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METABOLIC FRACTIONATION OF C^{13} & C^{12} IN PLANTS [1, 2]

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Nier and Gulbransen (10) observed 20 years ago that the C^{13}/C^{12} ratio of carbon in nature ($\sim$1/90) varies slightly depending on the source of the carbon analysed. These workers found that limestones, atmospheric carbon dioxide, marine plants, and land plants possess different and characteristic C^{13}/C^{12} ratios. Workers using improved analytical techniques (9) have confirmed and extended many of Nier and Gulbransen's early observations (4, 5, 7, 13, 14, 15). Our present knowledge of the C^{13}/C^{12} ratio ranges for various natural carbon containing materials is summarized in figure 1. The results in figure 1 are reported in terms of a δ value. The δ value indicates the difference in per mil of the C^{13}/C^{12} ratio of the sample relative to a standard and is defined as:

$$\delta \text{ in per mil } (‰) = \frac{C^{13}/C^{12} \text{ sample} - C^{13}/C^{12} \text{ standard}}{C^{13}/C^{12} \text{ standard}} \times 10^3$$

In this work the standard is CO_2 prepared from the fossil skeleton of a Cretaceous belemnite, *Belemnitella americana*, from the Peedee formation of South Carolina. This scale is called the PDB scale (6). A δ value of $+10$ means that the C^{13}/C^{12} ratio of the sample is greater than that of the standard by 10 per mil or 1 %. Conversely a δ value of -10 means that the C^{13}/C^{12} ratio of the sample is less than that of the standard by 10 per mil or 1 %. The precision of the data presented in figure 1 is about $\pm$0.01 per cent or $\pm$0.1 per mil of the ratio (9).

The data of figure 1 show that many steps in the carbon cycle are accompanied by isotope fractionation. One of the most important processes in nature which causes fractionation of carbon is photosynthetic carbon assimilation by green plants. Both terrestrial and marine plants have lower C^{13}/C^{12} ratios than their respective carbon sources, atmospheric CO_2 and ocean carbonates. This means that during photosynthetic CO_2 fixation there is preferential utilization of C^{12} and exclusion of C^{13}.

Investigations on the magnitude and mechanism of the C^{13}/C^{12} fractionation which occurs during photosynthesis have been made by Baertschi (1, 2) and by Park and Epstein (12). These workers observed that plants grown in carbon dioxide of controlled C^{13}/C^{12} ratio discriminated against C^{13} during photosynthetic CO_2 fixation. The fixed plant carbon was -26 per mil with respect to the atmospheric CO_2. Park and Epstein proposed and experimentally supported a model for photosynthetic fractionation of carbon which consisted of two stages. The first fractionation stage in favor of C^{12} was a kinetic effect which occurred during uptake of CO_2 from the atmosphere into the leaf cytoplasm. In rapidly photosynthesizing tomato plants, carbon dioxide dissolved in the cytoplasm was

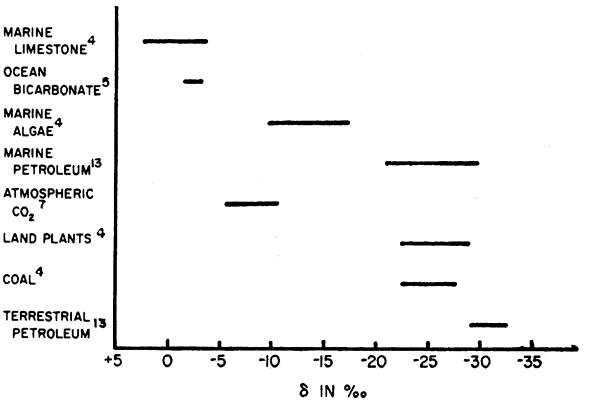

FIG. 1. Range of C^{13}/C^{12} ratios for various carbon reservoirs in nature.

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enriched in C^{12} compared to atmospheric CO_2 by 7 per mil. The second fractionation stage in favor of C^{12} occurred during fixation of dissolved CO_2 via the carboxydismutase enzyme into 3-phosphoglyceric acid (PGA). The experimentally observed fractionation between bicarbonate and carbon fixed into PGA by the isolated enzyme was 15 per mil. The sum of these two separate fractionations is about equal to the total fractionation observed during photosynthesis of experimentally grown tomato plants. These observations were used not only to explain C^{13}/C^{12} fractionation during photosynthesis, but also to explain the variations in C^{13}/C^{12} ratio of plants in nature.

The data in figure 1 have many other interesting features. For example, coals of all geological ages have about the same C^{13}/C^{12} ratios as present day plants. Appparently coal represents organic plant residue which is not fractionated during coal formation. On the other hand, petroleums of marine and terrestrial origin are both enriched in C^{12} with respect to their present day plant sources. Since coals and limestone of all geologic ages have about the same C^{13}/C^{12} ratio, the C^{13}/C^{12} ratio of atmospheric carbon dioxide has probably also been constant over geologic time. For these reasons, C^{12} enrichment in petroleum cannot be explained by geologic periods in which plant sources for petroleum were unusually enriched in C^{12}. A possible explanation for the C^{12} enrichment in petroleum hydrocarbons is that plants produce a C^{12} enriched fraction which is selectively preserved during sedimentation and petroleum formation while the remainder of the plant is oxidized. Metabolic fractionation of carbon compounds following photosynthetic carbon fixation would be necessary to produce a C^{12} enriched petroleum precursor. If measurable metabolic fractionation of carbon is occurring in plants, it should be possible to find chemical materials within the plant which differ in $C^{13}/^{12}$ ratio. This paper reports the results of a search for a C^{12} enriched plant fraction which might give rise to petroleum hydrocarbons.

Materials and Methods

Preparation of Lipid Fraction: The whole dried plant material was ground up with a mortar and pestle. The plant material was then extracted with ethanol in order to allow more complete subsequent extraction with chloroform. The ethanol extract was also extracted with chloroform and the two cholorform extracts were pooled. The following evidence indicates that no isotopic exchange between the solvent and solute took place during the extraction procedure.

Two samples, each containing 1 gm of dried Ulva sp. were extracted at room temperature with 10 cc of ethanol and 10 cc of chloroform, respectively. The extract was filtered after 6 hours and was transferred to a refluxing apparatus. At the time intervals shown in table I, 2 cc of solvent were removed from the reaction flask. After evaporation of the solvent, the residue was converted to CO_2 for analysis. During the interval 24 to 30 hours, the samples were refluxed. The consistency of the C^{13}/C^{12} ratios of the solutes in table 1 indicate that no measurable exchange of carbon between solvent and solute took place during this procedure.

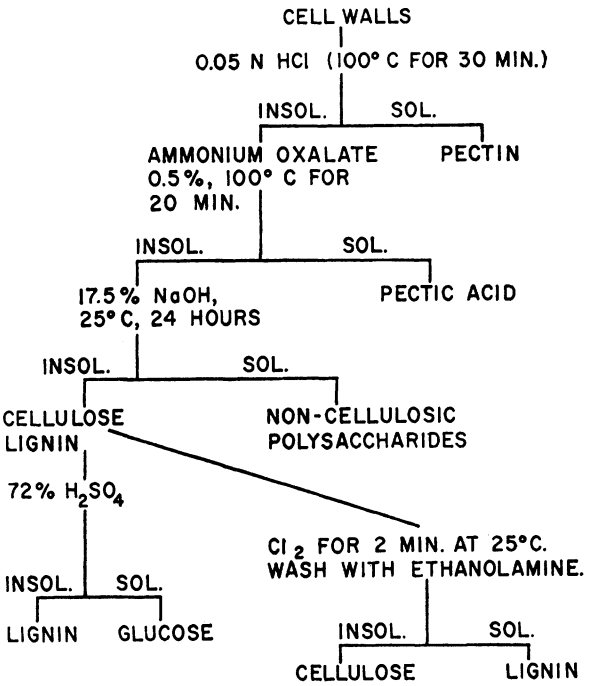

Fig. 2. Fractionation of cell wall material.

Fractionation of Cell Wall Material: The dried cell walls obtained as residue in the preparation of the lipid fraction were fractionated according to the scheme in figure 2. This method for plant cell wall fractionation is described by Ordin, Cleland, and Bonner (11) with the exception of the lignin and cellulose preparations which were prepared according to the methods cited by Bonner (3). The HCl and NaOH fractions were reduced to small volume in a desiccator and precipitated with 95 % ethanol.

Collection of Respired CO_2: Tomato plants (*Lycopersicon esculentum* Mill.) grown from CO_2 of known isotopic composition were used for the collec-

Table I

Exchange of Carbon Isotopes Between Solvent & Plant Lipid

Elapsed time, hr	δ of Ethanol soluble material, ‰	δ of Chloroform soluble material, ‰
6	−21.4	−22.5
24	−21.3	−22.5
30	−21.4	−22.4

tion of respired CO_2. The apparatus used for this study consisted of a respiratory chamber, about one liter in volume, followed by a dry ice trap and two liquid nitrogen traps in series. The plants were placed in a darkened respiratory chamber which was then flushed with 20 volumes of CO_2 free air prepared by washing with Ascarite. No CO_2 could be detected in air washed in this way either by an infrared analyzer, $Ba(OH)_2$ absorption, or absorption in a liquid nitrogen trap. Stopcocks were adjusted so that 70 cc of CO_2 free air swept through the system each minute. The respired CO_2 was then collected at desired time intervals. The respiratory chamber and dry ice trap were maintained at atmospheric pressure while the liquid nitrogen traps were at 1 cm mercury pressure. The respired CO_2 was completely removed by the first liquid nitrogen trap. Except for samples collected shortly after initiation of the experiment, samples were collected over a period of one hour. The collected CO_2 was transferred to the second trap and then to the mass spectrometer sample tube. The sample volumes were measured manometrically.

Combustion of Samples to CO_2: Plant materials were converted to CO_2 according to the procedure described by Craig (4). In this procedure samples are combusted over copper oxide in an oxygen atmosphere at 800 to 900° C. After cycling the gases through the combustion tube with an automatic Toepler pump for 30 minutes, the CO_2 produced is collected in a liquid nitrogen trap. The trap is then warmed in a dry ice bath and the CO_2 distilled into another trap at liquid nitrogen temperature. After several such transfers the CO_2 is free of water and most other contaminants. The CO_2 is then distilled into a sample tube and analyzed.

Mass Spectrometer Analysis: The CO_2 samples were analyzed in a Nier 60 ° sector type mass spectrometer which incorporates the improvements described by McKinney, et al for determining C^{13}/C^{12} ratios to $\pm$ 0.1 per mil (9). All samples are corrected for the O^{17} contribution to the mass 45 peak and for mixing of the sample and standard gas (6). Each of the δ values presented in the Results section represents an individual sample.

Results

C^{13}/C^{12} Ratios of Various Plant Fractions: In two experiments tomato plants were grown in air containing 1.5 % CO_2 by volume of known C^{13}/C^{12} ratio (12). A portion of the plants was separated into chemical fractions according to the scheme described above. These fractions were subjected to isotopic analysis (table II). The only fractions which differed greatly in C^{13}/C^{12} ratio from the plant as a whole were the chloroform soluble or non-polar lipid fractions. These fractions were of lower C^{13}/C^{12} ratio than the plant as a whole.

To establish the generality of C^{12} enrichment in the lipid fraction of various plant taxonomic groups, a number of other plant lipids were analyzed and compared to the isotopic composition of the plant as a whole. (table III). In all plants investigated, the C^{13}/C^{12} ratio of the lipid fraction was smaller than that of the whole plant.

Respired CO_2 Collected Over Long Intervals: The respired CO_2 from 19 thirteen-day-old tomato plants (δ = 25.2 per mil) was collected at various intervals during a three week dark period. After the first 14 hours, 14 plants were removed for isotopic analysis. Five plants were left in the respiratory chamber and respired CO_2 was collected and analyzed

Table II

C^{13}/C^{12} Ratios of Chemical Fractions Within Tomato Plants Grown in 1.5 % CO_2

Fraction	δ ‰ for AP series	δ ‰ for BP series
Cellulose	−23.6	−25.1
Lignin	−24.2	...
Non-cellulosic polysaccharides	−23.1	...
Pectin	...	−23.6
Ethanol soluble, $CHCl_3$ insoluble	−25.9	−25.1
$CHCl_3$ soluble	−30.2	−29.6

Table III

C^{13}/C^{12} Ratios of Plants & Their Respective Lipid Fractions as Found in Nature

Plant	δ of Carbon, ‰ Whole plant	Lipid	Residue	Amt. chloroform soluble material, % dry wt
Ulva sp.	−15.7	−23.5	−15.4	4.0
Gelidium sp.	−20.5	−24.3	−19.9	9.4
Macrocystis pyrifera (L.) Ag.	−12.6	−21.0	−12.7	1.7
Mixed Phyto plankton*	− 9.2	−14.2	− 8.3	6.8
Asplenium bulbiferum Forst.	−29.2	−37.9	−30.7	3.8
Triticum vulgare Vill.	−28.5	−31.1	−27.6	11.6
Sequoia gigantea (Lindl.) Dec.	−26.4	−28.3	−25.2	6.4
Solanum tuberosum L.	−25.6	−33.6	−25.6	0.2

* Kindly supplied by Dr. William Thomas of the Scripps Institution of Oceanography. Relative cell counts on the sample were as follows: *Prorocentrum micans* Ehrenb. 798, *Gymnodinium* sp. 13 with several other forms present in very minor amounts.

TABLE IV

C^{13}/C^{12} RATIO OF CO_2 RESPIRED FROM TOMATO PLANTS ($\delta = -25.2‰$) OVER LONG TIME INTERVALS

No. OF PLANTS	ELAPSED TIME FROM START OF EXP., hr	μmoles CO_2 RESPIRED BETWEEN COLLECTIONS	δ OF RESPIRED CO_2, ‰
19	14	1,230	−22.1
5	38	378	−23.9
5	86	462	−23.5
5	254	1,550	−25.7
5	518	1,880	−28.2

at the time intervals indicated in table IV. These plants ultimately began to decay. The CO_2 obtained near the end of the experiment was probably derived from bacterial decomposition of the plants. At the end of the experiment, the decomposed plant residue was collected and its isotopic composition measured. A material balance calculation for the respired CO_2 and residue as contrasted to the C^{13}/C^{12} ratio of the dried whole plants is made in table V. It may be concluded that there is good agreement between the isotopic composition of the plant as a whole and the value calculated from the respired CO_2 and the respiration residue.

TABLE V

MATERIAL BALANCE FOR RESPIRED CO_2 & RESIDUE AS COMPARED WITH INITIAL PLANTS

	MATERIAL (CALCULATED ON BASIS OF 1 PLANT)	AMOUNT OF C IN mmole	δ OF CO_2, ‰
1.	Intact plant (42.1 mg). Assume C = 43.6 % dry wt (8)	1.53	−25.2
2.	Respired CO_2	0.93	−26.2
3.	Residue. Assume C = 43.5 % dry wt (8)	0.73	−24.7
4.	Sum of lines 2 & 3	1.66	−25.6

RESPIRED CO_2 COLLECTED OVER SHORT INTERVALS: An experiment was done in which respired CO_2 was collected over much shorter time intervals than those in the first respiration experiment. Three 25-day-old, air grown tomato plants were placed in the respiratory chamber. The chamber was darkened and rapidly swept with CO_2-free air for 4 minutes. Carbon dioxide was then collected and analyzed at the intervals given in table VI. The rapid change in the δ values for the respired CO_2 over the first 10 minutes of the experiment probably represents the transition of dissolved CO_2 in the leaf from the photosynthetic state to the isotopically lighter respiratory state.

DISCUSSION

The results presented above show that the sugar polymers of the plant cell wall possess C^{13}/C^{12} ratios similar to the plant as a whole. This is not surprising, since cell wall material constitutes the bulk of the plant carbon. Lipids, on the other hand, are enriched in C^{12} compared to the whole plant, by as much as 8 per mil. The data in figure 3 show that the C^{13}/C^{12} ratio of plant lipids corresponds roughly to petroleum C^{13}/C^{12} ratios. The matching is especially good for the terrestrial petroleums. Contributions of terrestrial plant material to the marine environment may account for the somewhat low C^{13}/C^{12} ratio of marine petroleums. The fractionation, which enriched petroleums in C^{12} as compared to present day plants, may not have occurred by bacterial fractionation of plant cell wall carbon during sedimentation. Rather, the fractionation could have occurred within the living plant during the normal course of its fat synthesis. The difference in C^{13}/C^{12} ratio between present day plants and petroleums could result from the selective preservation of the C^{12} enriched plant lipid material during the decay of the plant organic matter. On the other hand, coal, which has about the same range of C^{13}/C^{12} ratios as present day plants, appears to represent preservation of representative plant organic material without preferential decomposition of either the lipid or carbohydrate fractions (see fig 1).

Any enrichment of C^{12} in one chemical component of the plant such as lipid must necessarily be accompanied by enrichment of C^{13} in some other component. The data of tables IV and VI show that respired CO_2 has a C^{13}/C^{12} ratio greater than the plant as a whole and is possibly the C^{13} enriched component which results from lipid formation. This proposal is supported by both biochemical and isotopic evidence. Respiration and lipid formation are closely related biochemical systems. The same two carbon fragment, acetate, is used both for lipid synthesis and for respiration in the tricarboxylic acid cycle. Isotope selection at the level of the two carbon unit acetate or the three carbon unit pyruvate provides an explanation for

TABLE VI

C^{13}/C^{12} RATIO OF CO_2 RESPIRED FROM TOMATO PLANTS ($\delta = 32.4$/mil) OVER SHORT TIME INTERVALS

SAMPLE NO.	ELAPSED TIME FROM START OF EXP.	μmoles CO_2 RESPIRED BETWEEN COLLECTIONS	δ, ‰
1	10 min	51	−24.3
2	2 hr	488	−25.8
3	4 hr	373	−27.2
4	6 hr	174	−27.6
5	16 hr	1,100	−28.2
6	40 hr	2,120	−30.0

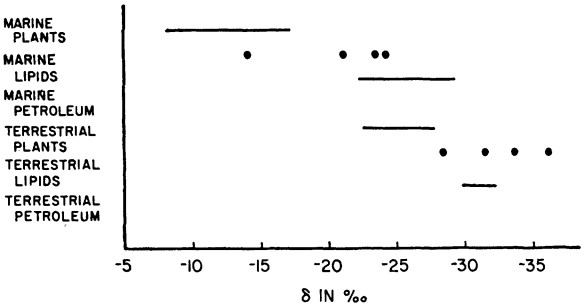

FIG. 3. C^{13}/C^{12} ratios of terrestrial and marine plants and petroleums as compared to the lipid fractions of terrestrial and marine plants.

the data obtained so far. Figure 4 outlines the biochemical pathways leading to lipid and respiratory CO_2 formation. That isotope selection actually does

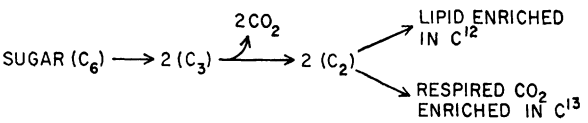

FIG. 4. The biochemical pathway leading from sugars to two carbon units and their subsequent metabolism.

occur at this point is indicated in figure 5, in which the degree by which the plant lipid is enriched in C^{12} above the level characteristic of the plant as a whole is plotted against the lipid content of the plant. It is noted that lipids are more enriched in C^{12} in those plants in which lipid is present in small amounts. This is consistent with the suggestion that C^{12} enriched C_2 units are selected for lipid synthesis. At the present time, the mechanism of this selection is a matter of speculation. It is interesting to note the wide taxonomic variety of plants which obey this relation.

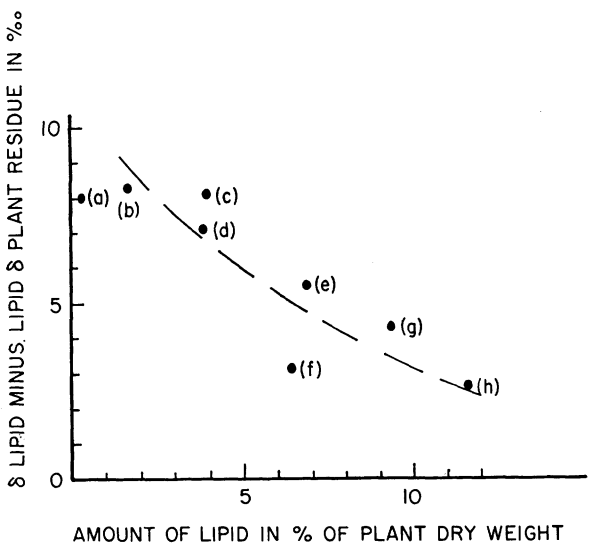

FIG. 5. C^{12} enrichment in lipid fraction as compared to the plant residue as a function of the amount of lipid in per cent of plant dry weight. (a) Potato, (b) brown alga, (c) green alga, (d) fern, (e) phytoplankton, (f) conifer, (g) red alga, (h) grass.

Baertschi (1) observed that CO_2 respired by germinating beans possessed the same C^{13}/C^{12} ratio as the whole seed. Our data indicate that CO_2 respired by tomato plants is initially of larger C^{13}/C^{12} ratio than the plant as a whole. These two results are not difficult to reconcile on the basis of the biochemical selection process proposed above. In the case of seeds germinating in the dark, lipids are not being formed, but are being oxidized along with the carbohydrates as an energy source. The C^{13}/C^{12} ratio of the respired CO_2 should be that of the sugars and lipid being respired as a whole. However, if C^{12} enriched lipid is being synthesized rather than oxidized, as in the case of growing tomato plants, the respired CO_2 would be expected to have a larger C^{13}/C^{12} ratio than the whole plant. The data of table IV show that CO_2 of higher C^{13}/C^{12} ratio than the plant as a whole is respired by tomato plants only during the initial portions of the experiment. This period can be interpreted as the time during which lipid synthesis is still occurring. When lipid is no longer being produced, the respired CO_2 would have a C^{13}/C^{12} ratio similar to that of the plant as a whole. CO_2 of lower C^{13}/C^{12} ratio than the plant, evolved during the decomposition of the plant, may result from preferential decomposition of the lipids, fractionation during bacterial respiration, or a combination of these two phenomena.

SUMMARY

C^{13}/C^{12} ratio analyses of chemical fractions from several plant phyla show that in all cases the lipid fraction is enriched in C^{12} compared to the whole plant. The C^{13}/C^{12} ratio of the plant lipids corresponds roughly to the C^{13}/C^{12} ratio of petroleums. The C^{12} enrichment in petroleums as compared to present day plants can be explained if selective preservation of plant lipids occurred during the sedimentation process. The degree of C^{12} enrichment in the plant lipid fraction is inversely related to the amount of lipid in the plant. The C^{12} enrichment which occurs in plant lipids may be balanced by the C^{13} enrichment which occurs in respired CO_2. Isotope selection at the level of acetate or pyruvate is a possible mechanism for explaining our results.

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