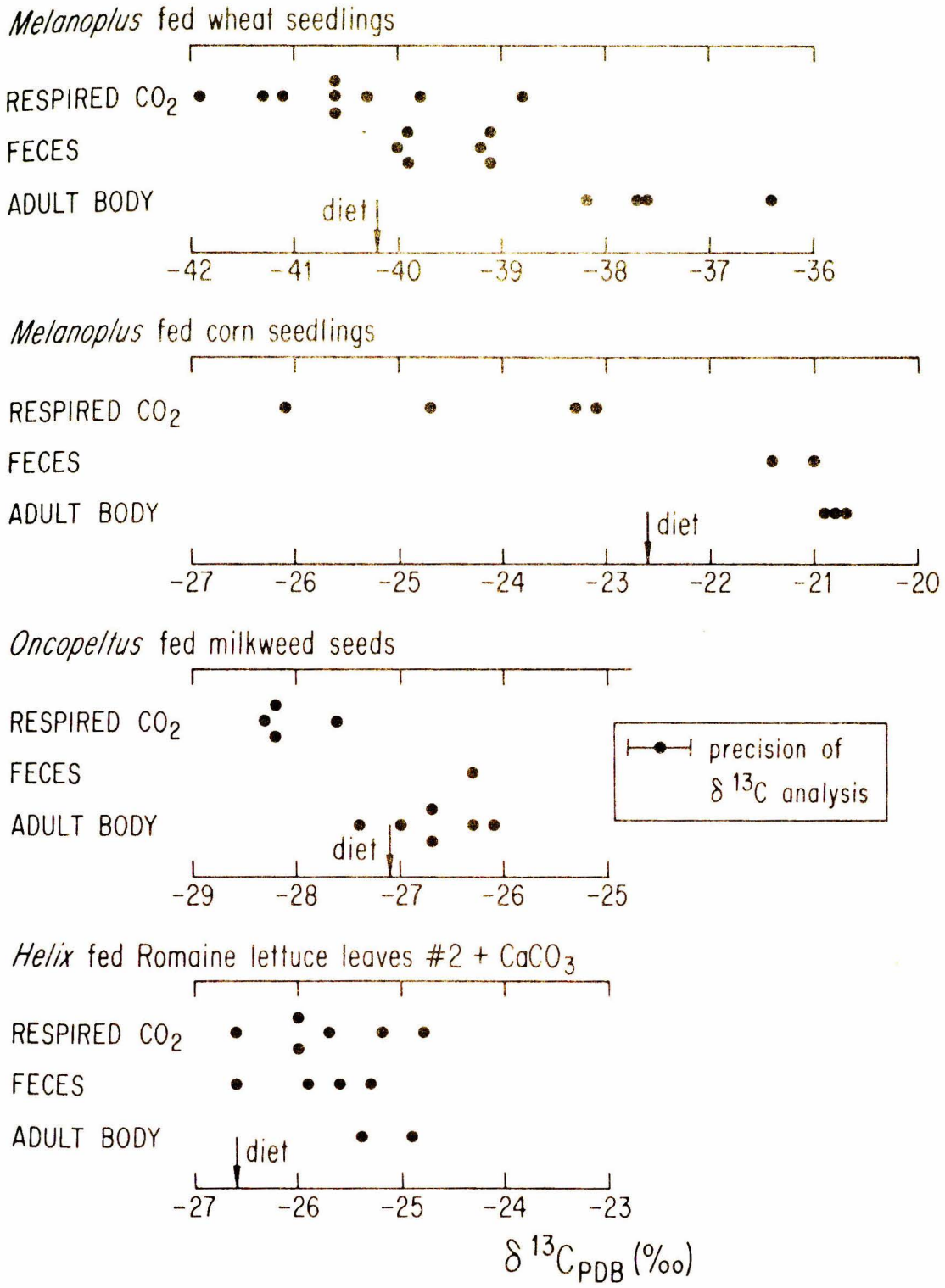

Melanoplus fed wheat seedlings
RESPIRED CO_2
FECES
ADULT BODY
diet
-42
-41
-40
-39
-38
-37
-36
Melanoplus fed corn seedlings
RESPIRED CO_2
FECES
ADULT BODY
diet
-27
-26
-25
-24
-23
-22
-21
-20
Oncopeltus fed milkweed seeds
RESPIRED CO_2
FECES
ADULT BODY
diet
-29
-28
-27
-26
-25
precision of
$\delta^{13}C$ analysis
Helix fed Romaine lettuce leaves #2 + $CaCO_3$
RESPIRED CO_2
FECES
ADULT BODY
diet
-27
-26
-25
-24
-23
$\delta^{13}C_{PDB}$ (‰)

Table 3-2. Carbon isotope mass balance for *Melanoplus* raised on wheat seedlings.

COMPONENT	Total weight for five-day period[a] (in mg.)	Conversion factor[b] (in μ moles CO_2 mg.$^{-1}$)	Total carbon for five-day period (in μ moles CO_2)	$\delta^{13}C$ (in ‰)
INPUT				
Food (wheat seedling)	148.0	34.5	5106	-40.2 ± 0.8
OUTPUT				
Respired (CO_2)	Not determined	--	1378[c]	-40.8 ± 0.6
Excreted (Feces)	112.5	30.3	3409	-39.5 ± 0.4
Assimilated (whole body)	8.1	39.4	319	-37.5 ± 0.8

[a] Smith, 1959.

[b] Average yield for samples from Figure 3-2a

[c] calculated by assuming $n_i = n_j$

$$\delta^{13}C_{OUTPUT} = \frac{\Sigma\ \delta^{13}C_j \cdot n_j}{\Sigma\ n_j}$$

$$= \frac{(-40.8\pm0.6)‰(1378\ \mu\text{moles})+(-39.5\pm0.4)‰(3409\ \mu\text{moles})+(-37.5\pm0.8)‰(319\ \mu\text{moles})}{(5106\ \text{moles})}$$

$$= -39.7\pm0.3‰$$

not determine, was assumed to make up the difference between the amounts of input and output carbon. The $\delta^{13}C$ value calculated for the output carbon, -39.7 ± 0.3 ‰, agrees reasonably well with the $\delta^{13}C$ value of the input carbon, -40.2 ± 0.8 ‰, indicating that an isotopic mass balance does exist for this system.

The $\delta^{13}C$ values of the input and output components for two other insect systems, Melanoplus adults raised on corn seedlings and Oncopeltus raised on milkweed seeds, are shown in Figure 3-2b and Figure 3-2c. The respired CO_2 samples have more negative $\delta^{13}C$ values while the body and the feces have more positive $\delta^{13}C$ values than the diet, which is the same pattern that was observed for Melanoplus adults raised on wheat seedlings. The isotopic mass balance expressions for these systems cannot be solved because data relating to the size of the input and output component pools are not available. However, qualitatively, it appears that there is isotopic mass balance in each of the systems.

The $\delta^{13}C$ values of some of the input and output components of Helix raised on Romaine lettuce leaves #2 plus $CaCO_3$ are shown in Figure 3-2d. In this case, the $\delta^{13}C$ values of all three output components -- respired CO_2, feces, and adult whole body -- are more positive than the $\delta^{13}C$ values of the diet. It should be noted that the Helix system is complicated by an additional source of input carbon, the $CaCO_3$ supplement, which had a $\delta^{13}C$ value 12.8 ‰ more positive than that of the diet, and by an additional output component, the $CaCO_3$ contained in the shell. The source of carbon for the shell carbonate is not known (see Section 3.8.2), so that it has not been possible to

solve, even qualitatively, the isotopic mass balance expression for this system. However, at least one aspect of the isotopic relationship among input and output components in the three insect systems was demonstrated not to hold for the Helix system. The respired CO_2 of Helix raised on Romaine lettuce leaves #2 plus $Ca_3(PO_4)_2$ as the calcium supplement has a $\delta^{13}C$ value, -25.6 ± 0.5 ‰ (n=5) which is more positive than the $\delta^{13}C$ value of the diet, -26.6 ‰. Thus, in the absence of a second source of input carbon, it can be seen that the isotopic relationship between the respired CO_2 and the diet of Helix raised on Romaine lettuce leaves #2 is the reverse of the relationship observed for these components in the three insect systems.

3.4. Effect of age on the isotopic composition of Melanoplus.

The data for Melanoplus raised on corn or wheat seedlings, shown in Figure 3-1, indicate that a small difference (on the order of 1 ‰) exists between the average $\delta^{13}C$ values of juvenile forms, known as nymphs in the case of Melanoplus, and adults raised on the same diet. The $\delta^{13}C$ values of nymphs are more similar to the diet isotopic composition than those of adults. These data, together with the weight and developmental stage* of each specimen, are listed in Table 3-3. Also listed are the $\delta^{13}C$ values and weights of the newly hatched 1st

*Insects have relatively inelastic cuticular exoskeletons which limit their growth. When an insect becomes too large for its exoskeleton, the animal sheds it and acquires a larger one through a process called moulting. The period between moults is termed a stadium, while the insect during a stadium is referred to as an instar. Our Melanoplus specimens fed on either corn or wheat seedlings went through five stadia prior to the final moulting to the adult stage.

Table 3-3. Effect of age on the $\delta^{13}C$ value of whole Melanoplus raised on corn or wheat seedlings. In some cases, several specimens of the same age were combined prior to combustion.

	DEVELOPMENTAL STAGE	WEIGHT (mg.)	^{13}C (‰)
Melanoplus Fed corn seedlings	1st instar, newly hatched	1	-25.8
	3rd instar	16	-22.0
	4th instar	22	-21.8
	5th instar	41	-21.6
	adult	76	-20.9
	adult	n.d.*	-20.8
	adult	n.d.	-20.6
corn seedlings	--	--	-22.6
Melanoplus fed wheat seedlings	1st instar, newly hatched	1	-22.6
	2nd instar	7	-37.8
	3rd instar	14	-37.8
	4th instar	32	-38.1
	5th instar	47	-38.9
	adult	n.d.	-38.2
	adult	n.d.	-37.7
	adult	56	-37.6
	adult	46	-36.4
wheat seedlings	--	--	-40.2

*not determined

instar nymphs which were used to start each culture. The eggs from which the nymphs were hatched were obtained from two sources who apparently maintained their Melanoplus cultures on diets of different isotopic composition. The data of Table 3-3 indicate that as the age of the nymph increases, the $\delta^{13}C$ value of its body trends away from the newly hatched nymph $\delta^{13}C$ value and toward a value somewhat heavier than the diet $\delta^{13}C$ value. This trend would result from mixing of carbon present in the newly hatched 1st instar nymph and carbon assimilated from the diet. As the age (and size) of the nymph increases, the contribution of carbon from the newly hatched 1st instar nymph is progressively diluted, so that beyond the 3rd instar nymph, this effect becomes negligible. The data of Table 3-3 also show that the $\delta^{13}C$ values of adults are more positive than the $\delta^{13}C$ values of the older nymphs. This observations suggests the possibility that there are differences in the isotopic composition of carbon incorporated into the body of Melanoplus at different stages of its life.

In order to understand the causes for the variations of the $\delta^{13}C$ value of Melanoplus whole bodies with age, the $\delta^{13}C$ values for moulted exoskeletons (exuviae) and whole rear legs taken from nymphs and adults were determined. During the period preceding moulting, part (up to 90% in some insects) of the old cuticle is adsorbed and reincorporated into the new exoskeleton (Wigglesworth, 1972). Analysis of the exuviae should demonstrate the mixing of carbon present in the newly hatched nymph and assimilated carbon. On the other hand, the rapid rate of weight gain in the rear leg of the grasshopper should reduce the effect of mixing on the $\delta^{13}C$ value of the specimen, permitting

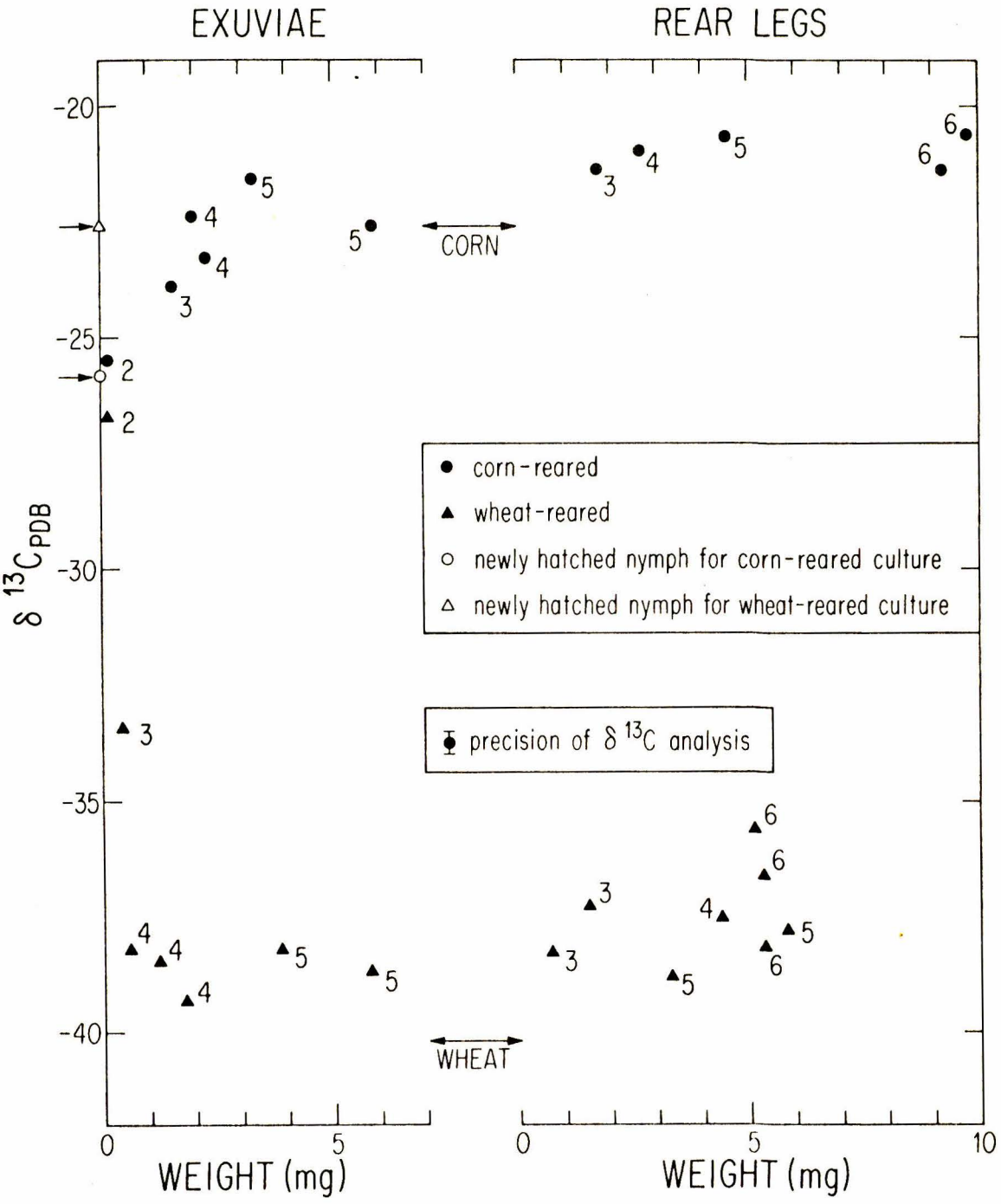
differences in the isotopic composition of carbon incorporated into the legs of nymphs and adults to be observed, if they exist. (In Melanoplus fed corn seedlings, the rear leg averaged 0.06 mg. in newly hatched 1st instar nymphs, 1.7 mg. in 3rd instar nymphs, 2.7 mg. in 4th instar nymphs, 4.5 mg. in 5th instar nymphs, and 9.5 mg. in adults.)

The $\delta^{13}C$ values of exuviae and rear legs are shown in Figure 3-3. The $\delta^{13}C$ values of exuviae demonstrate a mixing effect for moults from the 2nd and 3rd instar nymphs for Melanoplus raised on either corn or wheat seedlings. There is no mixing effect evident for moults from the 4th and 5th instar nymphs, presumably because the carbon present in the newly hatched 1st instar nymph has been sufficiently diluted by growth. No mixing effect is observed in the $\delta^{13}C$ values of the rear legs of Melanoplus raised on either diet. There may be a trend for legs from older specimens to have more positive $\delta^{13}C$ values, but the variation of $\delta^{13}C$ values for legs from specimens of the same age is nearly as large as the total range of values for specimens of all ages.

In summary, an age effect on the $\delta^{13}C$ value of carbon incorporated into the body of Melanoplus raised on either corn or wheat seedlings cannot be ruled out. However, the magnitude of the effect, on the order of 1 ‰, is small. The accuracy with which the $\delta^{13}C$ value of the diet can be determined from the $\delta^{13}C$ value of an animal's carbon will not be seriously affected by possible age effects of this size.

3.5. Relationship between the $\delta^{13}C$ values of biochemical fractions in animals and their diets.

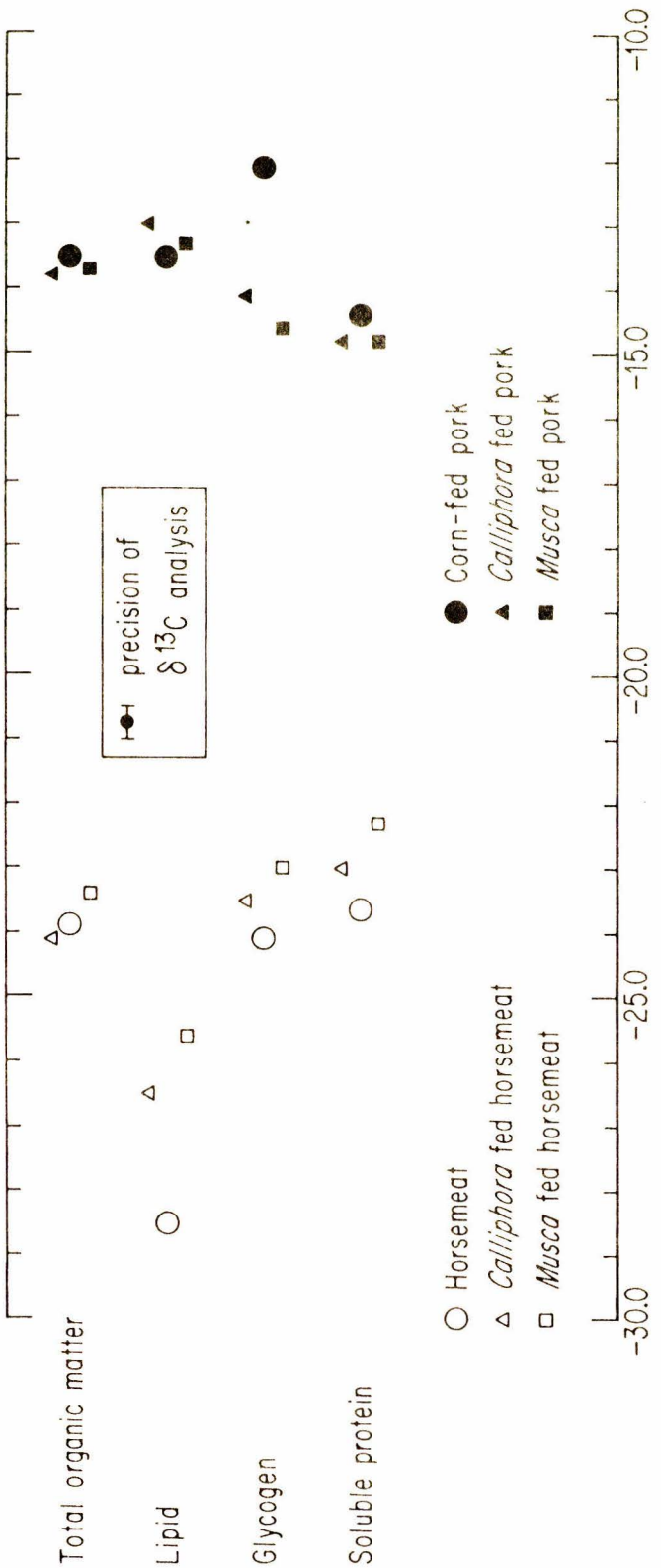
Figure 3-3. Effect of age on the $\delta^{13}C$ values of exuviae and rear legs of Melanoplus raised on corn or wheat seedlings. In some cases, several specimens of the same age were combined prior to combustion. The number near each point refers to the instar from which the specimen was taken (for rear legs) or the instar from which the animal was moulting (for exuviae). The number "6" identifies an adult specimen.


EXUVIAE
REAR LEGS
-20
-25
-30
-35
-40
δ 13C PDB
CORN
WHEAT
corn-reared
wheat-reared
newly hatched nymph for corn-reared culture
newly hatched nymph for wheat-reared culture
precision of δ 13C analysis
0
5
10
WEIGHT (mg)
WEIGHT (mg)

The $\Delta_{ANIMAL-DIET}$ values ($\delta^{13}C_{ANIMAL} - \delta^{13}C_{DIET}$) for an animal species raised on a single diet, as discussed in Section 3.2., range from -0.6 ‰ to +2.7 ‰. Differences in the relative proportions of the major biochemical fractions in different animals and their diets could contribute to the variability in the $\Delta_{ANIMAL-DIET}$ values, because the major biochemical fractions have characteristically different $\delta^{13}C$ values (see Section 5.1.). For example, if a diet with a high fat content were fed to an animal which incorporated only a small part of that fat, the $\Delta_{ANIMAL-DIET}$ value might be large because of the exclusion of the fat fraction, which is highly enriched in ^{12}C relative to the other components of the diet, from the animal body. Comparison of the $\delta^{13}C$ values of a biochemical fraction in the animal and its diet might avoid the complications resulting from differences in the concentration of the fractions in animals and their diets. Accordingly, the $\delta^{13}C$ values of several readily isolated biochemical fractions in animals and their diets were measured in order to determine if the accuracy of the isotopic method of dietary analysis could be improved by comparing the $\delta^{13}C$ values of animals and their diets at the suborganismic level.

The $\delta^{13}C$ values of the total organic matter and of the lipid, glycogen, and soluble protein fractions were determined for the following samples: horsemeat, pork and each of two species of flies, Calliphora and Musca, raised on each meat. The results, which are shown in Figure 3-4, indicate that the $\delta^{13}C$ value of a component in the fly generally reflects the $\delta^{13}C$ value of that component in the meat it was raised on. However, the $\delta^{13}C$ value of a component in a

Figure 3-4. $\delta^{13}C$ values of total organic matter and of the lipid, glycogen, and soluble protein fractions in Calliphora and Musca and in the horsemeat and pork they were raised on.


Total organic matter
Lipid
Glycogen
Soluble protein
precision of
δ 13C analysis
Horsemeat
Calliphora fed horsemeat
Musca fed horsemeat
Corn-fed pork
Calliphora fed pork
Musca fed pork
-30.0
-25.0
-20.0
-15.0
-10.0
δ 13CPDB (‰)

fly and the $\delta^{13}C$ value of that component in the diet may differ by up to 3.0 ‰, while at the same time the differences between the $\delta^{13}C$ values of the total organic matter of a fly and its diet are all less than 0.5 ‰

Evidently, the biochemical fractions of the diet are not incorporated into the animal without isotopic alteration. Differences between the $\delta^{13}C$ values of components in the animal and its diet could arise from isotope effects during assimilation of the component and/or in the de novo synthesis of the component in the animal. The fact that these isotope effects tend to balance one another when averaged over the whole of an animal's metabolism suggests that changes in the $\delta^{13}C$ value of one fraction occurs at the expense of the isotopic composition of some other component. It appears that the $\delta^{13}C$ analysis of the total organic matter of animals and their diets will result in a more accurate dietary analysis than the determination of the $\delta^{13}C$ values of major biochemical fractions.

The $\delta^{13}C$ values of the total organic matter and the biochemical fractions of horsemeat have the same relationships with one another as has been observed in autotrophs (see Section 5.1.): $\delta^{13}C_{LIPID} < \delta^{13}C_{TOTAL\ ORGANIC\ MATTER}$ and $\delta^{13}C_{LIPID} < (\delta^{13}C_{CARBOHYDRATE}, \delta^{13}C_{PROTEIN})$. The ^{13}C distribution in pork is the first case in which $\delta^{13}C_{LIPID} = \delta^{13}C_{TOTAL\ ORGANIC\ MATTER}$ and $\delta^{13}C_{LIPID} > \delta^{13}C_{PROTEIN}$. The different isotopic relationships in the two meats probably reflect differences in the feeding regimes of the animals they came from. Horses intended for slaughter are generally given a relatively constant diet throughout their lives (Carr, 1977). The $\delta^{13}C$ value of the horse-

meat, -23.9‰, indicates that the horse ate a diet composed primarily of C_3 plants. Corn-fed pigs, on the other hand, are fed mixed grains when they are young, and, in order to fatten them for market, are fed a diet rich in corn during the second half of their lives (Carr, 1977). Most grains except for corn come from C_3 plants, and therefore have more negative δ^{13} values than corn, which is a C_4 plant. If lipids and glycogen were formed preferentially during the fattening period, the $\delta^{13}C$ values of the lipids and glycogen would be more positive (corn-like) than that of the protein fraction. The ^{13}C depletion of the lipid fraction relative to the glycogen is the usual relationship observed between the $\delta^{13}C$ values of these two fractions (see Section 5.1.).

The limited data available (Figure 3-4) indicate that changes in the $\delta^{13}C$ value of a fraction in going from the diet to the animal are not large enough to affect the general isotopic relationships among the biochemical fractions, which appear to be inherited from the diet. The relationships of $\delta^{13}C_{LIPID} < \delta^{13}C_{TOTAL\ ORGANIC\ MATTER}$ and $\delta^{13}C_{LIPID} < (\delta^{13}C_{CARBOHYDRATE}, \delta^{13}C_{PROTEIN})$, which exist in autotrophs (Section 5.1.) and presumably in the plants eaten by the horse, are preserved, in turn, first in the horsemeat and then in the flies which were raised on the horsemeat. The flies raised on pork show the anomalous relationships, $\delta^{13}C_{LIPIDS} \geq \delta^{13}C_{TOTAL\ ORGANIC\ MATTER}$ and $\delta^{13}C_{LIPIDS} > \delta^{13}C_{PROTEINS}$, which are found in the pork. Apparently, the isotopic relationships among the biochemical fractions present in organisms at one trophic level are inherited by the organisms

at the next trophic level.

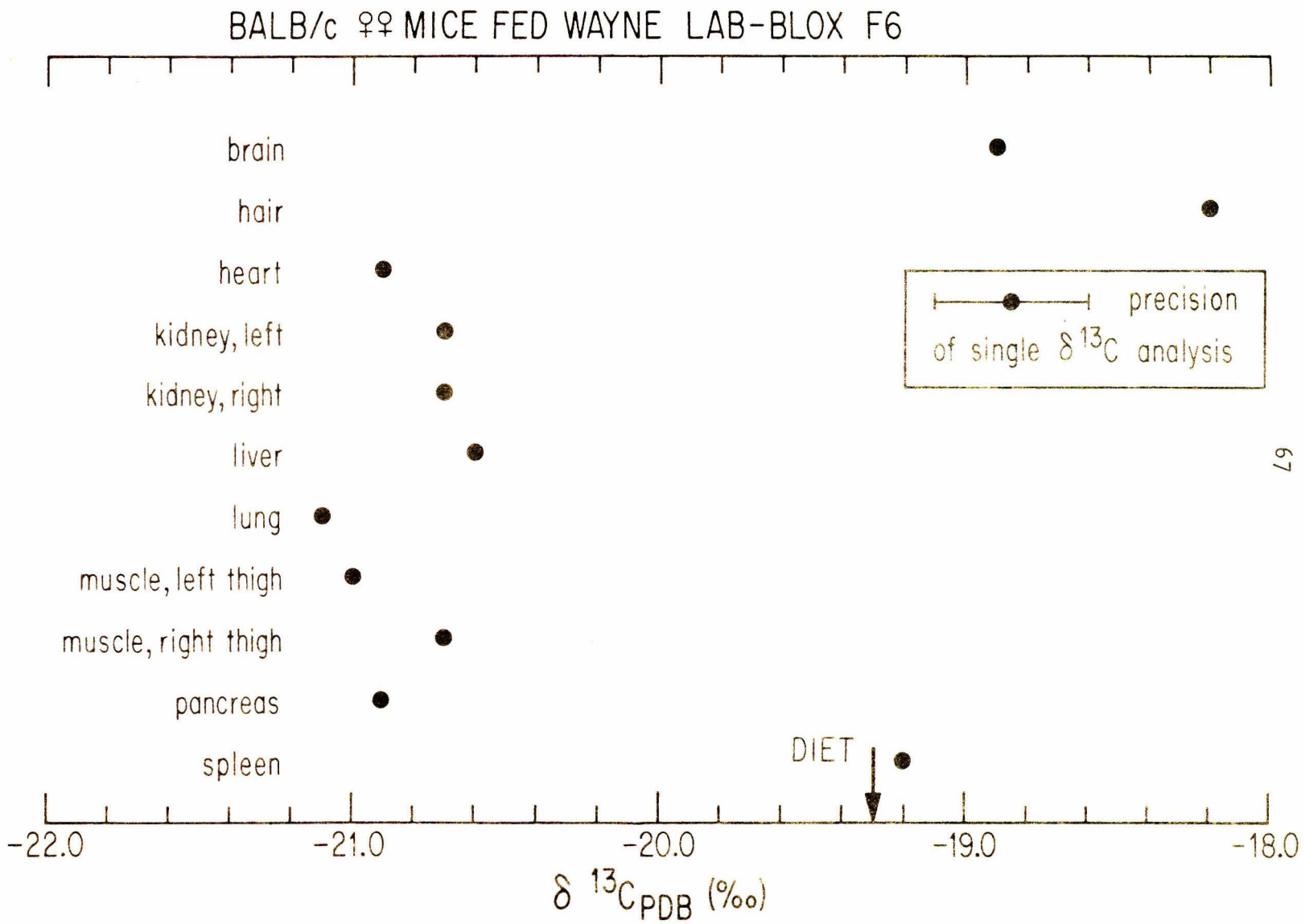
3.6. Relationship between the $\delta^{13}C$ values of the tissues of mice and their diets.

It is impractical to determine the $\delta^{13}C$ value of the total organic matter of a large animal for use in dietary analysis. An alternate approach is to determine the isotopic composition of some part of the animal which best reflects the $\delta^{13}C$ of the diet. The $\delta^{13}C$ values of various tissues of mice raised on diets of known isotopic composition were measured in order to determine which tissues might be suitable for this purpose.

In all other instances of this study, animals were raised on naturally occurring diets of constant $\delta^{13}C$ value. It was not possible to obtain mice or other vertebrates raised on a natural diet of constant $\delta^{13}C$ value, so the compromise approach of using mice raised on formulated diets of constant $\delta^{13}C$ values was adopted. Application of these results to interpretation of natural systems may involve more extrapolation than is required in those cases in which laboratory-raised animals were fed on natural diets.

In order to minimize differences among individuals, tissues from eleven BALB/c mice raised on Wayne Lab-Blox F6 were pooled and homogenized prior to combustion. The results of $\delta^{13}C$ analysis are shown in Figure 3-5. Most of the tissues have $\delta^{13}C$ values of approximately -21 ‰, which is ~1.5 ‰ more negative than the $\delta^{13}C$ value of the diet. However, the $\delta^{13}C$ values of brain, hair and spleen are all more positive than that of the diet by up to 1 ‰. Analysis of tissues

Figure 3-5. $\delta^{13}C$ values of tissues from BALB/c mice raised on Wayne Lab-Blox F6. The tissues of eleven animals were combined prior to combustion. The mice were 17 weeks old.


BALB/c ♀♀ MICE FED WAYNE LAB-BLOX F6
brain
hair
heart
kidney, left
kidney, right
liver
lung
muscle, left thigh
muscle, right thigh
pancreas
spleen
DIET
precision
of single δ 13C analysis
-22.0
-21.0
-20.0
-19.0
-18.0
δ 13CPDB (‰)

taken from individual mice raised on the same diet is shown in Figure 3-6a. For some tissues, considerable variation among samples taken from different mice was observed. Spleen and hair from individuals have $\delta^{13}C$ values which differ from the $\delta^{13}C$ value for the pooled sample by up to 3.6 ‰. The $\delta^{13}C$ values of brain, liver, or kidney from individual animals differ from the $\delta^{13}C$ value for the pooled sample by less than ~1 ‰.

$\delta^{13}C$ values of tissues from individual mice raised on other diets are also shown in Figure 3-6. Mice of a different strain and age raised on Purina Rat Chow (Figure 3-6b) have a distribution in the $\delta^{13}C$ values of their tissues similar to mice raised on Wayne Lab-Blox F6. Brain and hair generally have higher $\delta^{13}C$ values and the other organs have lower $\delta^{13}C$ values than the diet. The main difference between tissues of mice raised on the two diets is the absence of ^{13}C enrichment relative to the diet in the spleen samples of mice raised on Purina Rat Chow. Four mice raised on JAX 911A show two new features in the $\delta^{13}C$ values of their tissues (Figure 3-6c). First, the liver and kidney have more positive $\delta^{13}C$ values than the diet, whereas in the previous two cases these organs had $\delta^{13}C$ values which were 1-1.5 ‰ more negative than the diet $\delta^{13}C$ value. Second, the brain, instead of having the most positive $\delta^{13}C$ values as was observed in the other cases, now has $\delta^{13}C$ values that are slightly lower than the $\delta^{13}C$ values of the liver and kidney.

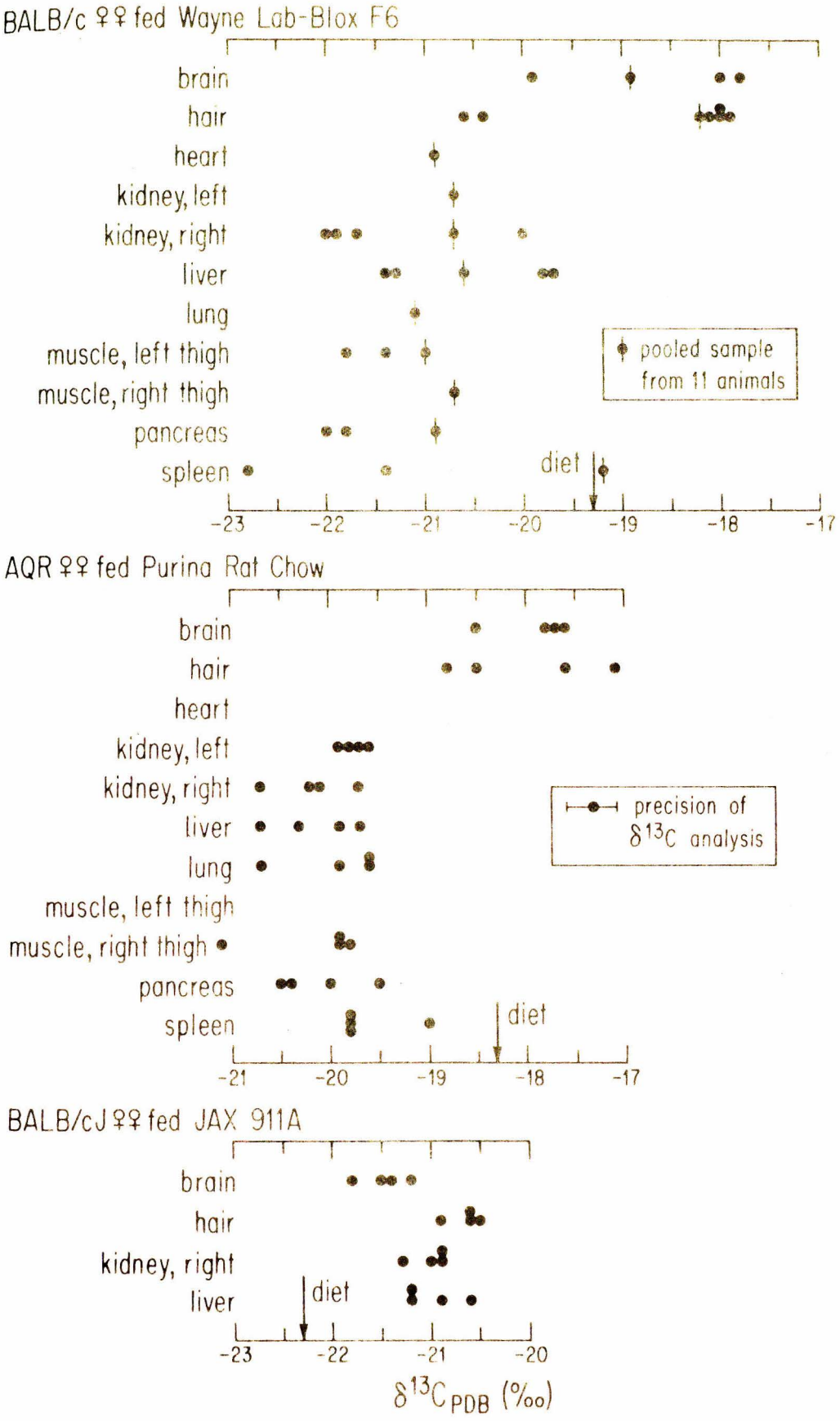
The results of Figure 3-5 and Figure 3-6 indicate that no single tissue can be analyzed in order to determine the $\delta^{13}C$ value of the diet by proxy. Because of the large range in the $\delta^{13}C$ values of a single

Figure 3-6. $\delta^{13}C$ values of tissues from mice raised on several diets. Each point, except as indicated, represents the analysis of tissue from an individual animal. The strain of mice, the diet, and the age of the mice in each figure are:

a) BALB/c, Wayne Lab-Blox F6, 14-17 weeks

b) AQR, Purina Rat Chow, 4-8 weeks

c) BALB/cJ, JAX 911A, 15 weeks.


BALB/c ♀♀ fed Wayne Lab-Blox F6
brain
hair
heart
kidney, left
kidney, right
liver
lung
muscle, left thigh
muscle, right thigh
pancreas
spleen
diet
pooled sample from 11 animals
-23
-22
-21
-20
-19
-18
-17
AQR ♀♀ fed Purina Rat Chow
brain
hair
heart
kidney, left
kidney, right
liver
lung
muscle, left thigh
muscle, right thigh
pancreas
spleen
diet
precision of δ13C analysis
-21
-20
-19
-18
-17
BALB/cJ ♀♀ fed JAX 911A
brain
hair
kidney, right
liver
diet
-23
-22
-21
-20
δ13C PDB (‰)

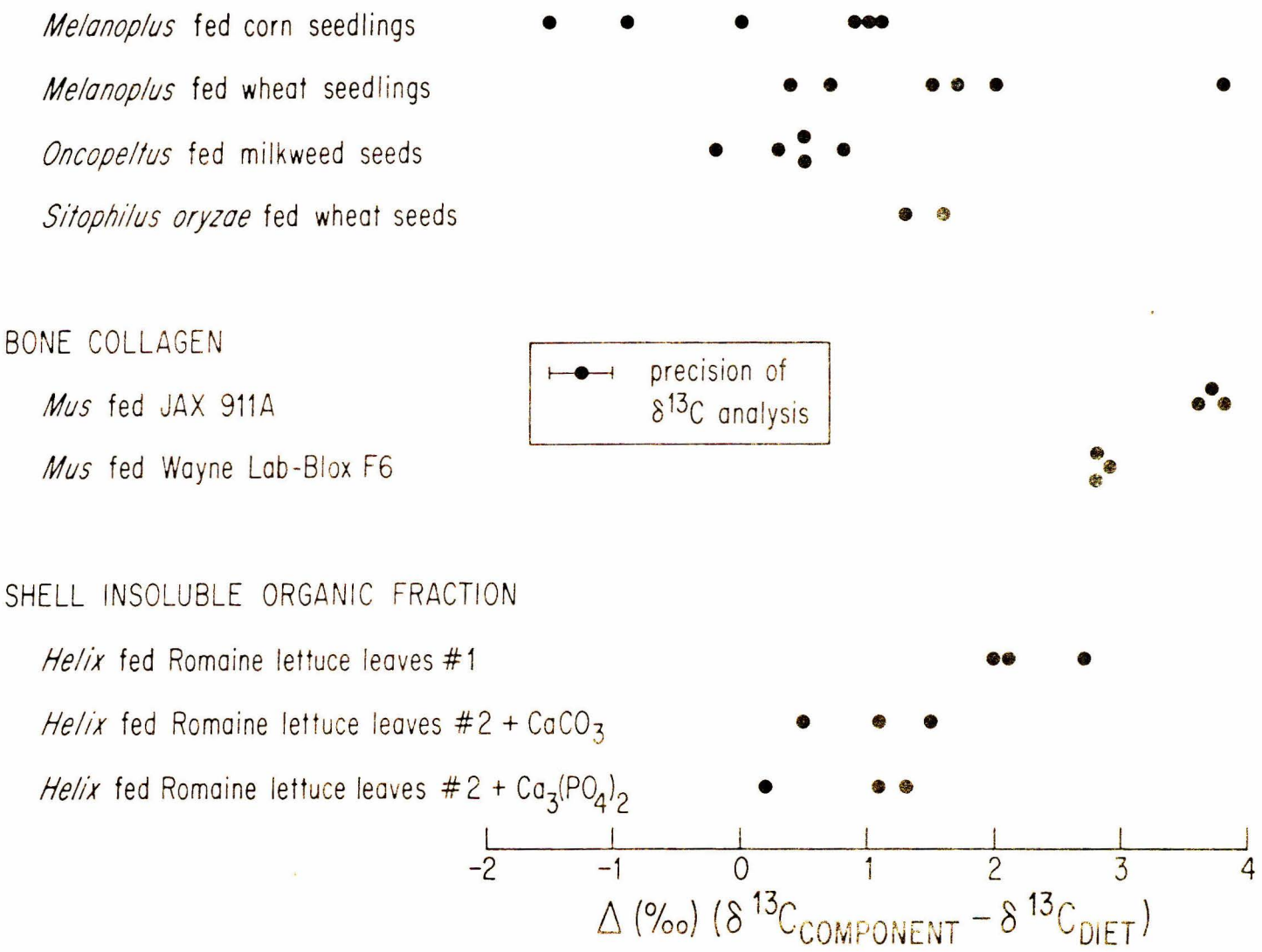
type of tissue taken from several specimens raised on the same diet, and because of the apparent influence of diet on the relationship between the $\delta^{13}C$ value of a tissue and the $\delta^{13}C$ value of the diet of the animal, it will be necessary to measure the $\delta^{13}C$ values of several tissues from an animal in order to estimate a $\delta^{13}C$ value for its diet.

3.7. Relationship between the $\delta^{13}C$ values of potentially fossil organic components of animals and their diets.

The method of dietary analysis by $\delta^{13}C$ determination of animal carbon may be applied to certain fossil organic material if two requirements are met. First, the $\delta^{13}C$ value of some component synthesized by the animal and preserved in the fossil material must be unaltered by diagenetic processes. Second, the relationship between the $\delta^{13}C$ value of such a component and the $\delta^{13}C$ value of the diet of the animal which synthesized it must be known. This first requirement is often met by several biochemical components of animals, such as collagen, chitin, and the organic fraction of intertebrate shells (see Section 3.8.2.). In order to satisfy the second requirement, the relationship between the $\delta^{13}C$ values of these components and the $\delta^{13}C$ values of the diets of the animals from which they were isolated was determined.

The $\Delta_{COMPONENT-DIET}$ values ($\delta^{13}C_{COMPONENT} - \delta^{13}C_{DIET}$) for chitin from insects, collagen from the bones of mice, and the insoluble organic fraction of Helix shells are shown in Figure 3-7. The $\Delta_{CHITIN-DIET}$ values are generally positive, with the variability in the $\delta^{13}C$ values of chitin isolated from individuals of the same species raised on a single diet ranging up to 3.5 ‰. Diet appears to affect

Figure 3-7. Relationship between the $\delta^{13}C$ values of some potentially fossil organic components of animals and the $\delta^{13}C$ values of their diets. The data are presented as $\Delta_{COMPONENT-DIET}$ values ($\delta^{13}C_{COMPONENT}-\delta^{13}C_{DIET}$).


Melanoplus fed corn seedlings
Melanoplus fed wheat seedlings
Oncopeltus fed milkweed seeds
Sitophilus oryzae fed wheat seeds
BONE COLLAGEN
Mus fed JAX 911A
Mus fed Wayne Lab-Blox F6
precision of $\delta^{13}C$ analysis
SHELL INSOLUBLE ORGANIC FRACTION
Helix fed Romaine lettuce leaves #1
Helix fed Romaine lettuce leaves #2 + $CaCO_3$
Helix fed Romaine lettuce leaves #2 + $Ca_3(PO_4)_2$
-2
-1
0
1
2
3
4
Δ (‰) ($\delta^{13}C_{COMPONENT} - \delta^{13}C_{DIET}$)

this isotopic relationship. Chitin samples isolated from Melanoplus raised on corn seedlings have a different distribution in their $\Delta_{CHITIN-DIET}$ values than chitin isolated from Melanoplus raised on wheat seedlings.

Collagen samples extracted from the bones of BALB/c mice raised on two different diets have similar distributions in their $\Delta_{COLLAGEN-DIET}$ values, the mean values being +3.7 ‰ and +2.8 ‰. The range of $\Delta_{COLLAGEN-DIET}$ values is only 0.1 ‰ for the collagen samples isolated from three mice on each diet.

The $\Delta_{COMPONENT-DIET}$ values for the insoluble organic fraction of snail shells isolated from individual animals whose diet was supplemented with $CaCO_3$ or $Ca_3(PO_4)_2$ range from approximately 0 ‰ to +1.5 ‰. There is an increase of about +1.5 ‰ in the average $\Delta_{COMPONENT-DIET}$ values for this component isolated from snails which received no calcium supplement. It is possible that the thin, almost carbonate-free, shells of snails raised without a calcium supplement have a different biochemical composition than the heavily calcified shells of snails which received a calcium supplement, and that this compositional difference results in the difference in the $\Delta_{COMPONENT-DIET}$ values of the insoluble organic fraction isolated from heavily calcified and poorly calcified shells. In any case, the heavily calcified shells are identical in appearance to the shells of Helix specimens grown in nature. The $\Delta_{COMPONENT-DIET}$ values determined for the insoluble organic fraction of shells of animals whose diets were supplemented with $CaCO_3$ or $Ca_3(PO_4)_2$ should be used in determining the $\delta^{13}C$ value of the diet of a terrestrial snail that grew in nature.

3.8. Dietary analysis by $\delta^{13}C$ determination of animal carbon.

3.8.1. Determining the $\delta^{13}C$ value of the diet from the $\delta^{13}C$ value of extant animal carbon. The isotopic composition of an animal's diet can be determined from the $\delta^{13}C$ value of animal carbon by the application of correction factors which take into account isotope effects during the incorporation of diet carbon into the animal. The $\delta^{13}C$ value of a whole animal was found to be 0.8 ± 1.0 ‰ more positive than its diet $\delta^{13}C$ value (Section 3.2), so that 1 ‰ should be subtracted from the $\delta^{13}C$ value of an animal's total carbon in order to estimate the $\delta^{13}C$ value of its diet. This diet $\delta^{13}C$ value is only accurate to 2 ‰, however, because the $\delta^{13}C$ values of individuals of a single species raised on the same diet can vary by that amount. If it is impractical to determine the $\delta^{13}C$ value of the whole animal, the $\delta^{13}C$ values of as many tissues as possible should be determined, and an average of these values taken for the $\delta^{13}C$ value of the diet. Because of the variability in the relationship between the $\delta^{13}C$ values of tissues and the $\delta^{13}C$ values of the diet for mice raised on several diets (Section 3.6), the $\delta^{13}C$ values of the diet estimated in this fashion is only accurate to within ±2 ‰.

Analysis of several specimens which are thought to have eaten the same diet should reduce the uncertainty in the diet $\delta^{13}C$ value resulting from individual variations.

The accuracy of the estimated $\delta^{13}C$ value for the diet apparently cannot be improved by determining the $\delta^{13}C$ values of the major biochemical fractions of the animal instead of the $\delta^{13}C$ value of the

whole animal, because, as was shown in Section 3.5, the isotopic relationships between these fractions in the animal and in its diet are generally more variable than the isotopic relationship between the total organic matter of the animal and the diet.

3.8.2. Determining the $\delta^{13}C$ value of the diet from the $\delta^{13}C$ value of fossil animal carbon. If the $\delta^{13}C$ value of fossil organic matter originally incorporated in an animal is to be related to the $\delta^{13}C$ value of the animal's diet, it is necessary to demonstrate that there has been no diagenetic alteration of the isotopic composition of the animal carbon. One method of avoiding possible effects of isotopic fractionation during diagenesis is the analysis of organic compounds which retain their integrity as fossils. There are several compounds with widespread fossil occurrence which have been shown to satisfy this requirement. Chitin and collagen are preserved without alteration of the chemical structure as fossil material extending back 400 m.y. (million years) (Rosenheim, 1905) and 350 m.y. (Isaacs et al., 1963) respectively. The organic fraction of a clam shell has been shown to contain inact proteins in specimens 80 m.y. old (Weiner et al., 1976), which suggests that this material might retain its original $\delta^{13}C$ value.

If it can be shown that there has been no diagenetic alteration of isotopic composition, $\delta^{13}C$ values of organic matter from animal fossil material can be related to the $\delta^{13}C$ values of the diet by the application of the appropriate correction factor, several of which have been determined (see Section 3.7). The $\delta^{13}C$ value of collagen is 3-4 ‰ more positive than the $\delta^{13}C$ value of the diet, with a difference

of only 0.1 ‰ encountered for collagen samples from several mice raised on a single diet. Therefore, 3.5 ‰ should be subtracted from the $\delta^{13}C$ value of collagen samples to determine the $\delta^{13}C$ value of the diet of the animal from which the collagen was isolated. The error in the inferred diet $\delta^{13}C$ value is presently estimated to be 0.5 ‰, but this estimate is probably low, since the correction factor is based on data from mice raised on only two different diets. Similar considerations for the insoluble organic fraction of terrestrial snail shells indicate that 1 ‰ should be subtracted from the $\delta^{13}C$ value of the fossil material to determine the $\delta^{13}C$ value of the diet; the estimated error is ±1 ‰. The correction factor to be subtracted from the $\delta^{13}C$ value of chitin samples is 1‰, but the considerable range in $\delta^{13}C$ values of chitin isolated from individuals of a single species raised on the same diet, coupled with the variability in the relationship between the $\delta^{13}C$ values of chitin and the $\delta^{13}C$ value of the diet for the same species raised on different diets, contribute to a large uncertainty in the estimated $\delta^{13}C$ value of the diet, on the order of ±3‰ .

Correction factors can be estimated for those organic components for which the relationship between the isotopic composition of the component and of the diet has not been experimentally established. Consideration of the distribution of ^{13}C in the major biochemical fractions of animals (Section 3.5 and Section 5.1) indicates that the $\Delta_{COMPONENT-DIET}$ values ($\delta^{13}C_{COMPONENT} - \delta^{13}C_{DIET}$) will be most positive for components rich in protein, positive but to a lesser degree for components with a large concentration of carbohydrate, and negative for lipid-rich components. Approximate correction factors can be

derived from these relationships, but the large uncertainties in such estimates, probably on the order of ±3-5 ‰, make it advisable to determine the $\Delta_{COMPONENT-DIET}$ value for the component of interest in laboratory-reared animals before attempting a fossil application.

The question arises at this point as to whether the $\delta^{13}C$ values of biogenically precipitated inorganic phases, specifically the carbonate fraction of invertebrate shells and of vertebrate bones, can be used to estimate the $\delta^{13}C$ value of the diet. The $\delta^{13}C$ values of some invertebrate shell carbonates are known to reflect the $\delta^{13}C$ values of carbon sources other than the diet and hence cannot be used for this purpose. The source of carbon in the shell carbonate of marine and freshwater mollusks is CO_2 dissolved in the water (Keith et al., 1964; Fritz and Poplawski, 1974); aquatic carbonate-depositing organisms from other phyla probably derive their carbon for shell formation from the same source.

The source of carbon for the shell carbonate of terrestrial snails is not known. Atmospheric CO_2 may make a small contribution, primarily through uptake of CO_2 dissolved in the water ingested by the snail. Carbon from solid $CaCO_3$ ingested along with the diet is known to be incorporated into the shell (Rubin et al., 1963), although this is also probably a minor source of carbon for the shell carbonate of snails living in the field, especially in environments with acidic soils (Crowell, 1973). The third and probably the major source of carbon for shell carbonate is the diet, presumably via metabolic CO_2 dissolved in the blood and in the extrapallial fluid from which the

shell is precipitated. Experiments designed to determine the dietary contribution to the shell carbonate could not be carried out, since it proved impossible to raise Helix on diets of different $\delta^{13}C$ values. However, even if it can be shown that there is a dietary influence on the isotopic composition of terrestrial snail shell carbonate, the prospects for using this value for estimating the $\delta^{13}C$ value of the diet appear dim, since relatively small contributions of carbon from atmospheric CO_2 or ingested $CaCO_3$, both of which have much more positive $\delta^{13}C$ values than diet sources, would result in a sizable shift in the $\delta^{13}C$ value of the shell carbonate away from the value determined by the diet.

The source of carbon in the carbonate fraction of vertebrate bones has not been established. However, even if it can be shown that there is a dietary contribution, the available evidence suggests that the $\delta^{13}C$ value of fossil bones cannot be used for the purposes of dietary analysis. Dating of bone samples, whose age has been independently established, from the ^{14}C content of the carbonate fraction has been shown to produce erroneous ages, presumably because carbon in the carbonate carbon is exchangeable with either or both CO_2 dissolved in groundwater (Olson, 1963) and atmospheric CO_2 (Tamers and Pearson, 1965). Thus, the original $\delta^{13}C$ value of bone carbonate is not likely to be preserved in the fossil. However, the amount of exchange which would affect the ^{14}C content of the carbonate is much smaller than that which would be necessary to significantly alter the ^{13}C content. Therefore, the possibility of using the $\delta^{13}C$ value of the bone carbonate to estimate the isotopic composition of a vertebrate

diet merits further investigation.

3.8.3. Potential applications of the method. The methods commonly used in determining what an animal eats involve either visual observation or analysis of undigested food fragments in the feces. These methods are time consuming and subject to considerably error, the first due to observer fatigue and the second due to differential digestibility of different foods. The first method is not applicable to fossil situations, while the second can sometimes be applied when the feces are preserved.

The method of dietary analysis by $\delta^{13}C$ determination of animal carbon provides a considerable improvement over these methods in some modern and fossil situations. The relative contribution of potential diet sources, either plant or animal, can be determined if the difference in $\delta^{13}C$ values of the diet sources are somewhat larger than the limits of accuracy on the $\delta^{13}C$ value of the diet as estimated from the animal $\delta^{13}C$ value. In many situations, the small range and overlap of the $\delta^{13}C$ values of potential diet sources would prevent the determination of the contribution of each source to an animal's diet. For example, five algae in a salt marsh had $\delta^{13}C$ values which range from -16.6 ‰ to -22.7 ‰ (Smith and Epstein, 1970). The relative amounts of each alga eaten by an herbivore subsisting solely on algae could not be determined from the $\delta^{13}C$ value of the animal's carbon. However, even in this case, some qualitative information concerning the consumption of the algae with the highest and lowest $\delta^{13}C$ values might be obtained.

In two cases, the potential diet sources come from groups which

differ sufficiently in $\delta^{13}C$ value so that the relative contribution of each group to the diet can probably be determined by analyzing the $\delta^{13}C$ value of the animal or one of its components. The first of these involves terrestrial vs. aquatic organisms as diet sources. The $\delta^{13}C$ value of the aquatic plants and animals rarely overlap those of terrestrial organisms (see Section 1.3.3.). Thus it should be possible to use this difference to determine the relative contribution of these organisms to the diets of animals living in near-shore environments. The second case involves C_3 vs. C_4 plants as potential diet sources. The $\delta^{13}C$ value of most C_3 plants range from -23 ‰ to -35 ‰, while most C_4 plants have $\delta^{13}C$ values which lie between -9 ‰ and -19 ‰ (see Section 1.3.3.1.). This difference is large enough so that the relative amounts of C_3 and C_4 plants eaten by an animal probably can be determined from the $\delta^{13}C$ value of its carbon.

4. SUMMARY

The distribution of stable carbon isotopes in animals has been largely ignored in previous investigations relating to the biogeochemistry of carbon isotopes. Application of stable isotope methodology to problems of extant and fossil animal biochemistry, physiology, and ecology requires an understanding of the factors affecting natural variations of the $^{13}C/^{12}C$ ratio in animals at the organismic and suborganismic levels. This study represents the beginning of a systematic investigation of these factors.

Eleven species of animals were each raised on one or more diets of constant $\delta^{13}C$ value. A total of thirteen diets were used. In some cases, an animal species was raised on two separate diets, and in other cases two animal species were raised on the same diet.

4.1. Principal findings.

1) The $\delta^{13}C$ value of an animal reflects the $\delta^{13}C$ value of its diet, but the animal is in most cases enriched in ^{13}C relative to the diet. The average difference between the $\delta^{13}C$ values of whole animals and their diets is $+0.8 \pm 1.0$ ‰.

2) There is appreciable variation in the $\delta^{13}C$ values of individuals of an animal species raised on a single diet. The maximum range observed is 1.8 ‰ for four individuals of a species raised on the same diet.

3) The species of the animal or the nature of the diet has little effect on the isotopic relationship between animal and diet.

4) A carbon isotopic mass balance exists between input (diet) and the sum of the total carbon either incorporated into or excreted and respired from an animal. For three cases, the ^{13}C enrichment of the body relative to the diet is balanced by a ^{13}C depletion of the respired CO_2. This relationship does not hold in all animals, however, since in one other case the respired CO_2 is enriched in ^{13}C relative to the diet.

5) The difference between the $\delta^{13}C$ values of a biochemical fraction such as lipid, glycogen, or soluble protein in an animal and its diet is generally larger than the difference between the $\delta^{13}C$ values of the animal and its diet.

6) The isotopic relationships among the biochemical fractions at one trophic level are preserved in the next trophic level.

7) The difference between the $\delta^{13}C$ values of the tissues of mice and the diet of the mouse varies depending on the tissue being analyzed and the diet.

8) The $\delta^{13}C$ values of collagen, chitin, and the insoluble organic fraction of terrestrial snail shells reflect the $\delta^{13}C$ value of the diet.

4.2. Conclusions.

The immediate application of the results of these studies is the proposal for a new method of dietary analysis based on the $\delta^{13}C$ value of animal carbon. The $\delta^{13}C$ value of the diet is first estimated from the $\delta^{13}C$ value of the animal or one of its components by the use of the isotopic relationships which were determined. The relative

contribution of diet sources of known isotopic composition are then calculated from this diet $\delta^{13}C$ value. The limits of accuracy on the estimated $\delta^{13}C$ value of the diet are such that the relative contributions to the diet of aquatic vs. terrestrial organisms or of C_3 vs. C_4 plants should be able to be determined from the $\delta^{13}C$ value of the animal.

II. MECHANISM OF CARBON ISOTOPE FRACTIONATION ASSOCIATED WITH LIPID SYNTHESIS

5. INTRODUCTION AND REVIEW OF THE LITERATURE

5.1. Distribution of carbon isotopes in the major biochemical fractions of organisms

Data from the first studies of the distribution of carbon isotopes at the suborganismic level (Abelson and Hoering, 1961; Park and Epstein, 1961) are plotted in Figure 5-1 as $\Delta_{COMPONENT}$ values ($\delta^{13}C_{COMPONENT} - \delta^{13}C_{WHOLE\ ORGANISM}$). A negative $\Delta_{COMPONENT}$ value indicates a ^{13}C depletion of the component relative to the $\delta^{13}C$ value of the whole organism. The Δ_{LIPID} values of all the lipid fractions are negative, ranging from -1.9 ‰ to -8.6 ‰. The $\Delta_{PROTEIN}$ values for the total amino acid (protein) fractions are positive, ranging from +0.1 ‰ to +3.9 ‰. The $\Delta_{CARBOHYDRATE}$ values for the limited number of carbohydrates are also positive, ranging from +0.1 ‰ to +1.6 ‰. The main feature of the distribution of carbon isotopes in these organisms is a depletion of ^{13}C in the lipid fraction relative to the $\delta^{13}C$ values of the whole organism and the other two major biochemical fractions, protein and carbohydrate. Park and Epstein (1961) also showed that there was a negative correlation between the dry weight percent of lipid in a plant species and the Δ'_{LIPID} value ($\delta^{13}C_{LIPID} - \delta^{13}C_{CHCl_3\text{-}EXTRACTED\ RESIDUE}$) for that species.

A similar pattern of carbon isotope distribution among the major biochemical fractions has been observed in nearly all organisms which have been analyzed. The $\delta^{13}C$ values of a number of organisms* and their

*The data for corn-fed pork and Calliphora and Musca raised on it are not included in Table 5-1 and the following discussion for reasons discussed in Section 3.5.

Figure 5-1. Carbon isotopic composition of the major biochemical fractions of organisms analyzed in the first studies of suborganismic distribution of carbon isotopes. The data are plotted as $\Delta_{COMPONENT}$ values ($\delta^{13}C_{COMPONENT} - \delta^{13}C_{WHOLE\ ORGANISM}$). The references are [1](Abelson and Hoering, 1961) and [2](Park and Epstein, 1961)

AUTOTROPHS (grown on CO_2)

Lycopersicon esculentum, series AP[2]

Lycopersicon esculentum, series BP[2]

Asplenium bulbiferum[2]

Triticum vulgare[2]

Sequoia gigantea[2]

Solanum tuberosum[2]

Ulva sp[2]

Gelidium sp[2]

Macrocystis pyrifera[2]

Mixed phytoplankton[2]

Chlorella pyrenoidosa[1]

Anacystis nidulans[1]

Scenedesmus quadricauda[1]

Chromatium sp[1]

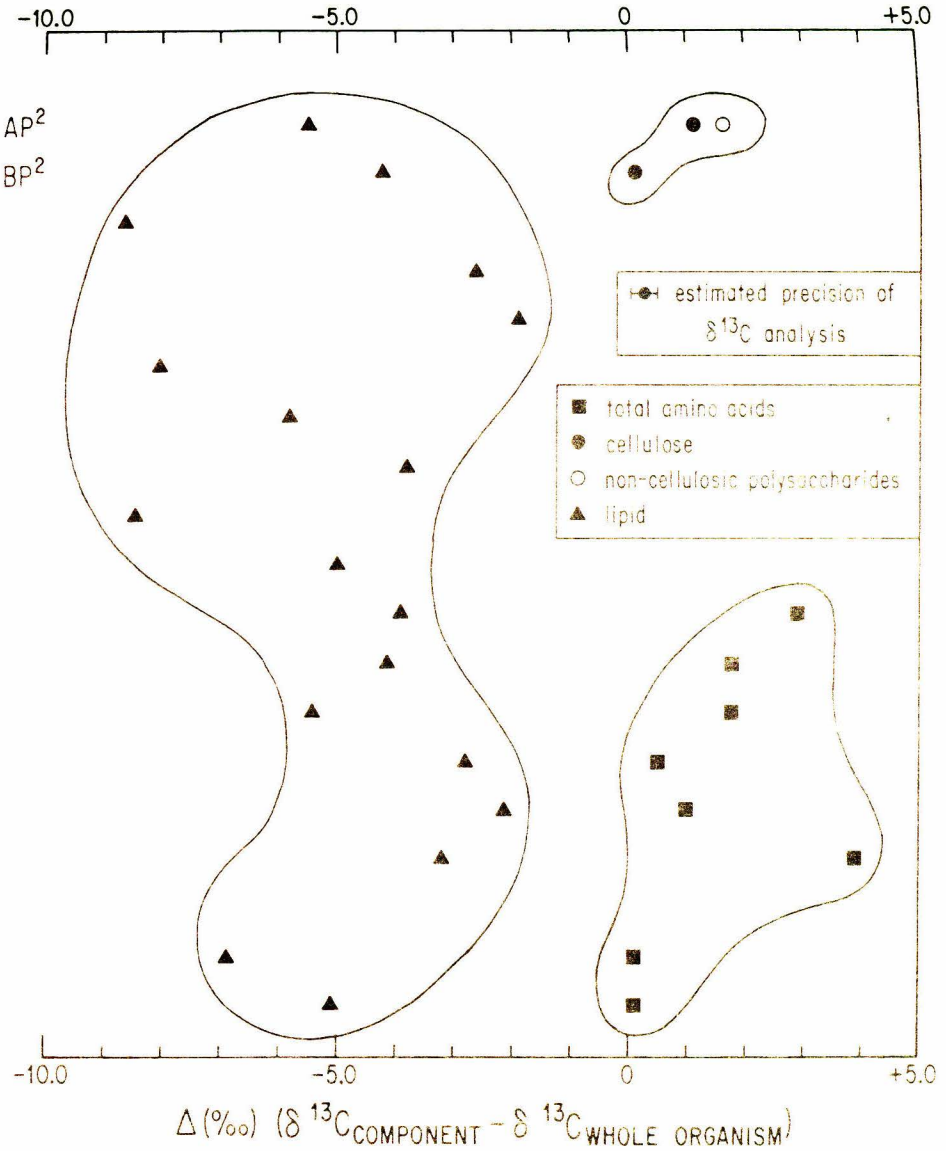
Euglena gracilis[1]

Ochromona danica[1]

HETEROTROPHS (grown on glucose)

Chlorella pyrenoidosa[1]

Escherichia coli[1]



protein, carbohydrate and lipid fractions are listed in Table 5-1. No $\delta^{13}C$ values have been published for nucleic acids, the other major biochemical fractions. The organisms in the table are grouped according to the mode of energy production they employ, either autotrophy (i.e., photosynthesis for all the organisms in the table under this classification) or heterotrophy (i.e. absorption or ingestion of reduced carbon compounds). Plankton samples are presented separately, since they represent mixtures of autotrophic and heterotrophic organisms. The autotrophs and heterotrophs are divided into unicellular and multicellular organisms, with the unicellular organisms further subdivided into prokaryotes (evolutionarily more primitive organisms lacking a nucleus) and eukaryotes (nucleated cells).

The Δ_{LIPID} values in all but one case (73. Leiostomas xanthuras) in Table 5-1 are negative. In all organisms which have been analyzed the $\delta^{13}C$ values of the lipid fraction is more negative than the $\delta^{13}C$ values of the protein or carbohydrate fractions. The $\delta^{13}C$ values of the carbohydrate fractions is generally within 1.5 ‰ of the $\delta^{13}C$ value of the whole organism, and the $\delta^{13}C$ value of the protein fraction is generally slightly more positive than that of the carbohydrate fraction. These relationships do not appear to be influenced by the manner in which an organism gathers its food or by the organizational complexity of the organism. The overriding feature in the distribution of carbon isotopes in all organisms is the ^{13}C depletion of the lipid fraction relative to the $\delta^{13}C$ value of the whole organism and the $\delta^{13}C$ values of the protein and carbohydrate fractions.

Table 5-1. Carbon isotopic composition of whole organisms and their protein, carbohydrate, and lipid fractions. The standard is the PDB carbonate, except as noted in the REFERENCE column.

ORGANISM	REFERENCE	$\delta^{13}C$ (‰)			
		WHOLE ORGANISM	PROTEIN	CARBOHYDRATE	LIPID
I. AUTOTROPHS					
I.a. Unicellular organisms					
I.a.i. Prokaryotes					
1. Agmenellum quadruplicatum	3[a]	-15.9			-21.5
2. Anacystis nidulans	3[a]	-13.1			-16.8
3. Anacystis nidulans	1[a]	-14.8	-13.0[c]		-18.9
4. Coccochloris elebens	3[a]	-12.3			-16.1
5. Oscillatoria willamsi	3[a]	- 5.0			- 8.5
6. Trichodesmium erythaeum	3[b]	-14.6			-20.6
7. Texas blue-green algal mat	3[b]	-14.9			-18.4
8. Chromatium sp.	1[a]	-17.8	-17.3[c]		-20.6
I.a.ii. Eukaryotes					
9. Chorella pyrenoidosa	10	-36.			-41.
10. Chorella pyrenoidosa	1[a]	-18.9	-16.0[c]		-22.8
11. Scenedesmus quadricada	1[a]	-11.9	-10.1[c]		-17.3
12. Euglena gracilis	1[a]	-16.4	-15.4[c]		-18.5
13. Ochromonas danica	1[a]	-19.5	-15.6[c]		-22.7

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ORGANISM		REFERENCE	$\delta^{13}C$ (‰)			
			WHOLE ORGANISM	PROTEIN	CARBOHYDRATE	LIPID
I.b.	Multicellular organisms					
I.b.i.	Algae					
14.	Ulva sp.	9	-15.7			-23.5
15.	Ulva sp.	10	-20.			-27.
16.	Gelidium sp.	9	-20.5			-24.3
17.	Macrocystis pyrifera	9	-12.6			-21.0
18.	Macrocystis pyrifera	14	-17.5			-24.0
19.	Laurencia poitei	10	-11.9			-27.
20.	Laurencia obtusa	10	-15.			-20.
21.	Diagenia simplex	10	-18.			-18.6
22.	Enteromorpha marginata	14	-16.6			-22.9
23.	Corallina chilense	14	-18.6			-24.9
24.	Grateloupia setchellii	14	-22.7			-25.2
25.	Gigartina cristata	14	-20.2			-27.2

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ORGANISM	REFERENCE	$\delta^{13}C$ (‰)			
		WHOLE ORGANISM	PROTEIN	CARBOHYDRATE	LIPID
I.b.ii. Higher plants					
26. Solanum tuberosum	7, 12	-25.8	-26.6[d] -27.1[c]	-23.8[f] -25.5[g]	-34.6
27. Solanum tuberosum	9	-25.6			-33.6
28. Lycopersicon esculentum	8, 9	-24.7		-23.6[h] -23.1[i]	-30.2
29. Triticum vulgare	9	-28.5			-31.1
30. Sequoia gigantea	9	-26.4			-28.3
31. Asplenium bulbiferum	9	-29.2			-37.9
32. Thalassia testudinum	10	-10.2			-18.6
33. Diplanthera wrightii	10	-10.6			-18.
34. Ruppia maritima	10	-10.8			-14.
35. Panicum maximum	13	-12.3	-13.9[c]	-13.9[g]	
36. Zostera marina	14	-10.0			-14.4
37. Monanthochloe littoralis	14	-15.3			-22.2
38. Distichlis spicata	14	-14.7			-23.2
39. Phyllospadix torreyi	14	-14.0			-25.5
40. Limonium commune	14	-23.2			-28.3
41. Salicornia bigelovii	14	-25.2			-29.8
42. Mesembryanthemum chilense	14	-23.6			-30.1
43. Frankenia grandifolia	14	-26.4			-31.1
44. Suaeda fruticosa	14	-26.5			-32.8

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LOCATION OF COLLECTION		REFERENCE	$\delta^{13}C$ (‰)			
			WHOLE SAMPLE	PROTEIN	CARBOHYDRATE	LIPID
II.	PLANKTON					
II.a.	Sample mostly phytoplankton					
45.	49°N 123°W Saanich Inlet British Columbia	2	-19.7	-15.8[c]		-22.0
46	12°S 77°W Pacific Ocean	4	-19.8	-16.1[c]	-20.2[h] -19.6[f]	-37.4
47.	0°S 92°W Pacific Ocean	4	-20.2		-23.2[h]	-31.5
48.	2°N 92°W Pacific Ocean	4	-20.4	-19.1[c]	-23.6[h] -19.2[f]	-30.5
49.	Location not specified Pacific Ocean (?)	9	- 9.2			-14.2
50.	61°07'S 40°08'W Atlantic Ocean	11	-28.3			-31.7

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LOCATION OF COLLECTION		REFERENCE	$\delta^{13}C$ (‰)			
			WHOLE SAMPLE	PROTEIN	CARBOHYDRATE	LIPID
11.b.	Sample mixture of phyto- and zooplankton					
51.	12°S 77°W Pacific Ocean	4	-21.2	-17.9[c]	-20.4[h]_f -19.0[f]	-23.0
52.	2°S 88°W Pacific Ocean	4	-19.9	-18.0[c]	-21.5[h]_f -18.4[f]	-23.5
53.	2°S 85°W Pacific Ocean	4	-20.1	-18.8[c]	-26.7[h]_f -21.2[f]	-34.6
54.	64°58'S 52°01'W Atlantic Ocean	11	-30.6			-33.0
11.c.	Sample mostly zooplankton					
55.	6°S 82°W Pacific Ocean	4	-20.2	-16.5[c]	-21.6[h]_f -18.1[f]	-28.3

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ORGANISM	REFERENCE	$\delta^{13}C$ (‰)			
		WHOLE ORGANISM	PROTEIN	CARBOHYDRATE	LIPID
III. HETEROTROPHS					
III.a. Unicellular organisms					
III.a.i. Prokaryotes					
56. Escherichia coli	1[k]	- 1.9	- 1.8[c]		- 7.0
57. Escherichia coli	5	-11.3	-12.6		-16.0
III.a.ii. Eukaryotes					
58. Saccharomyces carlsbergenesis	5	-23.3	-23.3[d]	-23.0[j]	-24.6
59. Chlorella pyrenoidosa	1[k]	- 0.1	0.0[c]		- 7.0
III.b. Multicellular organisms					
III.b.i. Organs from mice and rats					
60. Rat liver	6			-18.7[j]	-23.3
61. Mouse spleen	5	-19.2	-19.1[e]		-22.6
62a. Mouse kidney, left	5	-20.7	-18.4[e]		-22.8
62b. Mouse kidney, right	5	-20.7	-18.7[e]		-22.9
63. Mouse liver	5	-20.6	-18.8[e]		-23.2
64. Mouse brain	5	-18.9	-16.6[e]		-19.0

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ORGANISM	REFERENCE	$\delta^{13}C$ (‰)			
		WHOLE ORGANISM	PROTEIN	CARBOHYDRATE	LIPID
III.b.ii. Whole animal or unspecified part of animal					
65. Paleomonetes vulgaris	10	-15.			-20.
66. Penaeus aztecus	10	-12.			-15.
67. Uca pugilator	10	-14.			-19.
68. Callinectes sapidus	10	-18.			-20.
69. Cyprinodon variegatus	10	-10.4			-12.6
70. Baindiella chrysura	10	-12.			-14.
71. Mugil cephalus	10	-13.			-15.
72. Lagodon rhomboides	10	-14.			-17.
73. Leiostomas xanthuras	10	-14.			-14.
74. Cypraea spadicea	14	-19.3			-25.5
75. Navanax inermis	14	-14.0			-22.2
76. Mytilus californianus	14	-17.3			-22.9
77. Dendroaster excentricus	14	- 8.6			-18.6
78. Bulla gouldiana	14	- 9.1			-17.0
79. Cerithidia californica	14	-13.4			-21.6
80. Equus caballus	5	-23.9	-23.6[d]	-24.1[j]	-28.5
81. Musca domestica	5	-23.4	-22.3[d]	-23.0[j]	-25.6
82. Calliphora vicina	5	-24.1	-23.0[d]	-23.5[j]	-26.5
83. Helix aspersa	5	-25.2	-24.6[e]		-29.4
84. Oncopeltus fasciatus	5	-26.6	-24.7[d]		-28.6
85. Protoparce quinquemaculata	5	-29.0	-26.6[d]		-34.8

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Footnotes:

[a]Relative to tank CO_2, which was the carbon source

[b]Relative to oceanic CO_2, which was the carbon source

[c]Total amino acids

[d]Soluble protein

[e]Total protein

[f]Total sugars

[g]Starch

[h]Cellulose

[i]Non-cellulosic polysaccharides

[j]Glycogen

[k]Relative to glucose, which was the carbon source

References:

1. Abelson and Hoering, 1961.
2. Brown et al., 1972
3. Calder and Parker, 1973.
4. Degens et al., 1968.
5. DeNiro, this work.
6. Jacobson et al., 1972.
7. Jacobson et al., 1970.
8. Park and Epstein, 1960.
9. Park and Epstein, 1961
10. Parker, 1964.
11. Sackett et al., 1965.
12. Smith, 1972.
13. Smith and Benedict, 1974.
14. Smith and Epstein, 1970.

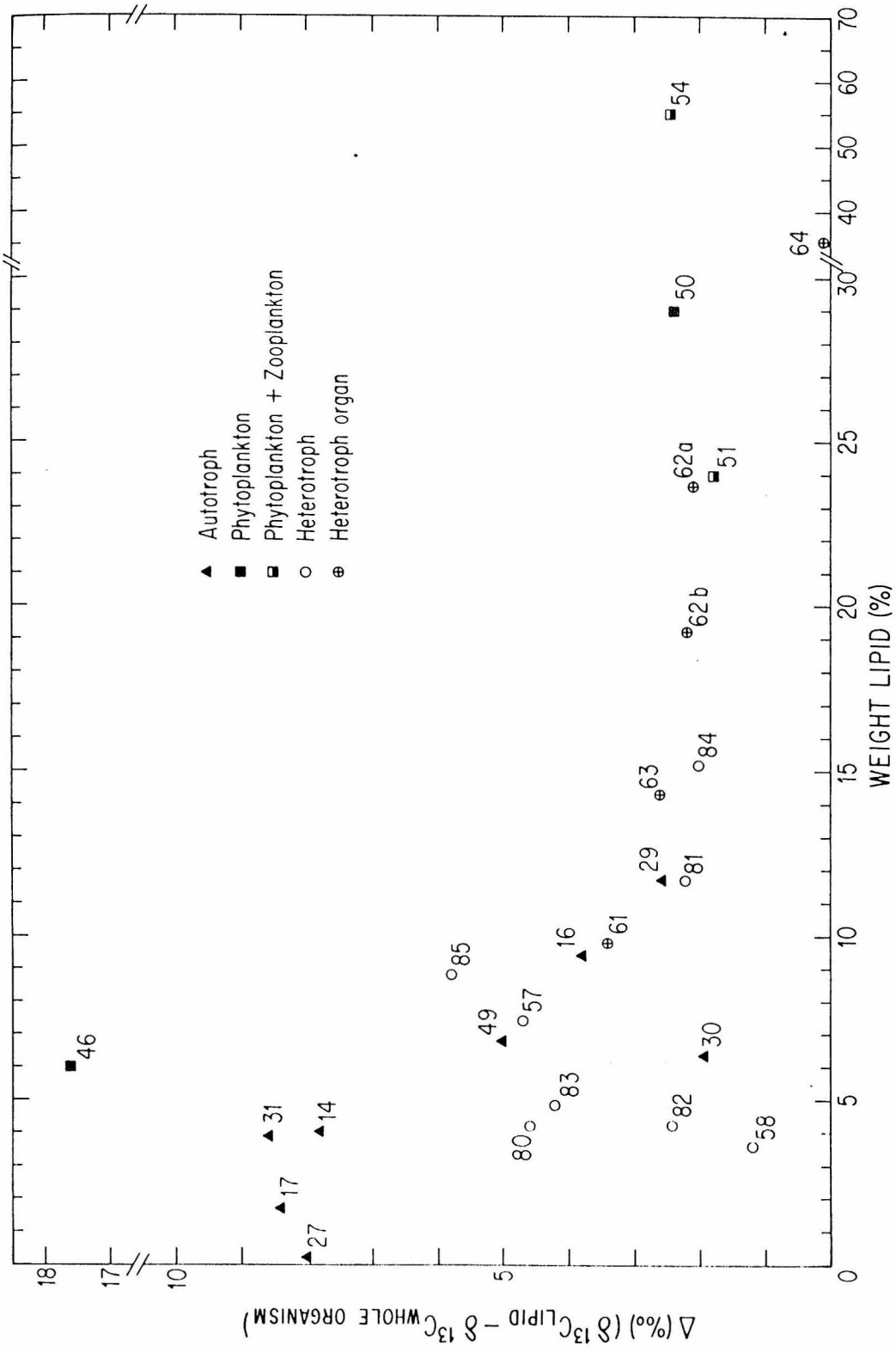
5.2. Relationship between the amount of lipid in an organism and the difference between the $\delta^{13}C$ values of the whole organism and the lipid.

An organisms's dry weight percent of lipid is compared with its Δ_{LIPID} value ($\delta^{13}C_{LIPID} - \delta^{13}C_{WHOLE\ ORGANISM}$) in Figure 5-2. There appears to be a negative correlation between the amount of lipid in an organism and the ^{13}C depletion of the lipid fraction. The data for autotrophs show this relationship more clearly than that for heterotrophs. The lipid fraction of a heterotroph contains components which are assimilated directly from the diet, as well as components synthesized de novo by the heterotroph. Since the $\delta^{13}C$ values of the assimilated lipid components are a function of the metabolic processes of the diet organisms and not of the heterotroph, it is not unexpected that the relationship between weight percent lipid and Δ_{LIPID} value is less well defined in heterotrophs than in autotrophs.

5.3. Isotopic composition of lipid fraction components isolated from a single organism.

The lipid fraction is composed of components, which, by definition, are soluble in chloroform or some other non-polar solvent. Any explanation of the basis of the ^{13}C depletion of the lipid fraction must actually explain the ^{13}C depletion of the components, the sum of whose $\delta^{13}C$ values determines the $\delta^{13}C$ value of the lipid fraction. However, since most of the carbon of the lipid fraction derives from a single intermediate, the acetyl group of acetyl CoA, and since the pathways involved in the synthesis of lipid fraction components are similar, it is expected that the components of the lipid fraction will have similar

Figure 5-2. The relationship between the amount of lipid in an organism and the difference between the $\delta^{13}C$ values of the whole organism and of the lipid. The isotopic data are plotted as Δ_{LIPID} values ($\delta^{13}C_{LIPID} - \delta^{13}C_{WHOLE\ ORGANISM}$). The number next to each point identifies the organism according to the code used in Table 5-1.


Autotroph
Phytoplankton
Phytoplankton + Zooplankton
Heterotroph
Heterotroph organ
WEIGHT LIPID (%)
Δ (‰) (δ 13C LIPID − δ 13C WHOLE ORGANISM)
0
5
10
15
20
25
30
40
50
60
70
10
17
18
46
31
14
17
27
85
49
57
16
61
80
83
29
63
84
81
82
30
58
62b
62a
51
54
50
64

$\delta^{13}C$ values.

The data relating to this point are limited. Parker (1964) has analyzed fatty acids, the major components of the lipid fraction, isolated by gas chromatography from three species of plants. His results are shown in Table 5-2. Within the limits of error of the procedure, stated to be ±1‰, the $\delta^{13}C$ values of fatty acids from a single organism generally do not differ from one another. Parker stated that the $\delta^{13}C$ value of the lipid fraction "is essentially that of the fatty acids", although he did not publish the values of the total lipid fractions. The only other data relating to this point has recently been published by Hoering (1975). He isolated general classes of lipid components by column chromatography from four plant species. His results are shown in Table 5-3. The limits of error of his procedure were not stated. The $\delta^{13}C$ values of the saturated and unsaturated fatty acids do not differ from one another and from the $\delta^{13}C$ value of the total saponified lipid fraction by more than 2 ‰, which is consistent with Parker's results. The $\delta^{13}C$ values of three minor lipid components, carotene pigments, sterols and phytol, have a wider range and are generally more positive than those of the fatty acids and hydrocarbons. The basis for this difference may be the significantly different pathways involved in the synthesis of these cyclic lipid components as compared with those involved in the synthesis of the aliphatic fatty acids and hydrocarbons (Richards and Hendrickson, 1964).

5.4. Previous proposals for the basis of the ^{13}C depletion of the lipid fraction.

Any explanation for the basis of the ^{13}C depletion of lipids must

Table 5-2. Carbon isotopic composition of fatty acids isolated from single plant species (Parker, 1964).

SAMPLE	$\delta^{13}C$ (‰)
Chlorella pyrenoidosa	
Total organism	-36
Palmitic acid	-41
Palmitoleic acid	-41
Stearic plus oleic acids	-41
Ulva sp.	
Total organism	-20
Palmitic acid	-27
Stearic acid	-27
Oleic acid	-27
Linoleic acid	-27
Linolenic acid	-27
Thallasia sp.	
Total organism	- 7.8
Palmitic acid	-21
Palmitoleic acid	-23
Stearic acid	-21.6
Oleic acid	-22
Linoleic acid	-22
Linolenic acid	-19

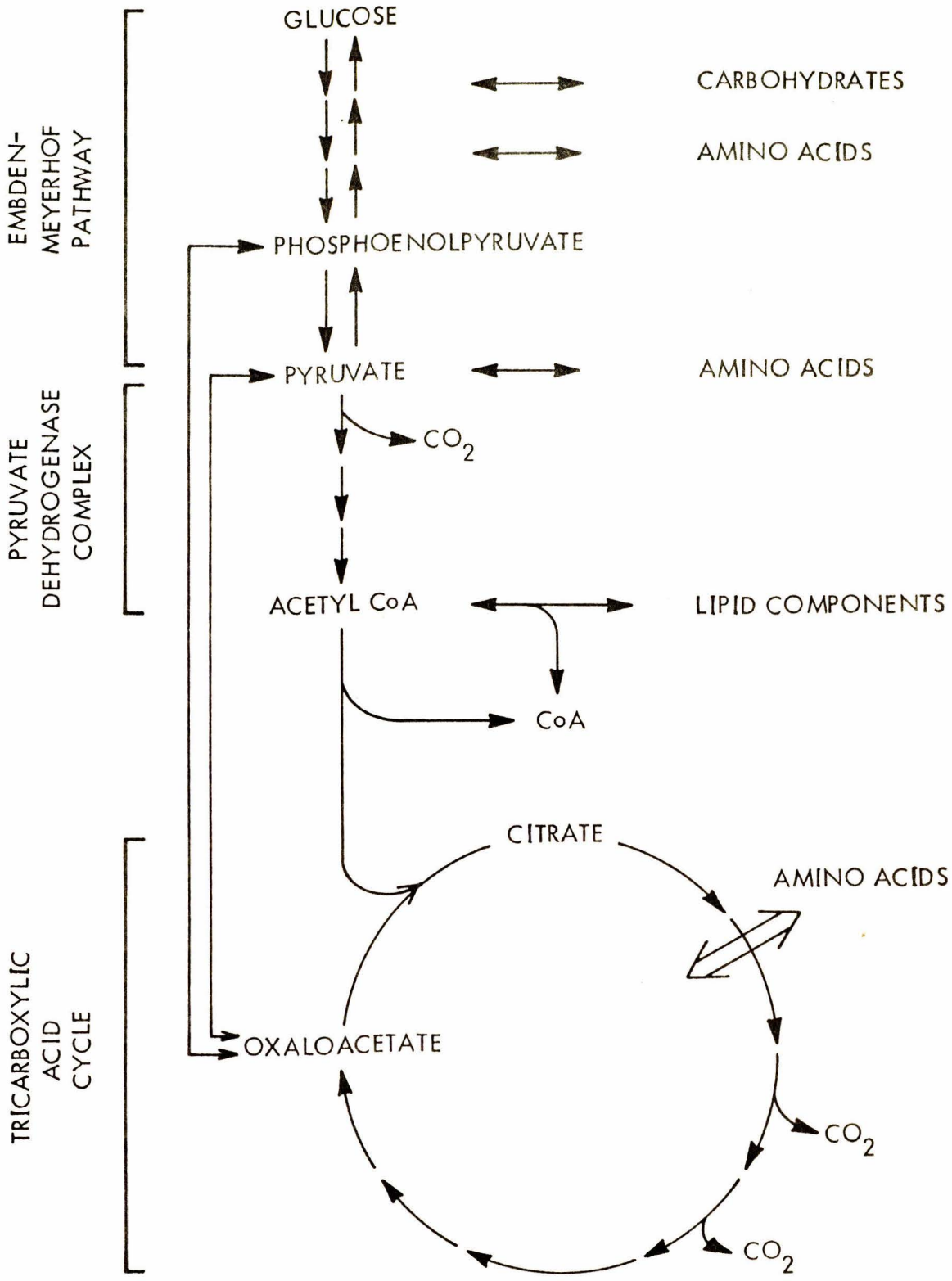
Table 5-3. Carbon isotopic composition of lipid fraction components isolated from single plant species (Hoering, 1975).

COMPONENT	$\delta^{13}C$ (‰)			
	Hypnea cornuta	Ulva fasciata	Gladitsia triacanthus	Spartina patens
Total saponified lipid	-20.3	-21.8	-34.5	-20.0
Saturated fatty acids	-19.9	-23.5	-33.9	-20.7
Unsaturated fatty acids	-19.7	-22.9	-35.7	-19.9
Total nonsaponified lipids	-20.3	n.d.*	-29.8	-17.3
Saturated hydrocarbons	-22.0	-25.6	-33.8	-22.0
Carotene pigments	-21.0	n.d.	n.d.	-17.3
Sterols	n.d.	n.d.	-31.9	-15.6
Phytol	-22.8	-20.9	-30.5	-17.9

*not determined

involve fractionation of the carbon isotopes at some step or steps during metabolism prior to the formation of lipid components. Two models have been proposed to explain this fractionation. An understanding of intermediary metabolism is necessary if these models are to be discussed. A schematic illustration of this topic is shown in Figure 5-3. Both models were presented to explain the ^{13}C depletion of lipids in autotrophs, so that the starting point of these considerations is glucose, which is the end product of photosynthesis. Glucose, a six-carbon molecule, is converted to two three-carbon molecules (pyruvate) by the Embden-Meyerhof pathway. Pyruvate is then oxidatively decarboxylated by the pyruvate dehydrogenase complex to give a two-carbon product, the acetyl group of acetyl CoA. Lipid components are formed primarily via polymerization of the acetyl group of acetyl CoA. Acetyl CoA can also be involved in other reactions, the most important of which is the incorporation of the acetyl group into the tricarboxylic acid (TCA) cycle. The static picture presented here is complicated by the presence of other reactions of the indicated intermediates. These reactions include the synthesis of carbohydrates from Embden-Meyerhof pathway intermediates and the synthesis of amino acids, which derive their carbon atoms from pyruvate, acetyl CoA, and several Embden-Meyerhof and tricarboxylic acid cycle intermediates. An additional set of important reactions are those involved with the anaplerotic replenishment of the tricarboxylic acid cycle from pyruvate and phosphoenolpyruvate, which serves to connect the Embden-Meyerhof pathway and the tricarboxylic acid cycle. Figure 5-3 also indicates that many of these reactions are functionally reversible, although

Figure 5-3. Simplified version of intermediary metabolism, emphasizing the source of carbon atoms for carbohydrate, protein, and lipid component synthesis.


EMBDEN-
MEYERHOF
PATHWAY
GLUCOSE
CARBOHYDRATES
AMINO ACIDS
PHOSPHOENOLPYRUVATE
PYRUVATE
AMINO ACIDS
PYRUVATE
DEHYDROGENASE
COMPLEX
CO_2
ACETYL CoA
LIPID COMPONENTS
CoA
CITRATE
AMINO ACIDS
TRICARBOXYLIC
ACID
CYCLE
OXALOACETATE
CO_2
CO_2

different pathways are often employed in the two directions.

Park and Epstein (1961) suggested that the basis of the ^{13}C depletion of lipids was the selection of ^{12}C-enriched C_2 units (the acetyl group of acetyl CoA) for lipid synthesis and of ^{13}C-enriched C_2 units for incorporation into the tricarboxylic acid cycle. As support, they cited the negative correlation between the ^{13}C depletion of the lipid fraction and the weight percent of lipid in a plant, implying that as a larger fraction of acetyl CoA was directed toward lipid synthesis, the fractionation at the selection point would be decreased. They also presented evidence that the $\delta^{13}C$ value of CO_2 respired by tomato plants has a more positive $\delta^{13}C$ value than that of the whole plant and suggested that the source of this respired CO_2 was ^{13}C-enriched C_2 units incorporated into the tricarboxylic acid cycle.

Ivlev et al. (1974) proposed that the ^{13}C depletion of the lipid fraction results from kinetic isotope effects during the decarboxylation of pyruvate. Abelson and Hoering (1961), using a crude extract of yeast, found that pyruvate with a ^{12}C in the carboxyl carbon atom is decarboxylated 0.2% faster than pyruvate with ^{13}C in that position, meaning that the CO_2 released in the reaction has a $\delta^{13}C$ value 2.0 ‰ less than that of the carboxyl carbon of pyruvate. Ivlev et al. proposed that as a result of this fractionation, which they mistook to be 20.0 ‰, the acetyl CoA formed from pyruvate by the pyruvate dehydrogenase complex would be depleted in ^{13}C relative to unreacted pyruvate, and that lipids synthesized from this acetyl CoA would be depleted in ^{13}C relative to other cell components.

5.5. Overview of work presented in this study.

The present study was undertaken to determine the basis of the ^{13}C depletion of the lipid fraction. Two basic points were established. First, the step during which the isotopic fractionation responsible for the ^{13}C depletion of lipids occurs was identified as the pyruvate dehydrogenase complex reaction; these experiments are discussed in Section 7.1. The kinetic isotope fractionations associated with the reaction of the complex were determined indirectly in a series of *in vitro* experiments with the enzyme pyruvate decarboxylase; these experiments are discussed in Section 7.2 and Section 7.3. Finally, a model for the ^{13}C depletion of the lipid fraction which incorporates these results is presented in Section 7.4.

6. ANALYTICAL TECHINQUES

6.1 Materials.

Sodium pyruvate and pyruvate decarboxylase from brewer's yeast were purchased from Sigma Chemical Company. The enzyme was purified by the method of Ullrich et al. (1966) (Broida, 1976). It was supplied as a crystalline suspension, in 3.2 M $(NH_2)_2SO_4$ at pH 6, which had an activity of 36 units per ml. (One unit of pyruvate decarboxylase converts to 1.0 μ mole of pyruvate to acetaldehyde at 25°C and pH 6.0.) 100% phosphoric acid was prepared by saturating 85% phosphoric acid with phosphorous pentoxide. Carbon monoxide, hydrogen sulfide, and methane were all CP grade and >99 ‰ pure. All other chemicals used were of reagent grade. Triply distilled water was used in all solutions except for bacterial growth experiments, in which commercially distilled water was used in the minimal media.

6.2. Growth of bacteria.

E. coli stain AB 1621 was used in all bacterial growth experiments. The composition of the minimal media is shown in Table 6-1. A single colony from an EHA plate was used to initiate each culture. The cultures were maintained at 25°C with vigorous aeration. Cells were harvested by centrifugation during log phase at 2×10^8 cells $ml.^{-1}$; at this concentration, less than 2% of the available carbon was assimilated into cell matter. The cells were stored at -20°C prior to lyophilization.

6.3. Isolation of lipid fraction.

The lipid fraction was isolated from bacteria by the technique described in Section 2.2.1.

Table 6-1. Composition of minimal media used for the growth of *E. coli*. The inorganic salt formulation is from Kornberg and Sadler (1961).

Inorganic salts	
$CaCl_2 \cdot 2H_2O$	27.0 mg.
$MnCl_2 \cdot 4H_2O$	2.0 mg.
$MgSO_4 \cdot 7H_2O$	40.0 mg.
$FeCl_3 \cdot 6H_2O$	2.1 mg.
K_2HPO_4	5.82 g.
KH_2PO_4	2.26 g.
NH_4Cl	2.68 g.
Vitamins	
thiamine HCl	1.0 mg.
Carbon sources (one of the following)	
glucose (28mM)	5.00 g.
Sodium pyruvate (56mM)	6.16 g.
sodium acetate $\cdot 3H_2O$ (84mM)	11.45 g.
Distilled water	to one liter

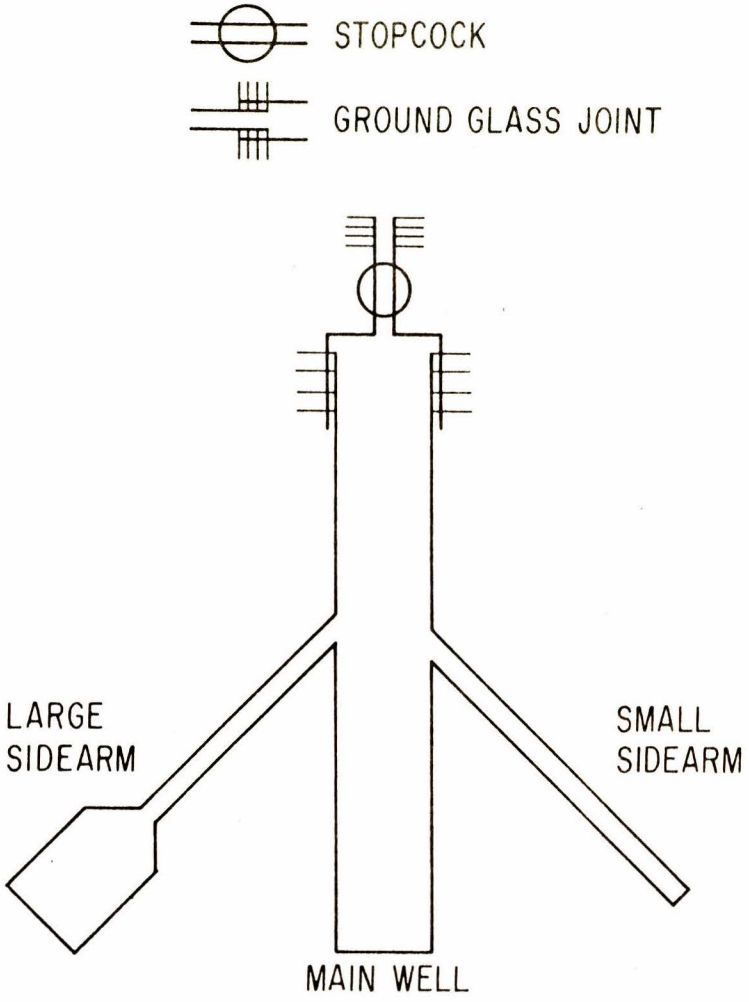
6.4. Preparation of reaction mixtures for the determination of kinetic isotope fractionations of pyruvate decarboxylase.

The preparation of several experimental and control reaction mixtures for the determination of the kinetic isotope fractionation of pyruvate decarboxylase is described in this section. The following abbreviations are used. ERV is an enzyme reaction vessel, a flask with two sidearms which can be connected to the vacuum line, shown in Figure 6-1. Dry ice-M17 is a slurry of powdered dry ice and M17, a commercial organic solvent. H_2O but not CO_2 will condense out at the temperature of the slurry, -80°C. LN_2 is liquid nitrogen. CO_2 will condense out at liquid nitrogen temperature, -180°C. Frozen n-pentane is a slurry of melting n-pentane, originally frozen by adding LN_2. Acetaldehyde but not CO_2 will condense out at the temperature of the slurry, -120°C. CO_2-free air is laboratory air scrubbed of CO_2 by passage over Ascarite (NaOH absorbed onto asbestos) and through a $Ba(OH)_2$ bath.

6.4.1. Reaction mixture for the hydrogen peroxide decarboxylation of pyruvate. Sodium pyruvate (from 10-30 mg.) was placed in the main well of an ERV and 3.0 ml. of 30% H_2O_2 was pipetted into the large sidearm. The ERV was capped and attached to the vacuum line. The H_2O_2 solution was frozen by immersing the sidearm in dry ice-M17, the ERV was evacuated, then filled with CO_2-free air. The cap stopcock was closed and the ERV placed in a 25°C water bath and equilibrated. The reaction was begun by tipping the H_2O_2 solution into the main well.

6.4.2. Reaction mixture for the enzymatic reaction with pyruvate. 1.0 ml. of a buffered solution containing pyruvate was pipetted into the

Figure 6-1. Enzyme reaction vessel (ERV). The reduction of diameter in the large sidearm is necessary to prevent a cylinder of frozen solution from being propelled, by the pressure from its own melted water vapor trapped behind it, out of the sidearm and into the main well.


STOPCOCK
GROUND GLASS JOINT
LARGE SIDEARM
SMALL SIDEARM
MAIN WELL

main well of an ERV, 2.0 ml. of the buffered solution containing 30 microliters of the pyruvate decarboxylase suspension was pipetted into the large sidearm, and 3.0 ml. of 100% phosphoric acid was pipetted into the small sidearm. The buffer was 100 mM sodium citrate, pH 6.0, except for the experiments reported in Table 7-12, in which the following buffers were used: pH 5.0, 48 mM citric acid and 103 mM sodium phosphate dibasic; pH 6.0, 37 mM citric acid and 126 mM sodium phosphate dibasic; pH 7.0, 18 mM citric acid and 165 mM sodium phosphate dibasic. The amount of pyruvate present in the reaction mixture was determined to within 0.1 mg. and was on the order of 1 mg. for the 100% enzymatic reaction and 110 mg. for the 1% enzymatic reaction. The ERV was capped and attached to the vacuum line. The large sidearm and the main well were immersed in dry ice-M17, the ERV was evacuated, then filled with CO_2-free air. The cap stopcock was closed and the ERV placed in a water bath and equilibrated. The reaction was begun by tipping the enzyme solution into the main well. The reaction was stopped by adding the phosphoric acid to the main well.

6.4.3. Reaction mixture for control experiments with added CO_2 and acetaldehyde of known isotopic composition. The ERV was set up in the usual fashion for the 1% enzymatic reaction with pyruvate, but after the evacuation step, the main well was immersed in LN_2. Samples of CO_2 and acetaldehyde of known quantity and $\delta^{13}C$ value were condensed into the main well. The ERV was then filled with CO_2-free air. The cap stopcock was closed and the ERV placed in a 25°C water bath and equilibrated. After 3 hours, the phosphoric acid

was added to the enzyme solution, and then this mixture was added to the pyruvate solution.

6.5. Mass spectrometer analysis.

The procedure described in Section 2.3 was followed.

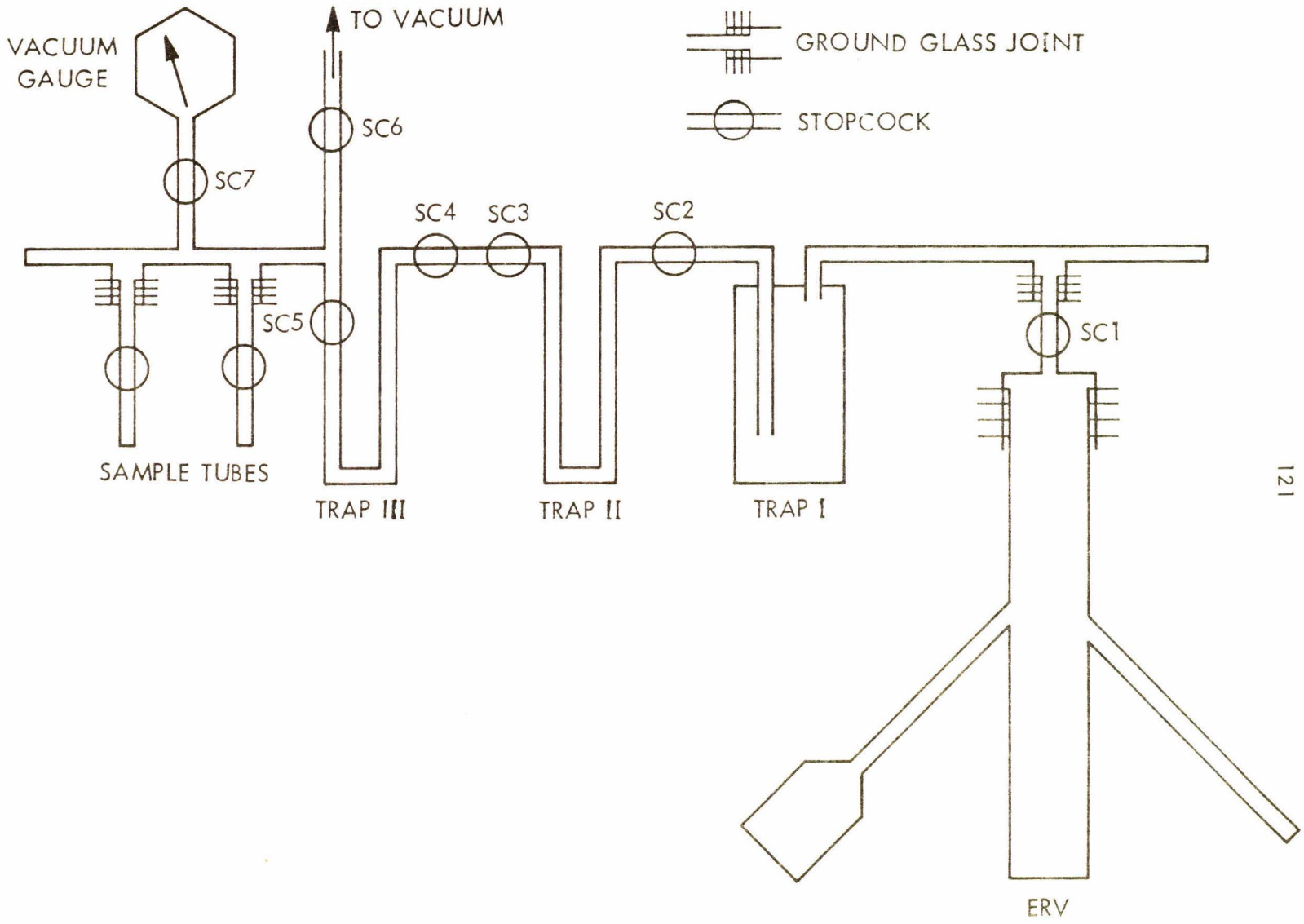
6.6. Carbon dioxide sample purification and yield determination.

All CO_2 samples were purified according to the procedure discussed in Section 2.4. These purification steps do not alter the carbon isotope composition of pure CO_2 samples, but are necessary to insure good traces on the mass spectrometer when impurities are present in the CO_2 samples.

6.7. Preparation of samples for mass spectrometer analysis.

6.7.1. Collection of CO_2 and acetaldehyde from reaction mixtures. CO_2 and acetaldehyde will pass through a trap maintained at dry ice temperature and condense out in a trap at liquid nitrogen temperature; they can be separated from one another with frozen n-pentane, which condenses acetaldehyde but not CO_2. That actual procedure that was used is as follows. The ERV was attached to the line shown in Figure 6-2 and the line was evacuated; SC (stopcocks) 2-6 were then closed. The main well of the ERV was immersed in dry ice-M17, Trap I in dry ice-M17, and Trap II and Trap III in LN_2. SC 1-6 were opened sequentially at one minute intervals. After the line was evacuated, SC 5 was closed and the reaction mixture was distilled out of the ERV by immersing the main well in hot (~80°C) water. The line was evacuated again through SC 6, which was then closed. The LN_2 condensates, all of which were contained in Trap II, were distilled to a sample tube immersed in liquid nitrogen.

Figure 6-2. High vacuum line for collection of CO_2 and acetaldehyde from reaction mixtures.


VACUUM
GAUGE
TO VACUUM
GROUND GLASS JOINT
STOPCOCK
SC6
SC7
SC4
SC3
SC2
SC5
SC1
SAMPLE TUBES
TRAP III
TRAP II
TRAP I
ERV

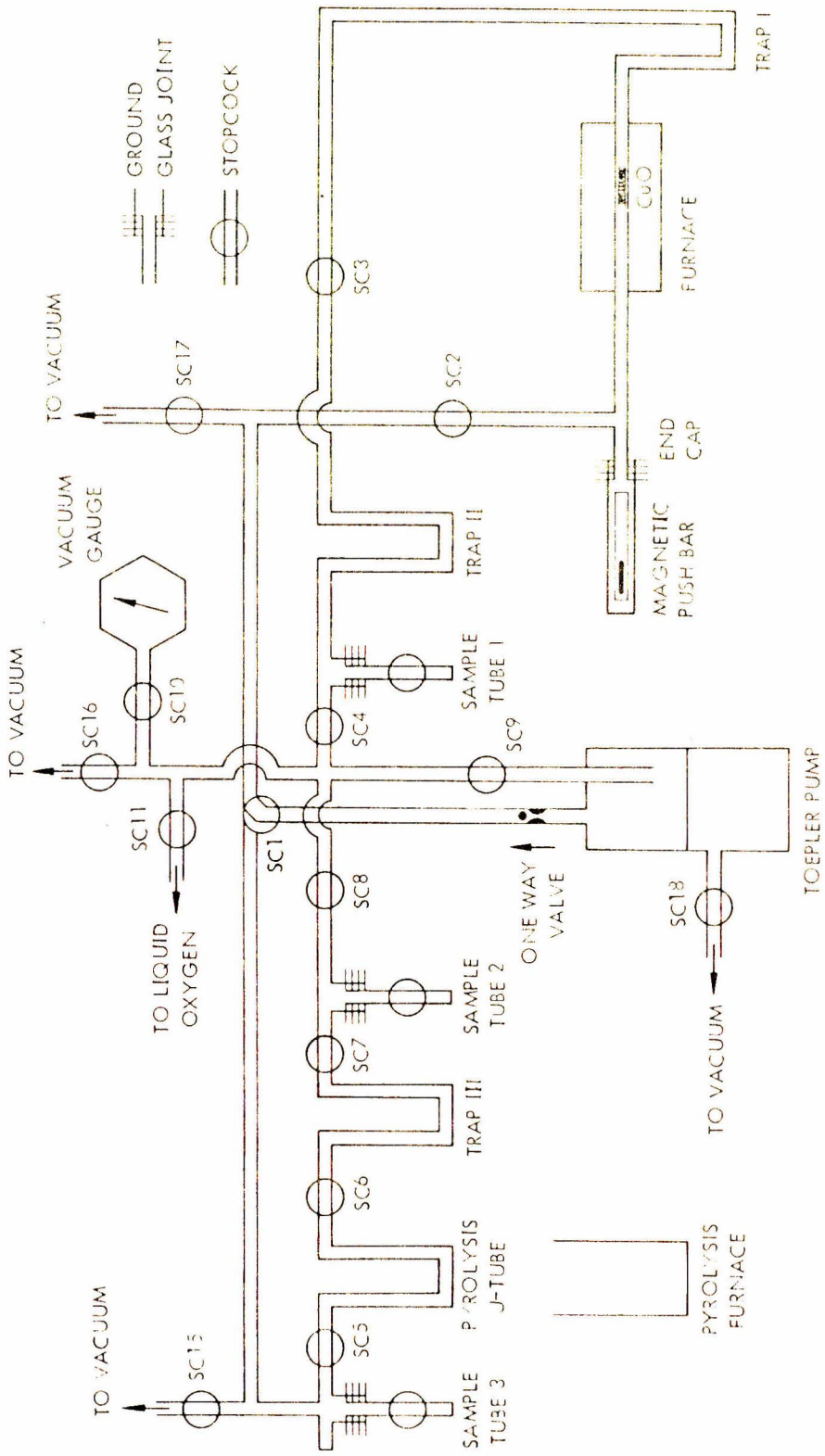
The sample tube was warmed to room temperature and then immersed in frozen n-pentane. Acetaldehyde condensed in this sample tube; CO_2 was condensed into the second sample tube which was immersed in liquid nitrogen. The CO_2 was purified and its volume determined prior to isotopic analysis. The volume of acetaldehyde was determined before further processing.

6.7.2. Combustion of organic samples to CO_2. Glucose, sodium pyruvate, sodium acetate, and the lipid fraction of E. coli were combusted to CO_2 by the procedure described in Section 2.5.3. Certain modifications of this procedure were required in order to combust acetaldehyde. A sample tube containing 6-15 μ moles of acetaldehyde was attached to the line shown in Figure 2-2. After the line was heated and evacuated, the acetaldehyde was condensed into Trap II with LN_2. After oxygen was admitted to the line, the LN_2 was removed from Trap II. The procedure described for the combustion of lipids (Section 2.5.3.) was then followed.

6.7.3. Conversion of each carbon atom of acetaldehyde to CO_2.

6.7.3.1. Pyrolysis of acetaldehyde followed by combustion of the pyrolysis products. Acetaldehyde was pyrolyzed in the line shown in the left half of Figure 6-3; the products of pyrolysis were then separated and combusted in the line shown in the right hand half of Figure 6-3. The pyrolysis was done according to the technique of Roth and Rollefson (1942), the separation and combustion according to the technique of Sakai et al. (1976). A sample tube containing 6-15 μ moles of acetaldehyde was attached at the sample tube 2 position, while a sample

Figure 6-3. High vacuum line for the pyrolysis-combustion of acetaldehyde.


GROUND GLASS JOINT
STOPCOCK
TO VACUUM
SC17
SC3
SC2
TRAP I
CuO
FURNACE
END CAP
MAGNETIC PUSH BAR
VACUUM GAUGE
TRAP II
SAMPLE TUBE I
SC4
SC9
SC16
SC10
TO VACUUM
SC11
SC1
SC8
ONE WAY VALVE
TOEPLER PUMP
TO LIQUID OXYGEN
SAMPLE TUBE 2
SC7
SC18
TRAP III
TO VACUUM
SC6
PYROLYSIS J-TUBE
PYROLYSIS FURNACE
TO VACUUM
SC5
SAMPLE TUBE 3

tube containing 13 μ moles of H_2S was attached at the sample tube 3 position. The line was heated with an annealing flame and evacuated; the stopcocks to the vacuum were then closed. SC 4 was closed and SC 1 was set in the left-hand position. The acetaldehyde and H_2S were condensed to the pyrolysis U-tube with LN_2; SC 5 and SC 6 were then closed. The LN_2 was removed from the pyrolysis U-tube and it was heated at 500°C for 2 hours. Trap III was then immersed in LN_2, SC 5 and SC 6 were opened, the automatic Toepler pump was activated, and the contents of the pyrolysis tube (H_2S, CO, CH_4) were circulated for 10 minutes, with H_2S being condensed out in Trap III. SC 1 was then closed resulting in the non-condensible CO and CH_4 being pumped out of the pyrolysis line by the Toepler pump and being contained between the one-way valve and SC 1. When the vacuum gauge indicated that the pyrolysis line was evacuated, SC 8 was closed and SC 1 was set at its right-hand position, admitting the CO and CH_4 to the combustion line. LN_2 was placed in Trap II. The gases were circulated through the combustion furnace at 425°C without added oxygen; under these conditions, CO is oxidized to CO_2 while CH_4 does not react. After thirty minutes, SC 1 was closed so that CH_4 was pumped out of the line by the Toepler pump and contained between the one-way valve and SC 1. SC 9 was closed when the gauge indicated the line was evacuated. Trap II was immersed in dry ice-M17 and the sublimed CO_2 condensed into sample tube 1 with LN_2. The temperature of the combustion furnace was raised to 850°C and Trap II was immersed in LN_2. SC 9 and SC 1 were opened, admitting the CH_4 to the combustion line. After forty minutes of circulation, during which CH_4 was oxidized to CO_2, the line was evacuated and Trap II was

immersed in dry ice-M17. The sublimed CO_2 was condensed into sample tube 2 with LN_2. The two CO_2 samples were purified and their volumes determined prior to isotopic analysis.

6.7.3.2. Control experiments for the pyrolysis-combustion procedure. Three control experiments were performed in order to determine if the pyrolysis of acetaldehyde and the subsequent combustion of the pyrolysis products introduced any error in the determination of the isotopic composition of each of the carbon atoms of acetaldehyde. These control experiments were performed using the line shown in Figure 6-3. In the first of these, 10-15 μ moles of acetaldehyde of known isotopic composition was subjected to the procedure to determine if the sum of the $\delta^{13}C$ values for the two carbon atoms equaled the $\delta^{13}C$ value of the acetaldehyde. In the second set of control experiments, CO and CH_4 of known isotopic composition were mixed and subjected to the pyrolysis-combustion procedure to determine if there were any isotopic exchange between the two pyrolysis products. A sample tube containing CO was attached at the sample tube 1 position, a sample tube containing CH_4 was attached at the sample tube 2 position, and a sample tube containing H_2S was attached at the sample tube 3 position. The line was heated with an annealing flame and evacuated. SC 3 was closed and SC 1 was set in the left-hand position. The stopcocks to the vacuum were closed. The stopcocks on the sample tubes containing CO, CH_4 and H_2S were opened and the automatic Toepler pump was activated. After ten minutes of circulation, SC 5 and SC 6 were closed. The amounts of CO, CH_4 and H_2S initially present in the sample tubes were such that the

the pyrolysis U-tube now contained similar amounts of three gases as would be present after the pyrolysis of an average size acetaldehyde sample. The rest of the line was then pumped down. The pyrolysis-combustion procedure was then followed, starting with the two hour heating of the pyrolysis U-tube at 500°C.

In the third control experiment, a mixture of acetaldehyde (the $\delta^{13}C$ value of each carbon atom of which had been determined in the first control experiment) and either CO or CH_4 of known isotopic composition was subjected to the pyrolysis-combustion procedure, as a further check to determine if there were any isotopic exchange between the two carbon atoms of acetaldehyde. A sample tube containing CO (or CH_4) was attached at the sample tube 1 position, a sample tube containing 12 μ moles of acetaldehyde was attached at the sample tube 2 position, and a sample tube containing 13 μ moles of H_2S was attached at the sample tube 3 position. The standard procedure was used to introduce the H_2S and acetaldehyde to the pyrolysis U-tube and SC 5 and SC 6 were then closed. SC 1 was closed, the sample tube containing CO (or CH_4) was opened, and the CO (or CH_4) was pumped out of the line by the Toepler pump and confined between the one-way valve and SC 1. SC 5 and SC 6 were then opened, and, with the LN_2 still on the pyrolysis U-tube, SC 1 was set in the left-hand position and the Toepler run through one cycle, filling the line with CO (or CH_4). SC 5 and SC 6 were then closed, and the line was pumped down. The pyrolysis - combustion procedure was then followed, starting with the two hour heating of the pyrolysis U-tube at 500°C.

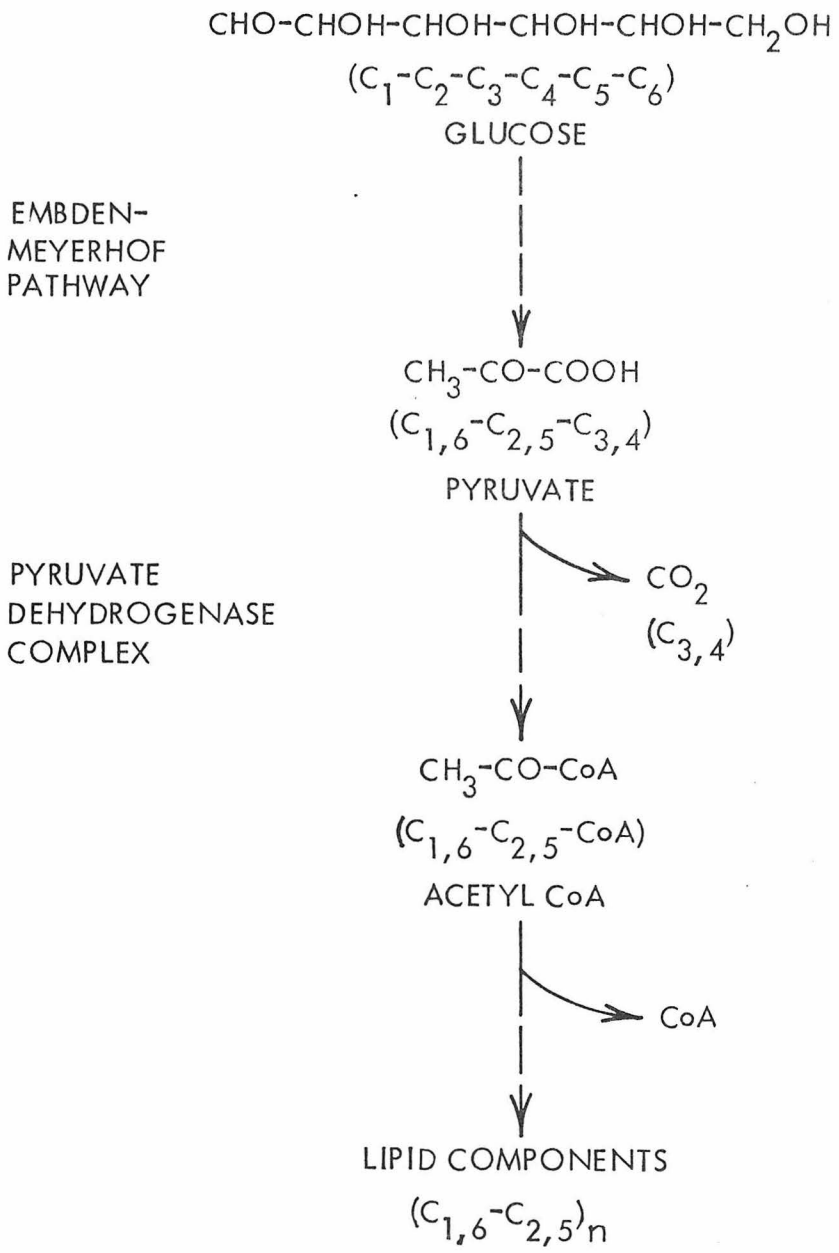
7. RESULTS AND DISCUSSION

7.1 Identification of the metabolic step during which the carbon isotope fractionation leading to the ^{13}C depletion of lipids occurs.

The data of Abelson and Hoering (1961), which showed that both E. coli and Chlorella grown on glucose as the sole carbon source had lipid fractions with $\delta^{13}C$ values 7.0 ‰ less than that of the glucose, established that the ^{13}C depletion of the lipid fraction must be due to isotope effects during the catabolic metabolism of glucose. Since most of the carbon atoms of the lipid fraction are derived from carbon atoms 1, 2, 5 and 6 of glucose (see Figure 7-1), a lower ^{13}C content in these carbon atoms relative to carbon atoms 3 and 4 of glucose could explain the ^{13}C depletion of the lipid fraction without recourse to isotope effects in the synthetic pathway from glucose to lipid components. However, since the glucose used in these experiments was isotopically homogeneous (i.e. there were not large differences in the isotopic composition of the individual carbon atoms of the glucose), the ^{13}C depletion of the lipid fraction must be due to isotopic fractionation during the metabolic conversion of glucose to lipid components.

Microorganisms grown in minimal media (inorganic salts, trace quantities of vitamins, and a single source of carbon) provide a simple system for identifying the metabolic steps during which the fractionation of carbon isotopes leading to a ^{13}C depletion of the lipid fraction occur. The pathways involved in lipid synthesis from glucose in microorganisms growing on glucose as the sole carbon source are shown in simplified form in Figure 7-1. Glucose is metabolized via the Embden-Meyerhof pathway to pyruvate. Pyruvate is then de-

Figure 7-1. Simplified version of lipid synthesis in microorganisms growing on glucose as the sole carbon source.


CHO-CHOH-CHOH-CHOH-CHOH-CH2OH
(C1-C2-C3-C4-C5-C6)
GLUCOSE
EMBDEN-
MEYERHOF
PATHWAY
CH3-CO-COOH
(C1,6-C2,5-C3,4)
PYRUVATE
PYRUVATE
DEHYDROGENASE
COMPLEX
CO2
(C3,4)
CH3-CO-CoA
(C1,6-C2,5-CoA)
ACETYL CoA
CoA
LIPID COMPONENTS
(C1,6-C2,5)n

carboxylated and oxidized by the pyruvate dehydrogenase complex to acetyl 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 the fractionating 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 7-1. Cells grown on glucose and pyruvate have lipid fractions which are depleted in $\delta^{13}C$ by 6.5 ‰ and 8.4 ‰ relative to their respective carbon sources, while growth on acetate produces a lipid fraction which is depleted by only 1.0 ‰ relative to the carbon source.

Abelson and Hoering (1961) concluded from indirect evidence that there is little isotopic fractionation during the metabolism of glucose to pyruvate. Their datum that in Chlorella grown on glucose as the sole carbon source, alanine has a $\delta^{13}C$ value only 0.5 ‰ more positive than the glucose supports this conclusion, since alanine derives its carbon atoms directly from pyruvate via a transamination reaction (Umbarger and Davis, 1962). This conclusion is consistent with the above results which indicate that growth on both glucose and pyruvate give ^{13}C depletions in the lipid fraction of similar magnitude. Growth on acetate,

Table 7-1. Effect of carbon source on the carbon isotopic composition of the lipid fraction of *E. coli*.

CARBON SOURCE	$\delta^{13}C$ (‰)	
	Carbon Source	Lipid
Glucose	- 9.5	-15.7
		-16.3
Na pyruvate	-20.5	-28.9
		-28.9
Na acetate	-20.1	-21.5
		-20.7

which is metabolized directly to acetyl CoA (Doelle, 1975) 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 by the pyruvate dehydrogenase complex.

The results of these experiments in which E. coli was grown on several carbon sources indicate that the model of Park and Epstein (1961) for the basis of the ^{13}C depletion of lipids is not correct. They proposed (see Section 5.4) that there is a fractionation after acetyl CoA formation, with ^{12}C-rich acetyl CoA going for lipid synthesis and ^{13}C-rich acetyl CoA going for respiration via the TCA cycle. If this were the case, growth on acetate, which enters before their proposed fractionation step, should have resulted in the usual ^{13}C depletion of the lipid fraction relative to the carbon source.

7.2. The use of pyruvate decarboxylase to determine the isotope effects during the oxidation of pyruvate to acetyl CoA.

Pyruvate is decarbyoxylated and oxidized to acetyl CoA by the pyruvate dehydrogenase complex (PDC), a multienzyme complex containing three enzymes, in which the following reactions occur (Lowenstein, 1971).

(1) $E_1\text{-TPP} + CH_3COCOOH \longrightarrow E_1\text{-TPP-}CHOHCH_3 + CO_2$

(enzyme-coenzyme)$_1$ (pyruvate) (hydroxyethyl-enzyme)

(2) $E_1\text{-TPP-}CHOHCH_3 + E_2\text{-lipoic acid}_{OX.} \longrightarrow E_1\text{-TPP}+E_2\text{-lipoic acid}_{RED}\text{-}COCH_3$

(enzyme-coenzyme)$_2$

(3) $E_2\text{-lipoic acid}_{RED.}\text{-}COCH_3 + CoA \longrightarrow E_1\text{-lipoic acid}_{RED.} + CH_3CO\text{-}CoA$

(acetyl CoA)

(4) E_2-lipoic $acid_{RED.}$ + E_3-FAD $\longrightarrow$ E_2-lipoic $acid_{OX.}$ + E_3FADH_2

(enzyme-coenzyme)$_3$

(5) E_3-$FADH_2$ + $NAD^{\oplus}$ $\longrightarrow$ E_3 - FAD + NADH + $H^{\oplus}$

After the initial step of the pyruvate dehydrogenase complex reaction, the enzyme-bound hydroxethyl group will be completely converted to acetyl CoA, since the intermediates of the PDC reaction do not dissociate from the complex (Reed and Oliver, 1969). Therefore, the ^{13}C depletion of the lipid fraction must result from kinetic isotope effects during this initial step producing hydroexyethyl 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 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. The enzyme pyruvate decarboxylase from yeast was selected for the study of the isotope effects in question. It catalyzes the same reaction as equation (1) above, but the CH_3CHOH-E complex then breaks down to E and CH_3CHO (acetaldehyde) (Utter, 1962). This allows precise measurement of the kinetic isotope effects of the enzymatic reaction, since both carbon dioxide and acetaldehyde can be isolated by vacuum technique, permitting their $\delta^{13}C$ values to be determined by simple and direct methods. Direct determination of the isotope effects involved in the pyruvate dehydrogenase complex reaction probably could not be done as accurately as the determination with pyruvate decarboxylase since isolation of the product of the PDC reaction, acetyl CoA, and determination of the isotopic composition of the acetyl group would require the use of numerous wet chemical techniques which might introduce

large errors in analysis.

7.3. Kinetic isotope effects of pyruvate decarboxylase.

The determination of the isotope effects of the pyruvate decarboxylase reaction involves comparison of the $\delta^{13}C$ values of the products of the reaction run to ~1% of completion with the $\delta^{13}C$ values of the homologous carbon atoms of pyruvate from which the products are derived. The basic experiment for determining these $\delta^{13}C$ values involves isolation of the products of the enzymatic reaction, CO_2 and acetaldehyde, followed either by direct conversion of the acetaldehyde to CO_2 or by separation and conversion of each carbon atom of acetaldehyde to CO_2. It is necessary to demonstrate that the experimental procedures do not introduce any artifacts to the isotope effects which are being determined. Consequently, a large part of the following discussion of isotope effects of pyruvate decarboxylase is concerned with control experiments. Such extensive control on the techniques is required, however, since the isotope effects are so small (<2%) that any error introduced during the procedure is likely to obscure the true effect.

7.3.1. Control experiments demonstrating that there are no significant isotope effects during the collection, separation and analysis of CO_2 and acetaldehyde. In order to demonstrate that the procedure used to analyze CO_2 and acetaldehyde produced in the enzymatic reaction does not introduce any significant alterations in the $\delta^{13}C$ values of the two molecules, known amounts of CO_2 and acetaldehyde of known isotopic composition were added to standard enzymatic reaction mixtures, the enzyme was denatured so that no CO_2 or acetaldehyde could be generated

from pyruvate, and the reaction mixtures were subjected to the standard collection procedure. The results are shown in Table 7-2.

The $\delta^{13}C$ values of CO_2 collected from the reaction mixtures do not differ significantly from the $\delta^{13}C$ values of the CO_2 samples that were added. The differences between initial and collected values range from 0.0 - 0.8 ‰, with the average difference being 0.3 ‰ . The same finding holds for the acetaldehyde. The differences between initial and collected $\delta^{13}C$ values ranges from 0.1 - 0.7 ‰ with the average difference being 0.4 ‰. These errors, possibly introduced during collection of CO_2 and acetaldehyde, are small compared with the magnitude of the isotope effects to be measured, 6 - 10 ‰, and hence will not affect the determinations.

The yields of CO_2 in Table 7-2, which indicate the fraction of added CO_2 that was recovered, range from 92 - 109 %. The amounts of CO_2 and acetaldehyde commonly measured in this study were on the order of 10-20 μ moles; the manometric determinations are accurate to within ±10% in this range. Thus, the recovery of CO_2 from the reaction mixtures is complete, within the limits of detection of our system. The recovery of acetaldehyde, however, is generally on the order of 50%. The basis for this low recovery of acetaldehyde, which was observed consistently throughout these experiments in both control and experimental runs, is not known; however, whatever process is involved does not cause a significant isotope effect. Most probably, the acetaldehyde is being polymerized to paraldehyde by the catalytic action of phosphoric acid (Hatcher and Brodie, 1931). Paraldehyde, with a melting point of +12.5°C, would not pass through a dry ice trap whereas acetaldehyde does.

Table 7-2. $\delta^{13}C$ values of CO_2 and acetaldehyde added to ($\delta^{13}C_{INITIAL}$) and collected from ($\delta^{13}C_{COLLECTED}$) enzyme reaction mixtures in which the enzyme was denatured so that there could be no enzymatic production of CO_2 and acetaldehyde.

EXPERIMENT	CO_2			ACETALDEHYDE		
	YIELD (%)	$\delta^{13}C$ COLLECTED (‰)	$\delta^{13}C$ INITIAL (‰)	YIELD (%)	$\delta^{13}C$ COLLECTED (‰)	$\delta^{13}C$ INITIAL* (‰)
A	100	-28.9	-28.9	50	-29.8	-29.7
B	92	-29.3	-28.8	50	-29.0	-29.7
C	93	-28.8	-28.7	54	-29.3	-29.7
D	94	-28.7	-28.6	54	-29.2	-29.7
E	109	-22.2	-21.4	50	-29.5	-29.7
F	100	-29.7	-29.8	25	-30.0	-29.7

*The acetaldehyde was drawn from a large reservoir;

its $\delta^{13}C = -29.7 \pm 0.0$ ‰ (n=4)

The yield of CO_2, rather than that of acetaldehyde, will be used to determine the extent of the enzymatic reaction, since CO_2 is completely recovered from the reaction mixtures.

7.3.2. Control experiments demonstrating that there are no significant isotope effects during the pyrolysis-combustion of acetaldehyde. It became necessary to determine the kinetic isotope effects on each of the three carbon atoms of the products of the enzymatic reaction. A procedure was developed which permitted the acetaldehyde to be quantitatively pyrolyzed to CH_4 and CO (which derive from the methyl and carbonyl carbon atoms of acetaldehyde respectively)(Roth and Rollefson, 1942); the CO and CH_4 were separated from one another and converted to CO_2 for isotopic analysis by combustion at 425°C and at 850°C (Sakai et al., 1976). The following experiments were done in order to demonstrate that there are no isotopic artifacts introduced during this procedure.

Acetaldehyde of known isotopic composition and quantity was processed by the pyrolysis-combustion procedure. The results are indicated in Table 7-3. The yields of CH_4 and CO are all nearly 100%, indicating quantitative conversion of acetaldehyde to products. The $\delta^{13}C$ values calculated for acetaldehyde from the sum of the $\delta^{13}C$ values for the CO and CH_4 generated in each experiment are all within 0.2‰ of the $\delta^{13}C$ value of the acetaldehyde used. Finally, the $\delta^{13}C$ values for CO or CH_4 generated in each experiment agree well with one another. Thus, if there is no exchange between CO and CH_4, the $\delta^{13}C$ values of the CO and CH_4 represent the isotopic composition of the carbonyl and methyl carbon atoms of acetaldehyde respectively.

Table 7-3. $\delta^{13}C$ values of CO and CH_4 generated from the pyrolysis-combustion of acetaldehyde of known isotopic composition.

EXPERIMENT	CO		CH_4		ACETALDEHYDE*
	$\delta^{13}C$ (‰)	YIELD (%)	$\delta^{13}C$ (‰)	YIELD (%)	$\delta^{13}C$ (‰)
A	-35.4	103	-25.2	103	-30.3
B	-35.4	103	-24.9	100	-30.2
C	-35.3	100	-25.3	106	-30.2
D	-35.2	103	-24.9	100	-30.0
AVERAGE	-35.3 ± 0.1		-25.1 ± 0.2		-30.2±0.1

*These values were calculated from the $\delta^{13}C$ values of CO and CH_4 generated in each experiment. The acetaldehyde used was drawn from a large reservoir; its $\delta^{13}C$ = -30.2 ± 0.3 ‰ (n = 4).

Two experiments were done to determine if there were any exchange between CO and CH_4 during the pyrolysis-combustion procedure. In the first of these, CO and CH_4 of known isotopic composition were mixed and subjected to the procedure. The results are shown in Table 7-4. The $\delta^{13}C$ of CO collected from two experiments is within 0.3 ‰ of that of the initial CO; the $\delta^{13}C$ of CH_4 collected is within 0.2 ‰ of the starting CH_4.

A second experiment was done in which a sample of acetaldehyde, which had been analyzed in the experiment reported in Table 7-3, was pyrolyzed in the presence of either CO or CH_4 of known isotopic composition. The added CO and CH_4 had $\delta^{13}C$ values 7 ‰ and 15 ‰ lighter than the CO and CH_4 generated from the acetaldehyde; they were present in excess so that any exchange should have shifted the $\delta^{13}C$ value of the carbon atom generated solely from acetaldehyde away from that determined previously. This did not occur, as is shown in Table 7-5. In experiment A, the presence of a 3.3X excess of CO did not change the $\delta^{13}C$ value of the CH_4 generated from acetaldehyde. Similarly, in experiment B, the presence of a 2.5X excess of CH_4 did not affect the $\delta^{13}C$ value of CO generated from acetaldehyde. There is no exchange between CO and CH_4, and since the pyrolysis-combustion procedure was shown to give quantitative yields of CO and CH_4, the procedure can be used to determine the $\delta^{13}C$ values of the two carbon atoms of acetaldehyde.

7.3.3. Isotopic composition of the individual carbon atoms of pyruvate. The products of the pyruvate decarboxylase reaction, CO_2 and acetaldehyde, are derived from the carboxyl carbon atom and from the methyl and

Table 7-4. $\delta^{13}C$ values of CO and CH_4 of known isotopic composition which were mixed and then subjected to the pyrolysis-combustion procedure.

EXPERIMENT	CO	CH_4
	$\delta^{13}C$ (‰)	$\delta^{13}C$ (‰)
A	-42.2	-38.4
B	-42.4	-38.4
Initial*	-42.1	-38.6

*The $\delta^{13}C$ values of the CO and CH_4 that were mixed together are indicated in this line.

Table 7-5. $\delta^{13}C$ values of CO and CH_4 generated by the pyrolysis-combustion of acetaldehyde of known isotopic composition in the presence of CO or CH_4 of different isotopic composition.

EXPERIMENT	COMPONENTS	CO		CH_4	
		$\delta^{13}C$ (‰)	Yield (%)*	$\delta^{13}C$ (‰)	Yield (%)*
A	Acetaldehyde + CO	-40.7	430	-25.2	100
B	Acetaldehyde + CH_4	-35.8	104	-35.0	350
	Added Acetaldehyde	-35.3**	---	-25.1**	---
	Added CO	-42.1***	---	---	---
	Added CH_4	---	---	-38.6***	---

*Based on acetaldehyde

**This value was determined in Table 7-3.

***This value was determined in Table 7-4.

carbonyl carbon atoms of pyruvate respectively:

$$E + \underset{\text{(methyl-carbonyl-carboxyl)}}{CH_3 - CO - COOH} \longrightarrow E + \underset{\text{(methyl-carbonyl)}}{CH_3 - CHO} + \underset{\text{(carboxyl)}}{CO_2}$$

In order to determine the isotope effects on each product of the reaction it is necessary to know the $\delta^{13}C$ values of the homologous carbon atoms of pyruvate from which the products are derived. These values were determined either for the pyruvate carboxyl carbon atom and for its methyl and carbonyl carbon atoms taken together (designated as methyl-carbonyl carbon atoms), as described in Section 7.3.3.1., or for each of the carbon atoms of pyruvate, as described in Section 7.3.3.2.

7.3.3.1. <u>$\delta^{13}C$ values of the methyl-carbonyl and carboxyl carbon atoms of pyruvate</u>. Pyruvate is decarboxylated by H_2O_2 (Holleman, 1904) in the reaction:

$$CH_3COCOOH + H_2O_2 \longrightarrow CH_3\,COOH + CO_2 + H_2O$$

Analysis of the CO_2 formed in this reaction permits the $\delta^{13}C$ value of the carboxyl carbon of pyruvate to be determined. The acetate formed does not react with H_2O_2. A control experiment in which H_2O_2 was added to 27 mg. of sodium acetate yielded no CO_2. The $\delta^{13}C$ values of the sum of the three carbon atoms of pyruvate can be determined by combustion of pyruvate. The $\delta^{13}C$ value of the methyl plus carbonyl carbon atoms of pyruvate can be calculated from the mass balance equation. The data for this experiment are presented in Table 7-6. The three carbons of pyruvate have a $\delta^{13}C$ of -21.0 ‰ , while the carboxyl carbon has a $\delta^{13}C$ of -22.1 ‰. By using the mass balance equation, the $\delta^{13}C$ of the

Table 7-6. Determination of the $\delta^{13}C$ values of the carboxyl and methyl-carbonyl carbon atoms of pyruvate by H_2O_2 decarboxylation.

Combustion of Na pyruvate

EXPERIMENT	YIELD (%)	$\delta^{13}C$ (‰)
A	102	-21.0
B	103	-21.0
C	105	-21.0
	$\delta^{13}C_{PYRUVATE}$ =	-21.0 ± 0.0 (n=3)

Hydrogen peroxide decarboxylation of Na pyruvate

EXPERIMENT	YIELD (%)	$\delta^{13}C$ (‰)
A	103	-22.3
B	105	-22.4
C	98	-22.0
D	98	-22.0
E	97	-21.9
	$\delta^{13}C_{CARBOXYL}$ =	-22.1 ± 0.2 (n=5)

$$\delta^{13}C_{PYRUVATE} = 2/3\ \delta^{13}C_{METHYL\text{-}CARBONYL} + 1/3\ \delta^{13}C_{CARBOXYL}$$

$$\delta^{13}C_{METHYL\ -\ CARBONYL} = 20.4 \pm 0.1$$

methyl-carbonyl carbon atoms of pyruvate is calculated to be -20.4 ‰.

The $\delta^{13}C$ value for the different carbon atoms of pyruvate can be determined directly by running the enzymatic reaction to completion. Results of this experiment are shown in Table 7-7. The CO_2 yield is 96% for all experiments, indicating that the reaction has run to completion. The carboxyl carbon of pyruvate has a $\delta^{13}C$ value of -22.5 ‰, while the methyl-carbonyl carbon atoms have a $\delta^{13}C$ value of -20.6 ‰. These values are in close agreement with those determined by hydrogen peroxide decarboxylation of pyruvate as described in the previous paragraph.

7.3.3.2. $\delta^{13}C$ values of the methyl, carbonyl, and carboxyl carbon atoms of pyruvate. The isotopic composition of each of the reactant carbon atoms of pyruvate was determined by first running the enzymatic reaction to completion; the CO_2 and acetaldehyde were separated; and the acetaldehyde was then subjected to the pyrolysis-combustion procedure. The results are shown in Table 7-8. The CO_2 yields are all around 90-95%, confirming that the reaction has run to completion. The yields for CO and CH_4 are generally within 10% of one another, as expected since they are generated in equimolar amounts by the pyrolysis of acetaldehyde. The $\delta^{13}C$ value for the carboxyl carbon of pyruvate as determined here, -22.3 ‰, is in good agreement with the values determined previously in Table 7-6, -22.1 ‰, and in Table 7-7, -22.5‰. The $\delta^{13}C$ value of the methyl-carbonyl carbon atoms of pyruvate, calculated from the average of the $\delta^{13}C$ values of the methyl and the

Table 7-7. Determination of the $\delta^{13}C$ values of the carboxyl and methyl-carbonyl carbon atoms of pyruvate by the 100% enzymatic reation.

EXPERIMENT	CO_2		ACETALDEHYDE	
	$\delta^{13}C$ (‰)	YIELD (%)	$\delta^{13}C$ (‰)	YIELD (%)
A	-22.5	96	sample lost	
B	-22.7	96	-21.1	60
C	-22.1	96	-20.2	54
D	-22.6	96	-20.6	60
Average	-22.5 ± 0.3		-20.6 ± 0.4	

$\delta^{13}C_{CARBOXYL} = -22.5 \pm 0.3$ (n = 4)

$\delta^{13}C_{METHYL\text{-}CARBONYL} = -20.6 \pm 0.4$ (n = 3)

Table 7-8. Determination of the $\delta^{13}C$ values of the methyl, carbonyl, and carboxyl carbon atoms of pyruvate by the 100% enzymatic reaction.

EXPERIMENT	CH_3		CO		COOH	
	$\delta^{13}C$(‰)	YIELD(%)	$\delta^{13}C$(‰)	YIELD(%)	$\delta^{13}C$(‰)	YIELD (%)
A	-20.3	49	-20.2	57	-22.3	90
B	-20.2	51	-20.3	73	-22.2	90
C	-18.9	41	-20.2	57	-22.4	90
D	-20.2	41	-20.1	52	-22.1	95
E	-21.0	48	-19.7	48	-22.4	88
F	-20.9	41	-19.8	48	-22.1	95
G	-20.3	41	-19.9	48	-22.5	95
H	-19.6	41	-19.5	48	-22.6	95
I	-19.8	41	-19.6	41	-22.1	95
Average	-20.1 ± 0.6		-19.9 ± 0.3		-22.3 ± 0.2	

$\delta^{13}C_{METHYL}$ = -20.1 ± 0.6 (n = 9)

$\delta^{13}C_{CARBONYL}$ = -19.9 ± 0.3 (n = 9)

$\delta^{13}C_{CARBOXYL}$ = -22.3 ± 0.2 (n = 9)

calculated $\delta^{13}C_{METHYL-CARBONYL}$ = -20.0 ± 0.7

carbonyl carbon atoms determined here, is -20.0 ‰, which compares reasonably well with the values of -20.4 ‰ calculated in Table 7-6 and -20.6 ‰ measured in Table 7-7.

7.3.4. Kinetic isotope fractionation during the pyruvate decarboxylase reaction. The isotopic fractionations associated with the pyruvate decarboxylase reaction were determined at several temperatures and pH values. The reaction was run to ~1% of completion and the $\delta^{13}C$ values of the products compared with those of the homologous carbon atoms of pyruvate. A 1% completion of a reaction with a 20 ‰ fractionation (which is larger than any effect to be measured here) changes the $\delta^{13}C$ value of the reactant pool by only 0.1 ‰, so that the fractionation effects obtained in this fashion are the maximum values attainable. As larger fractions of reactant are converted to products, the difference between the $\delta^{13}C$ values of the original reactant pool and of product carbon atoms decreases until, at 100% reaction, there is no fractionation.

7.3.4.1. Reaction at 25°C and pH 6.0. The kinetic isotope fractionations at 25°C and pH 6.0 were determined; the results are shown in Table 7-9. The CO_2 formed in the 1% reaction has a $\delta^{13}C$ value, -28.8 ‰, which is 6.3 ‰ less than that of the carboxyl carbon of pyruvate, which has a $\delta^{13}C$ value of -22.5 ‰. The acetaldehyde formed in the 1% reaction has a $\delta^{13}C$ value which is 7.9 ‰ less than the $\delta^{13}C$ value of the methyl-carbonyl carbon atoms of pyruvate from which it is derived, i.e., -28.5 ‰ compared with -20.6 ‰. Thus, not only is the CO_2 released in the decarboxylation reaction depleted in ^{13}C, but the acetaldehyde

Table 7-9. Kinetic isotope fractionations of pyruvate decarboxylase at 25°C and pH 6.0 for CO_2 and for acetaldehyde.

EXPERIMENT	CO_2		ACETALDEHYDE	
	$\delta^{13}C$ (‰)	YIELD (%)	$\delta^{13}C$ (‰)	YIELD (%)
A	-28.9	1.2	-28.6	0.6
B	-28.6	1.6	-28.2	0.7
C	-28.8	1.2	-28.6	0.7
D	-28.8	1.6	-28.7	0.7
Average	-28.8 ± 0.1		-28.5 ± 0.2	
100% enzymatic reaction, Table 7-7	-22.5 ± 0.3		-20.6 ± 0.4	

formed in the reaction is depleted in ^{13}C relative to the homologous carbon atoms of pyruvate from which it is derived.

The bonding to the methyl carbon of pyruvate does not change during the pyruvate decarboxylase production of acetaldehyde (Utter, 1961). Thus it was anticipated that the ^{13}C depletion of acetaldehyde would be restricted to its carbonyl carbon atom, since secondary isotope effects, which are isotope effects on atoms which do not undergo a change in bonding during the reaction, are generally small for carbon (Fry, 1970). This hypothesis was confirmed by comparing the $\delta^{13}C$ values of each of the three carbon atoms of the enzymatic products with the $\delta^{13}C$ values of the homologous carbon atoms of pyruvate. The data are shown in Figure 7-10. The CO_2 formed in the 1% reaction is depleted in $\delta^{13}C$ by 6.5 ‰ relative to the carboxyl carbon of pyruvate, which compares well with the value of 6.3 ‰ determined in Table 7-9. The carbonyl carbon atom of acetaldehyde formed in the 1% reaction has a $\delta^{13}C$ value which is 14.1 ‰ lower than the carbonyl carbon atom of pyruvate, i.e., -34.0 ‰ compared with -19.9 ‰. As expected, the change is the isotopic composition of the methyl carbon atom in going from pyruvate to acetaldehyde is small, being only 1.0 ‰. The ^{13}C depletion of acetaldehyde formed in this reaction, determined by comparing the $\delta\ ^{13}C$ values of the methyl and carbonyl carbon atoms of acetaldehyde with the same carbon atoms in pyruvate, is 7.6 ‰, which compares well with the value of 7.9 ‰ determined in Table 7-9. In summary, the ^{13}C depletion of the acetaldehyde formed in the 1% reaction of pyruvate with pyruvate decarboxylase is confined largely to the carbonyl carbon atom of acetaldehyde. However, the 1‰ lowering of the $\delta^{13}C$ value of the

Table 7-10. Kinetic isotope fractionations of pyruvate decarboxylase at 25°C and pH 6.0 for CO_2 and for each carbon atom of acetaldehyde.

EXPERIMENT	ACETALDEHYDE				CO_2	
	METHYL CARBON		CARBONYL CARBON			
	$\delta^{13}C$ (‰)	YIELD (%)	$\delta^{13}C$ (‰)	YIELD (%)	$\delta^{13}C$ (‰)	YIELD (%)
A	-21.0	1.1	-34.4	1.2	-28.8	2.1
B	-20.8	1.0	-34.1	1.2	-28.7	2.1
C	-22.0	0.8	-33.9	0.8	-28.8	1.9
D	-21.0	0.6	-33.3	0.9	-28.7	1.9
E	-20.5	0.7	-34.3	0.9	-28.9	1.7
Average	-21.1 ± 0.6		-34.0 ± 0.4		-28.8 ± 0.1	
100% enzymatic reaction, Table 7-8	-20.1 ± 0.6		-19.9 ± 0.3		-22.3 ± 0.2	

methyl carbon atom of the acetaldehyde is statistically significant by Student's t test (at the P = 0.05 level).

7.3.4.2. Reaction at 15°C, 25°C, or 35°C and pH 6.0. The results of experiments in which the effects of temperature on the kinetic isotope fractionation of pyruvate decarboxylase were determined are shown in Table 7-11. The $\delta^{13}C$ depletion of the CO_2 produced in the 1% reaction relative to the carboxyl carbon of pyruvate changes from -5.6 ‰ at 15°C to -6.3 ‰ at 25°C to -7.7 ‰ at 35°C. The small differences in the extent of reaction, as determined from the yield of CO_2, do not affect these values. For example, if experiment C at 15°C had been run to 1.6% of completion (the highest value in the table) instead of 0.4% of completion (the lowest value in the table), the $\delta^{13}C$ value of the product CO_2, which can be calculated from the expression given by Bigeleisen (1949), would not have changed. The difference between the $\delta^{13}C$ value of the acetaldehyde produced in the 1% enzymatic reaction and the $\delta^{13}C$ value of the methyl-carbonyl carbon atoms of pyruvate also shows a temperature effect, changing from -5.9 ‰ at 15°C to -7.9 ‰ at 25°C to -9.6 ‰ at 35°C. Since the small isotope effect on the methyl carbon atom of acetaldehyde will not vary significantly with temperature, the temperature effect on the ^{13}C depletion of acetaldehyde 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 acetaldehyde produced in the 1% reaction must increase by 0.4 ‰ per 1°C, i.e., twice the 3.7 ‰ increase observed for the two carbon atoms of acetaldehyde over the 20°C range of the experiments of Table 7-11.

Table 7-11. Kinetic isotope fractionations of pyruvate decarboxylase at 15°, 25° or 35°C and pH 6.0 for CO_2 and for acetaldehyde. The data for the reaction at 25°C are reprinted from Table 7-9.

EXPERIMENT	CO_2		ACETALDEHYDE	
	$\delta^{13}C$ (‰)	YIELD (%)	$\delta^{13}C$ (‰)	YIELD (%)
A, 15°C	-27.9	0.6	-26.2	0.3
B, 15°C	-28.4	0.5	-26.6	0.3
C, 15°C	-27.6	0.4	-26.8	0.2
D, 15°C	-28.6	0.5	-26.5	0.3
Average, 15°C	-28.1 ± 0.5		-26.5 ± 0.2	
E, 25°C	-28.9	1.2	-28.6	0.6
F, 25°C	-28.6	1.6	-28.2	0.7
G, 25°C	-28.8	1.2	-28.6	0.7
H, 25°C	-28.8	1.6	-28.7	0.7
Average, 25°C	-28.8 ± 0.1		-28.5 ± 0.2	
I, 35°C	-30.4	0.8	-30.2	0.4
J, 35°C	-30.9	0.8	-29.3	0.3
K, 35°C	-29.9	0.6	-30.9	0.3
L, 35°C	-29.8	1.1	-30.3	0.6
Average, 35°C	-30.2 ± 0.5		-30.2 ± 0.7	
100% enzymatic reation, Table 7-7	-22.5 ± 0.3		-20.6 ± 0.4	

7.3.4.3. Reaction at 25°C and pH 5.0, 6.0 or 7.0. The results of experiments in which the effects of pH on the kinetic isotope fractionations of pyruvate decarboxylase were determined are presented in Table 7-12. The composition and ionic strength of the buffers used in these experiments were different from those of the buffer used in all other experiments of this study (see Section 6.4.2).

However, these changes in the buffer do not have a significant effect on the kinetic isotope fractionations. The values determined at pH 6.0 in this experiment are in good agreement with the values determined at pH 6.0 in Table 7-9. The $\delta^{13}C$ depletion of CO_2 determined in Table 7-12 had a value of -6.5 ‰, which compares well with the value of -6.3 ‰ determined in Table 7-9. The difference between the kinetic isotope fractionation of acetaldehyde determined using the different buffers is also small, with the value of -7.6 ‰ in Table 7-12 compared to the value of -7.9 ‰ from Table 7-9.

The effect of pH on the kinetic isotope fractionations appears to be small. There may be a smaller fractionation in the CO_2 as pH is increased, but the average values differ by only 0.9 ‰ between pH 5.0 and 7.0. There may also be some effect of pH on the fractionation associated with the formation of acetaldehyde, but again the averages at the three pH values differ by less than 0.8 ‰.

7.4. A model for the ^{13}C depletion of lipids.

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. Refer to Figure 7-1 for an outline of the

Table 7-12. Kinetic isotope fractionations of pyruvate decarboxylase at 25°C and pH 5.0, 6.0, or 7.0 for CO_2 and for acetaldehyde.

EXPERIMENT	CO_2		ACETALDEHYDE	
	$\delta^{13}C$ (‰)	YIELD (%)	$\delta^{13}C$ (‰)	YIELD (%)
A, pH 5.0	-29.6	0.5	-27.5	0.3
B, pH 5.0	-29.3	1.0	-27.2	0.6
C, pH 6.0	-29.0	2.4	-28.2	1.3
D, pH 6.0	-29.0	2.9	-28.3	1.4
E, pH 7.0	-28.8	2.6	-27.8	1.2
F, pH 7.0	-28.4	2.1	-27.6	1.0
100% enzymatic reaction, Table 7-7	-22.5 ± 0.3		-20.6 ± 0.4	

metabolic steps discussed below. There is little isotope fractionation when glucose is converted to pyruvate via the Embden-Meyerhof pathway. The 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 depends 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. The ^{13}C depletion of the lipid fraction results from the incorporation of ^{13}C-depleted acetyl groups into lipid components; 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.

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 in an organism (Figure 5-2) can thus be explained by this feature of the model.

The model explains the observation by Abelson and Hoering (1961) that among nine amino acids isolated from Chlorella and E. coli grown on glucose, leucine has the carboxyl carbon atom with the most negative $\delta^{13}C$ value, 9-17 ‰ lower than the $\delta^{13}C$ value of the carboxyl carbon atoms of the other amino acids. The carboxyl carbon atom of leucine is derived from the carbonyl carbon atom of the acetyl CoA, while the carboxyl carbon atoms of the eight other amino acids are derived from either Embden-Meyerhof pathway or tricarboxylic acid cycle intermediates (Umbarger and Davis, 1962). Abelson and Hoering found that, relative to the glucose carbon source, the carboxyl carbon atom of leucine has a $\delta^{13}C$ value of -14.0 ‰ for Chlorella and -18.5 ‰ for E. coli, while the lipid fractions of both organisms are -7.0 ‰. 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 could account for the ^{13}C depletion of the lipid fraction observed in both autotrophs and heterotrophs* as they exist in nature. Species-specific

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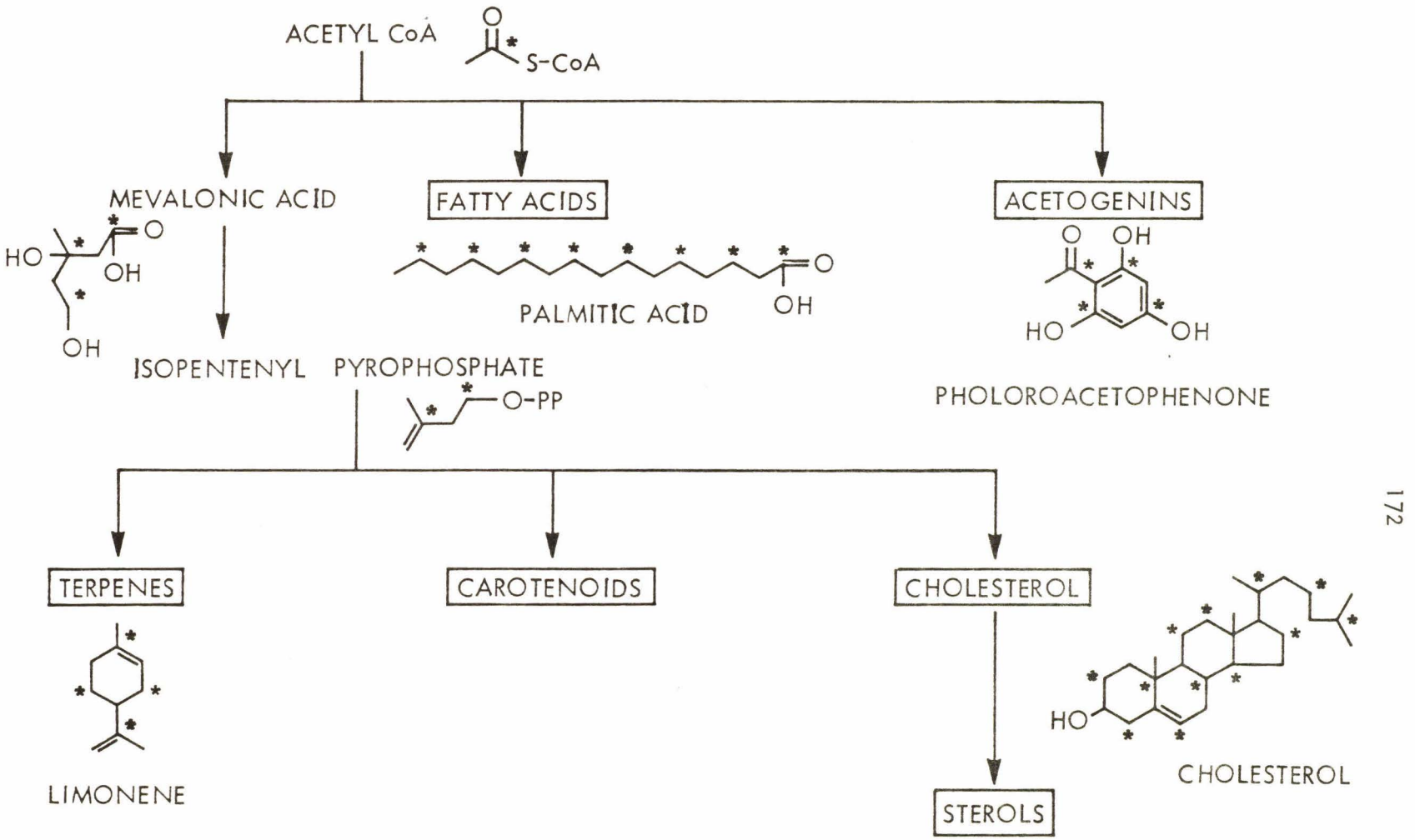
*The situation in heterotrophs is complicated by the fact that their biochemical fractions consist of components assimilated directly from the diet and those synthesized de novo in the heterotroph. Newly synthesized lipid components will be depleted in ^{13}C according to the model. The addition of ^{13}C-depleted lipid components from the diet will, in general, result in the usual pattern of isotope distribution in the biochemical fractions of the animal (Table 5-1).

differences in the magnitude of the ^{13}C depletion could arise from different kinetic isotope effects during the specific pyruvate dehydrogenase complex reaction and from the different flow rates of pyruvate to its various metabolic reactions. The ^{13}C depletion would be limited primarily to the carbonyl carbon atoms of acetyl CoA.

The main complication in observing intramolecular carbon isotopic heterogeneity in lipid components in these natural systems is the possibility of intramolecular isotopic heterogeneity in the carbon atoms of pyruvate, which in turn is a function of the isotopic distribution in the metabolic precursors of pyruvate. In plants, the cyclic nature of the Calvin cycle produces random incorporation of ^{14}C into photosynthetic carbohydrate in the long run (Arnoff and Vernon, 1950; Calvin et al., 1951), 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 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 composition of its pyruvate.

Techniques which allow for the 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 (see Figure 7-2 for some examples) 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

Figure 7-2. Origin of the carbon atoms of some lipid fraction components. Those carbon atoms marked C* derive from the carbonyl carbon atom of acetyl CoA; unmarked carbon atoms derive from the methyl carbon atom of acetyl CoA.


ACETYL CoA
S-CoA
MEVALONIC ACID
HO
OH
OH
FATTY ACIDS
PALMITIC ACID
OH
ACETOGENINS
OH
HO
OH
ISOPENTENYL PYROPHOSPHATE
O-PP
PHOLOROACETOPHENONE
TERPENES
CAROTENOIDS
CHOLESTEROL
LIMONENE
HO
CHOLESTEROL
STEROLS

provide a method which might yield information as to the source and early thermal history of fossil lipids.

8. SUMMARY

The mechanism of the carbon isotope fractionation associated with lipid synthesis is determined.

8.1. Principal findings.

1) The oxidative decarboxylation of pyruvate to give acetyl CoA by the pyruvate dehydrogenase complex is identified as the metabolic step during which the carbon isotope fractionation leading to the low $^{13}C/^{12}C$ ratio of the lipid fraction occurs. The identification of this step was accomplished by determining the effect that growth of E. coli on different carbon sources had on the ^{13}C depletion of the lipid fraction. With glucose or pyruvate serving as the sole carbon source, the lipid fraction is depleted in $\delta^{13}C$ relative to the carbon source by 6.5 ‰ and 8.4 ‰ respectively, while growth on acetate results in a lipid fraction which is depleted in $\delta^{13}C$ by only 1.0 ‰ relative to the acetate. Since acetate is metabolized directly to acetyl CoA before lipid synthesis, most of the ^{13}C depletion must occur during the oxidative decarboxylation of pyruvate to acetyl CoA.

2) The kinetic isotope fractionations occurring during the pyruvate dehydrogenase complex reaction were studied using yeast pyruvate decarboxylase, which produces CO_2 and acetaldehyde from pyruvate. The reaction catalyzed by the yeast enzyme is identical with the initial step of the pyruvate dehydrogenase complex reaction. It is during this step that all the isotopic fractionation occurring during the formation of acetyl CoA from pyruvate must occur. The experiments involving pyruvate decarboxylase show:

a) There is an isotopic fractionation of the proper direction and magnitude occurring during the reaction. The acetaldehyde formed on the 1% reaction at 25°C has a $\delta^{13}C$ value which is 7.9 ‰ lower than the $\delta^{13}C$ value of the carbon atoms of pyruvate from which it was derived.

b) The ^{13}C depletion of the acetaldehyde is not uniformly distributed over the two carbon atoms of the molecule. The ^{13}C depletion of the acetaldehyde formed in the 1% reaction at 25°C is concentrated almost entirely in the carbonyl carbon atom, although there is also a small ^{13}C depletion in the methyl carbon atom.

c) The isotopic fractionations show a temperature dependence. The ^{13}C depletion of the acetaldehyde formed in the 1% reaction, relative to the $\delta^{13}C$ value of the carbon atoms of pyruvate from which it was derived, increases by 3.7 ‰ over the range 15°-35°C. The observed difference between the $\delta^{13}C$ values of the methyl and carbonyl carbon atoms of the acetaldehyde must increase by 0.4 ‰ per 1°C, since the temperature effect on the small ^{13}C depletion of the methyl carbon atom is negligible.

8.2. Conclusions.

The results of the experiments described above are incorporated into the following model for the ^{13}C depletion of lipids in micro-organisms grown on glucose. There is no isotopic fractionation during the conversion of glucose to pyruvate. The fractionation of carbon

isotopes during the pyruvate dehydrogenase complex reaction results in the formation of acetyl CoA in which the acetyl group has a $\delta^{13}C$ value more negative than that of the pyruvate from which it was derived. This ^{13}C depletion is confined to the carbonyl carbon atom of acetyl CoA, with the methyl carbon atom retaining the $\delta^{13}C$ value of the pyruvate. Incorporation of these ^{13}C-depleted acetyl groups into lipid components results in the low $^{13}C/^{12}C$ ratio of the lipid fraction. The difference in the $\delta^{13}C$ values of the methyl and carbonyl carbon atoms of acetyl CoA is preserved in the carbon atoms of lipid components which derive from them.

The model also explains the cause of the low $^{13}C/^{12}C$ ratio of the lipid fraction relative to the rest of the organism for both autotrophs and heterotrophs as they exist in nature.

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