I. THE BIOSYNTHESIS OF OPEN CHAIN TERPENES IN PLANTS

II. FRACTIONATION OF THE STABLE CARBON ISOTOPES IN PLANTS

Thesis by

Roderic Bruce Park

In Partial Fulfillment of the Requirements

For the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1958

ACKNOWLEDGMENTS

The author wishes to express his appreciation to Dr. James Bonner for the guidance and enthusiasm he imparted toward the completion of this work. The author is indebted to Dr. Samuel Epstein of the Division of Geology who supervised much of the second portion of this thesis. Dr. Epstein was not only generous with his advice and laboratory equipment, but also contributed much to the author's understanding of the formulation and execution of scientific problems. The author also wishes to express his appreciation to Dr. Frits Went and Dr. Walter McNutt for their valuable advice concerning the execution of these experiments.

The author is indebted to the California Institute of Technology, the Arthur McCallum Fellowship Fund and The Woods Hole Oceanographic Associates for financial support during his graduate work at the Institute.

ABSTRACT

I. Open chain terpene synthesis in plants was studied by measurement of the incorporation of potential intermediates into the rubber of the rubber producing plants Taraxacum kok saghyz and Hevea brasiliensis. Intact plants incorporate 1-C^{14}-acetate and 2-C^{14}-acetate into rubber without randomization of the label. β-Methylcrotonic acid was found to be an ineffective rubber precursor in intact plants.

Enzymatic experiments were performed using Hevea latex as a source of enzyme. C^{14}-acetate is rapidly incorporated into a volatile, non-acidic, non-polar substance in this system under anaerobic conditions. C^{14}-acetate is not incorporated into rubber. Mevalonic acid is rapidly incorporated into rubber in this system. Partial degradation of the rubber indicates that no randomization occurs during incorporation. This result suggests that mevalonic acid is on the pathway of terpene synthesis in plants.

II. The two stable carbon isotopes, C^{12} and C^{13}, occur in nature in the ratio of about ninety to one. Various workers have shown that this ratio is not fixed, but may vary by as much as 5%. Interestingly enough, this variation is not random. Carbon reservoirs such as limestone, atmospheric CO_2, land plants, algae and coal all exhibit characteristic C^{13}/C^{12} ratios. This section of the dissertation is concerned with the differences between the C^{13}/C^{12} ratios of plants and those of the carbon sources from which such plants have grown.

Both algae and terrestrial plants have smaller C^{13}/C^{12} ratios than those of dissolved carbonates and atmospheric CO_2 respectively. The magnitude of this fractionation was determined for tomato plants by growing the plants from seed in CO_2 of known isotopic composition. Separation of the plant material into its component chemical constituents showed that only the lipid fraction differed markedly in C^{13}/C^{12} ratio from that of the plant as a whole. The lipid fraction is enriched in C^{12} and possesses a C^{13}/C^{12} ratio similar to that of petroleums derived from land plants. A similar relation was found to exist between marine algae, their lipids, and petroleums of marine origin. The CO_2 evolved by plant respiration is slightly enriched in C^{13} as compared to the plant. This process is apparently closely related to the C^{12} enrichment in lipid fractions.

A possible mechanism for fractionation of C^{13} and C^{12} in photosynthesis is suggested. This suggestion is supported by observations of the C^{13}/C^{12} ratio of CO_2 dissolved in higher plants and by determination of the fractionation which occurs during fixation of CO_2 by the photosynthetic carboxylation enzyme.

TABLE OF CONTENTS

SECTION PAGE

GLOSSARY OF ABBREVIATIONS

ATP	-	Adenosine triphosphate
DPN	-	Diphosphopyridine nucleotide
TPN	-	Triphosphopyridine nucleotide
CoA	-	Coenzyme A
GSH	-	Glutathione
AcAc	-	Acetoacetate
BOG	-	β-Methyl-β-hydroxyglutaric acid
BMC	-	β-Methylcrotonic acid
EDTA	-	Ethylenediaminetetraacetic acid
TCA	-	Trichloroacetic acid
MgFDP	-	Magnesium fructose 1,6-diphosphate
RuDP	-	Ribulose 1,5-diphosphate
PGA	-	3-Phosphoglyceric acid
p-p	-	Pyrophosphate

I. THE BIOSYNTHESIS OF OPEN CHAIN TERPENES IN PLANTS

GENERAL INTRODUCTION

All green plants contain terpenes. Phytol, carotenoids, the side chain of vitamin K and rubber are a few of the terpenes synthesized by higher plants. Terpenes are characterized by their structure which consists of two or more isoprene units joined by 1,4 carbon to carbon bonds. This basic structure is shown in Figure 1. Terpenes consisting of two isoprene units are referred to as simple terpenes. Those consisting of three, four, six, eight and many isoprene units are known as sesqui-, di-, tri-, tetra-, and polyterpenes respectively. Terpenes may be either open chain or cyclic compounds. The latter include the steroids and many of the constituent terpenes of the essential oils.

This thesis is concerned with the biosynthesis of open chain terpenes in higher plants. The biosynthesis of these compounds is probably similar, in initial stages, to that of the more elaborate cyclic terpenes. This is apparently true in animals in which the open chain terpene squalene has been shown to be a cholesterol precursor (1).

The particular terpene chosen for this study is the plant polyterpene rubber. Rubber is an open chain polyterpene which occurs in about 4,000 species of plants. Rubber was chosen as a material for the study of terpene biosynthesis for the following reasons:

a. Some plants are known to make rubber quite rapidly. In particular, Arisz (2) has calculated that the commercial rubber

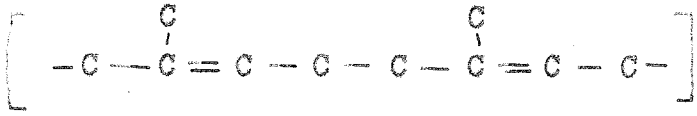


Figure 1. The carbon skeleton of two isoprene units joined by 1,4 carbon to carbon bonds in an open chain terpene.

tree, Hevea brasiliensis, produces more than 300 mg of rubber per 100 cc of latex per hour under optimal conditions.

b. Rubber can be degraded so as to obtain fairly unambiguous information as to the position of the C^{14} atoms incorporated into the terpene carbon skeleton.

c. Latex obtained by tapping an Hevea tree may be used as a crude, cell free system for studying the incorporation of C^{14}-labeled intermediates into rubber.

Rubber has one definite shortcoming as a compound for the study of terpene synthesis. There is no easy way to purify rubber nor any unambiguous criterion of purity other than the information obtained by chemical degradation. The solubility of rubber in non-polar solvents is shared by many other lipid materials. In the presence of C^{14}-labeled intermediates, these other materials often become much more highly labeled than the rubber. These materials may be fatty acids or other non-terpenes. Degradation of the rubber to levulinic acid circumvents this difficulty since non-terpenaceous materials do not yield this product. The degradation, however, introduces a new uncertainty. It is no longer known whether the labeled levulinic acid arose from rubber or from some other highly labeled open chain terpene present in small amounts. Since the systems in which terpene synthesis have been studied here make primarily the open chain terpene rubber, it is quite probable that it is actually rubber biosynthesis and not the synthesis of some other terpene which is studied in this dissertation.

Some initial experiments were done on lemon grass which produces the simple terpene citral. The rate of incorporation of the C^{14} of $C^{14}O_2$ into citral by intact plants was so low as to discourage further work on this plant. Experiments were then carried out with two rubber producing plants, the Russian dandelion, Taraxacum kok saghyz and the rubber tree, Hevea brasiliensis. These plants are shown to incorporate C^{14}-acetate into rubber with a distribution of labeling consistent with the carbon pathway suggested by Bonner (3). BMC is shown to be a poor rubber precursor in intact plants. Enzymatic incorporation of proposed intermediates into rubber is also studied. It will be shown that rubber is synthesized enzymatically in good yield from the 6 carbon branched chain compound, mevalonic acid.

REVIEW

During the past 15 years, much effort has been directed toward identification of the monomer which polymerizes in terpene biosynthesis. It was thought, until two years ago, that terpenes arose from the polymerization of one of several postulated five carbon intermediates. Recent evidence indicates, however, that the immediate terpene precursor contains not five, but six carbons. Evidence which forms the basis for our present thoughts concerning biosynthesis of open chain terpenes is reviewed below.

Figure 2 gives the various steps in terpene biosynthesis as they are now thought to occur. Not all these reactions have been demonstrated in biological systems. Nevertheless, all the reactions have precedent in other enzyme mediated reactions. Most of these steps have been studied more thoroughly in animals than in plants. The same intermediates, however, are involved in both animal and plant systems, and the experimental evidence obtained in both systems is usually complementary. The first steps of this carbon pathway involve acetate. Acetate labeled with C^{14} in the one or two position is incorporated by intact tissues and by enzymatic systems into a number of terpenes and terpene intermediates. The incorporation of C^{14}-acetate into the triterpene squalene has been studied extensively in animal and yeast systems. Langdon and Block (4) observed that squalene is synthesized

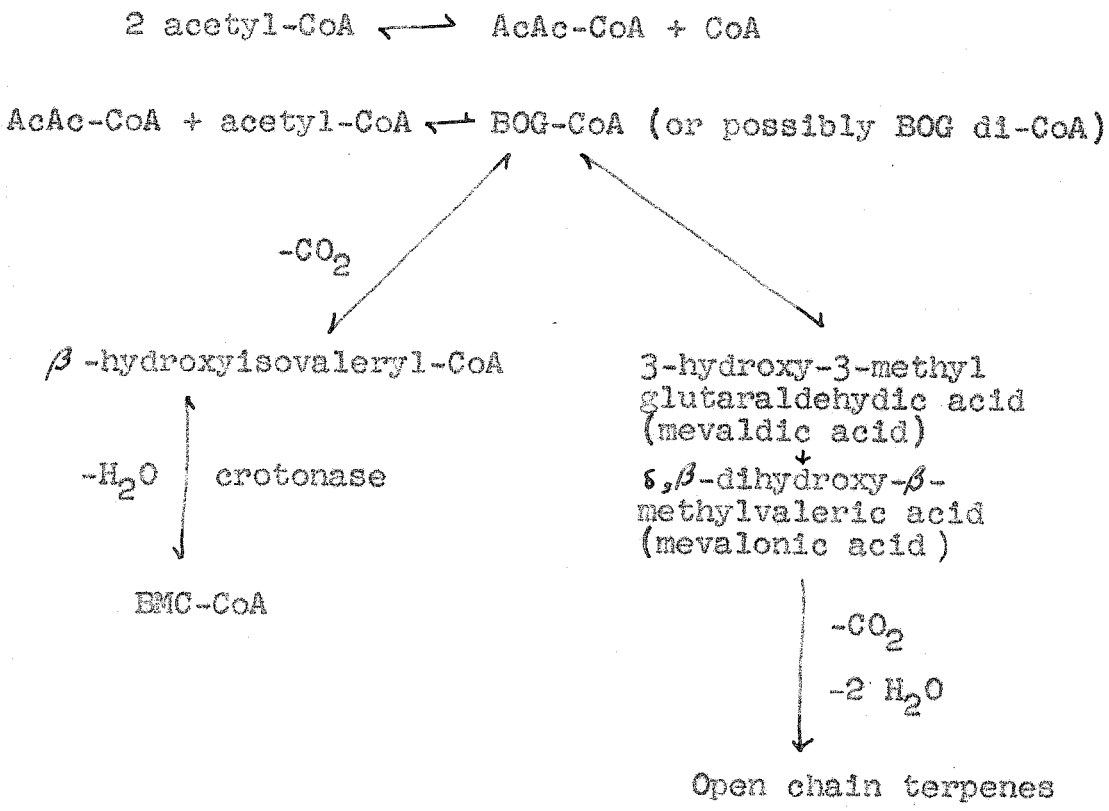
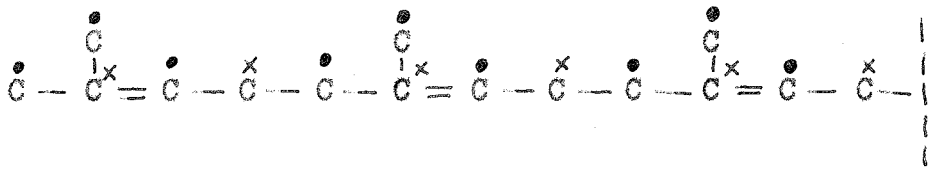


Figure 2. The probable carbon pathway leading terpene synthesis in plants and animals.

from both 1-C^{14}-acetate and 2-C^{14}-acetate in the whole rat. In 1954 Cornforth and Popjak (5), in a magnificent piece of work, completely degraded squalene which had been synthesized from 2-C^{14}-acetate in a liver mince and found the distribution of labeling shown in Figure 3. This supports Bloch's (6) findings on the incorporation of 1-C^{14}-acetate and 2-C^{14}-acetate into the isooctyl side chain of cholesterol and conforms to the general scheme for terpene synthesis in Figure 2. Dituri et al. (7), Bucher et al. (8,9), and Rabinowitz et al. (10) have all reported enzymatic systems which incorporate acetate into animal terpenes. Corwin et al. (11) have reported the incorporation of acetate into squalene in yeast. In plants, acetate has been shown to enhance the rate of rubber formation in guayule (12). Acetate is also incorporated into rubber, assayed as the rubber tetrabromide, in whole guayule plants (13). Teas and Bandurski (14) have recently reported acetate incorporation into rubber in a crude enzymatic system from Hevea. Zabin (15) has investigated the incorporation of C^{14}-acetate into lycopene in tomatoes. He finds complete randomization of the label which, due to the long time periods (12 days) used, might be expected. The formation of β-carotene from specifically C^{14}-labeled acetate in bacteria has been investigated by Grob and Bütler (16). They find a pattern of labeling identical to that described by Cornforth and Popjak for squalene (5).



• - labeled carbons

x - unlabeled carbons

Figure 3. The distribution of C^{14} in the carbon of squalene enzymatically labeled from 2-C^{14}-acetate according to Cornforth and Popjack (5).

The above evidence indicates strongly that acetate gives rise to terpenes without the randomization which would occur if acetate were reassimilated into the carbohydrate pool.

Before the role of CoA in lipid metabolism was realized, a number of carbon pathways were proposed for terpene biosynthesis which, in the light of present knowledge, are probably incorrect (12,17,18). It is now known that acids such as acetate must be activated before they can participate in lipid metabolism. This activation consists of formation of acyl-CoA compounds at the expense of ATP hydrolysis. This reaction occurs in many animal tissues. That such reactions also occur in plants has been shown by Millerd and Bonner (19), and Patrick (20). The reaction may be formulated as shown below.

$$\text{ATP} + \text{CoA} + \text{carboxylic acid} \rightleftharpoons \text{acyl-CoA} + \text{AMP} + \text{p-p}$$

The first step in terpene synthesis is the condensation of two acetyl-CoA units to form AcAc-CoA as shown in Figure 2. This step has been demonstrated in animal systems by Rudney (21) and Bachhawat et al. (22). Acetoacetate formation from acetyl-CoA in a spinach leaf system has been studied by Millerd and Bonner (19) and in a flax enzyme system by Johnston, Racusen and Bonner (3). According to subsequent work of Rudney (21), this reaction is apparently formulated as shown below.

2 acetyl-CoA ⟷ acetoacetyl-CoA + CoA

The next step in terpene biosynthesis is the addition of another acetate to acetoacetate to form the six carbon intermediate β-methyl-β-hydroxyglutaric acid (BOG). This reaction has been studied both with respect to terpene synthesis and to leucine degradation (22). Acetyl-CoA reacts with acetoacetate as shown in Figure 2. The reaction is analogous to the acetylation of oxalacetate to form citrate. Rudney points out that whether BOG exists as the mono- or di-CoA derivative at this point is not known. It is interesting to note that BOG does not exist as a free acid in nature but is bound as a diester in both flax and in the Crotalaria alkaloid dicrotaline (23,24). This is not inconsistent with the idea that BOG may exist as a di-activated acid. The biosynthesis of BOG has been studied by Rudney (21,25), Bachhawat, Robinson and Coon (22), Rabinowitz and Gurin (10), and Johnston, Racusen and Bonner (23).

The distribution of labeling in BOG synthesized by a rat liver homogenate from 2-C^{14}-acetate as determined by Rudney (25) is shown in Figure 4. The three labeled carbons represent the three methyl carbons of acetate. This scheme is consistent with the carbon pathway shown in Figure 2. Bloch (26) has shown that 3-C^{14}-BOG is incorporated into cholesterol in the rat. Since the labeling of fatty acids was small, he concluded that the conversion is fairly direct.

Figure 4. The distribution of C^{14} in the carbon of BOG enzymatically labeled from 2-C^{14}-acetate according to Rudney (25).

Dituri et al. (7) found that methyl-C^{14}-BOG is incorporated into squalene in a rat liver homogenate. Coon (22) has shown that BOG-CoA undergoes decarboxylation to form β-hydroxyisovaleric acid. The reverse decarboxylation reaction requires ATP and in fact the active carboxylating agent appears to be carbonyl phosphate. The β-hydroxyisovaleric acid in turn is acted upon by crotonase to yield BMC. Johnston, Racusen and Bonner (23) report a similar system in flax seedlings. Both Rudney (27) and Rabinowitz (28) have investigated the incorporation of C^{14}-acetate into BMC in enzymatic systems. The distribution of labeling found by Rudney (27) for BMC with 2-C^{14}-acetate as the substrate is shown in Figure 5. This pattern of labeling is consistent with that found in BOG. Bonner (12), on the basis of evidence obtained with guayule seedlings nutrition experiments, proposed that BMC might be an intermediate in terpene biosynthesis. Though BMC is closely related to other terpene intermediates, in the light of recent evidence, it is not on the major pathway of terpene synthesis.

In 1956, Folker's group, at the Merck laboratories, isolated a factor capable of replacing acetate in the nutrition of a strain of lactobacillus (29,30). This compound was shown by synthesis to be δ,β-dihydroxy-β-methylvaleric acid (mevalonic acid) (31,32,33). Mevalonic acid labeled with C^{14} in the two position was synthesized and incubated with an enzymatic rat liver cholesterol synthesizing

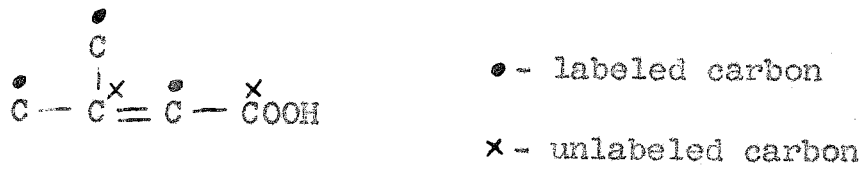


Figure 5. The distribution of C^{14} in the carbon of BMC enzymatically labeled from 2-C^{14}-acetate according to Rudney (27).

system (34). Ninety percent incorporation of one of the two isomers of mevalonic acid into cholesterol was obtained. Table I gives the relative effectiveness of BOG, BMC and mevalonic acid as precursors for cholesterol in this system. It may be seen that mevalonic acid is by far the most effective cholesterol precursor. Tavormina and Gibbs (35) have shown that the carboxyl carbon of 1-C^{14}-mevalonic acid is not incorporated into cholesterol. On the contrary, all of the C^{14} activity is recovered as CO_2 lost from the system. This suggests that the carboxyl carbon is lost during the incorporation of mevalonic acid into cholesterol.

Mevalonic acid may be thought of as BOG in which one carboxyl group has been reduced to the level of a primary alcohol. Analogous reductions in biochemistry occur in two steps in which the aldehyde occurs as an intermediate oxidation state. Shunk et al. (36) showed that DL-3-hydroxy-3-methylglutaraldehydic acid (mevaldic acid) inhibits C^{14}-acetate incorporation into cholesterol on a molar basis almost to the same extent as mevalonic acid. Incorporation of C^{14}-mevaldic acid into terpenes has not yet been reported.

There are three reports of incorporation of C^{14}-mevalonic acid into terpenes from other than the Merck laboratory. Wright (37) reported that liver homogenate supernatant is highly active in the conversion of mevalonic acid into a non-saponifiable material. Addition of a microsomal fraction was necessary for conversion of this substance into

TABLE I

The relative effectiveness of BOG, BMC and mevalonic acid as cholesterol precursors according to Tavormina *et al.* (34).

Substrates fed in equimolar amounts	% Conversion to cholesterol
3-C^{14}-BOG	0.16
4-C^{14}-BMC	3.8
DL-2-C^{14}-mevalonic acid	43.4

cholesterol. Amdur, Rilling and Bloch (38) reported the enzymatic conversion of mevalonic acid into squalene. This conversion requires enzyme, Mn^{++}, ATP and DPN or TPN. Dituri, Gurin and Rabinowitz (39) also reported incorporation of 2-C^{14}-mevalonic acid into squalene. These workers carried out the degradation of squalene, and the distribution of labeling which they found is shown in Figure 6. Unsaturation is achieved by the removal of water from carbon atoms three and four of mevalonic acid.

No one has, as yet, demonstrated the reduction of BOG to mevaldic or mevalonic acid in a biochemical system. This is the most severe criticism and the weakest point of the scheme proposed in Figure 2. Mevalonic acid is, however, a natural product. In this dissertation it is shown that mevalonic acid is incorporated enzymatically into rubber. The fact that mevalonic acid is incorporated so effectively into both animal and plant terpenes suggests that if mevalonic acid is not a true intermediate, it must certainly be very similar to the real compound. Verification of this point awaits the isolation of C^{14}-mevalonic acid from a C^{14}-acetate fed, terpene producing system.

The scheme of terpene synthesis presented in Figure 2 is in accord with all of the major bodies of evidence. Much work is still necessary, however, to verify the scheme for plant systems.

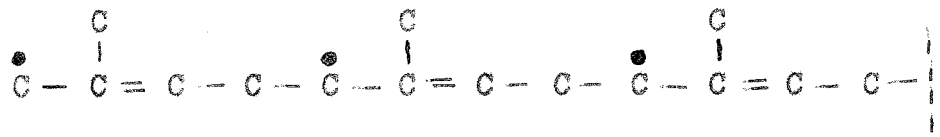


Figure 6. The distribution of C^{14} in the carbon of squalene enzymatically synthesized from DL-2-C^{14}-mevalonic acid according to Dituri *et al.* (39).

• - labeled carbons

EXPERIMENTAL APPROACH

In the initial experiments, intact plants were fed C^{14}-acetate, or C^{14}-BMC through the roots. The rubber from these plants was isolated, partially degraded and the distribution of labeling determined. An effort was then made to effect the incorporation of C^{14}-labeled intermediates into rubber in a cell free system from Hevea. The only substrate incorporated at a sufficiently high level to permit of partial degradation and determination of labeling was 2-C^{14}-mevalonic acid.

METHODS AND PROCEDURES

Three plants (Lemon grass, Cymbopogon citratus, Russian dandelion, Taraxacum kok saghyz, and the rubber tree, Hevea brasiliensis) were used to study open chain terpene biosynthesis in intact systems. The plants were fed $C^{14}O_2$ in the light for short time periods to determine the rate of terpene synthesis in the plant. In further experiments the plants were fed various C^{14}-labeled intermediates through the roots and the terpene was isolated and degraded. This method was used to compare the relative effectiveness of possible rubber precursors in intact systems. Enzymatic incorporation of intermediates into rubber was also studied.

Isolation of citral.

Lemon grass plants were broken up and placed in a 1,000 ml distilling flask with 600 ml of water and steam distilled. The distillate was then extracted five times with 200 ml of $CHCl_3$. The $CHCl_3$ extract was then refluxed with excess 2,4-dinitrophenylhydrazine for four hours. The 2,4-dinitrophenylhydrazone derivative was chromatographed on an activated alumina column 0.5 x 18 cm. The column was washed with petroleum ether and developed with a mixture containing 70% petroleum ether and 30% benzene. The band corresponding to the known derivative was collected and crystallized to constant specific activity.

Isolation of rubber from whole plants.

The plant was ground up and extracted four or five times with boiling ethanol. The residue was dried and then extracted with either chloroform or benzene. The filtered non-polar extract was then washed with 10% KOH until no more C^{14}-labeled material could be extracted by this method. The non-polar extract was reduced to small volume and precipitated with five times that volume of ethanol. The precipitated rubber was then collected and dried. This constitutes the fraction termed crude rubber in this dissertation. Rubber from enzymatic reaction mixtures was isolated by essentially the same procedure except that the initial grinding step was omitted.

Preparation of rubber tetrabromide.

Crude rubber was dissolved in $CHCl_3$. Bromine dissolved in $CHCl_3$ was then added dropwise until no more bromine was taken up. The derivative was precipitated with ethanol and washed several times with an ethanol, H_2O mixture. The derivative was then weighed and counted. This is essentially the method described by Willits, Swain and Ogg (40).

Purification and degradation of rubber.

Crude rubber as defined above still contains many C^{14}-labeled contaminants. It was also found that precipitation of rubber as the tetrabromide derivative does not free rubber from C^{14} contamination by other sources. To be certain that the material counted is actually terpenoid in nature, it is

necessary to degrade the rubber and isolate and purify the degradation products. The degradation used was that of ozonolysis followed by hydrolysis, outlined in Figure 7.

Twenty-five milligrams of crude rubber were found to be the minimum amount of material which could be successfully degraded to levulinic acid. The ozonolysis reaction was carried out as follows. The rubber was dissolved in about 10 ml of $CHCl_3$ and placed in a tube 1.5 cm in diameter through which ozone could be bubbled. The $CHCl_3$ solution was cooled to 0°C on an ice bath and the outlet bubbled through a trap containing KI. Oxidation of iodide ion to free iodine occurred when ozone was no longer absorbed by rubber and passed into the KI trap. The appearance of free iodine indicated that the ozonization of rubber was complete.

After ozonization, the $CHCl_3$ solvent was distilled off under vacuum. Two milliliters of distilled water were added and the flask contents were refluxed for one hour. The solution was then filtered. Oxidation of the aldehydes present was carried out by first acidifying the solution with H_2SO_4 and then adding a dilute $K_2Cr_2O_7$ solution dropwise. As the yellow color disappeared, more dichromate was added until, after heating 10 minutes at 80°C, no further change in color was noted. Excess chromic acid was reduced by addition of sodium sulfite. The acid solution was extracted with ether for six hours. The extract was placed on Whatman #1 or #3 filter paper and chromatographed in ethanol : NH_4OH : H_2O, 40 : 4 : 8. In this

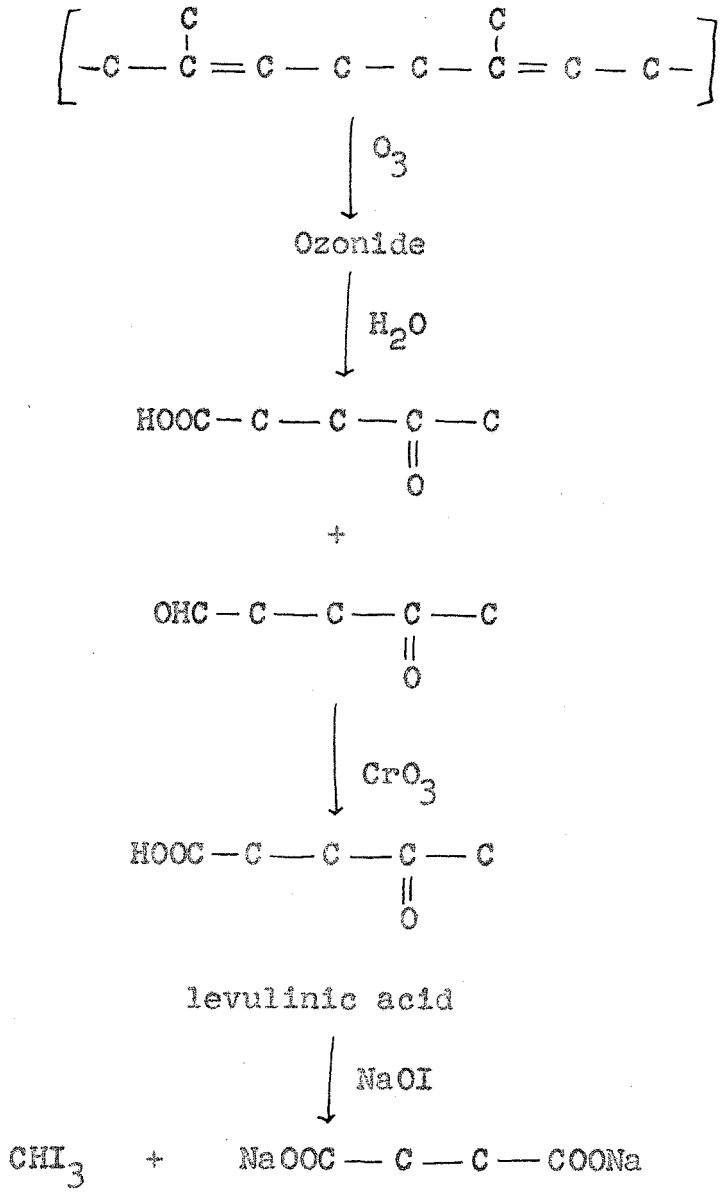


Figure 7. Procedure used for degrading rubber.

solvent levulinic acid possesses an Rf value of about 0.65. The levulinic acid band was eluted with water and treated with 2,4-dinitrophenylhydrazine. The precipitate usually formed rather slowly and was recrystallized by dissolving it in dilute ammonia and neutralizing with HCl. The solution was placed at 0°C and the crystals separated in the cold. These crystals have the melting point of authentic levulinic 2,4-dinitrophenylhydrazone. Chromatography of the 2,4-dinitrophenylhydrazone of levulinic acid against authentic material in $(NH_4)_2CO_3$: NH_4OH, 1:1 or in n-butanol : ethanol : 0.5 N NH_4OH, 7:1:2, also indicated the identity of the two compounds. The yield of levulinic acid by this degradation is usually 10-20%. Table II shows the specific activity of rubber isolated from a C^{14}-acetate fed kok saghyz plant at various stages of purification. These results indicate that both the crude rubber and tetrabromide derivative contain some C^{14}-labeled material which does not yield levulinic acid upon degradation.

In this thesis it is assumed that the specific activity of purified levulinic acid derived from rubber is the only criterion which can be used to judge the incorporation of C^{14} intermediates into rubber.

Degradation of levulinic acid.

The levulinic acid in aqueous solution was made basic with NaOH and treated with an iodine-potassium iodide solution. Iodoform separated and was collected and purified by subli-

TABLE II

Specific activity of kok saghyz rubber labeled with 1-C^{14}-acetate at various stages of purification.

Material	CPM/10^{-4} mol rubber*
Crude rubber	782
Rubber tetrabromide	421
Levulinic acid counted as 2,4-dinitrophenylhydrazone after chromatography in ethanol, NH_4OH, H_2O.	320
Levulinic acid 2,4-dinitrophenyl-hydrazone after chromatography in $(NH_4)_2CO_3$, NH_4OH.	313

* A mol of rubber is defined as one 5 carbon unit (MW = 68).

mation. The succinate in the supernatant was then isolated as follows: the solution was acidified and free iodine was formed. The solution was then treated with sufficient sodium sulfite so that all the iodine was reduced to iodide ion. The acidified solution was then ether extracted for six hours. The ether extract was chromatographed in ethanol : NH_4OH : H_2O, 40:4:8 and the band corresponding to succinate was eluted and counted as the crystalline acid after crystallization from an ether ligroin solution.

Latex collection.

The Hevea trees available for latex collection were only about 1 m high. The stem diameter was about 1 cm. Latex was obtained by tapping the trees. This was done by cutting diagonally through the bark with a razor blade. A 1 mm capillary was then placed against the drops of latex which form along the cut. The latex was allowed to flow into the capillary of its own accord by capillary and gravitational attraction. Care must be taken with latex collected in this way to prevent coagulation. Coagulation tends to occur upon physical maltreatment, dilution of the latex or prolonged exposure to temperatures greater than 30°C.

Preparation of reaction mixtures.

Since Hevea latex tends to coagulate upon dilution, the ratio of latex volume to the volume of other components in the reaction mixture was kept as large as possible. A ratio of 1.5 to 1 or greater was necessary for the latex obtained from these trees.

Isolation of rubber from reaction mixtures.

At the end of the incubation, 2 ml of ethanol were added to the reaction mixture which usually had a volume of less than 0.5 ml. The precipitate was extracted three times with 2 ml volumes of ethanol. The reaction mixture was then dried and dissolved in $CHCl_3$. Purification and degradation were then carried out as described above.

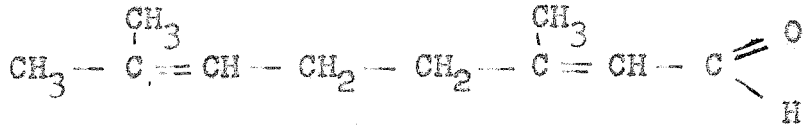
Radioactive assay of samples.

All samples were counted at infinite thinness in a micromil gas flow scaler (Nuclear Chicago Model 181). The aluminum planchets used were 2.9 cm in diameter and sand blasted on the inside surface. All samples were counted for 10^3 or more counts. The counting error with this equipment under these conditions is less than 5%.

RESULTS

Lemon grass.

Lemon grass contains large quantities of the simple terpene citral whose structure is shown below.



Citral

A simple, easily isolated terpene such as citral would provide an excellent model for the study of terpene formation in plants. Two C^{14}-labeling experiments were therefore done to determine the suitability of lemon grass for studies on terpene biosynthesis. In the first experiment $C^{14}O_2$ was fed to a four week old plant for 48 hours under natural photoperiod. In a second experiment, a lemon grass plant was fed 1-C^{14}-acetate through the roots. These experiments are described below.

$C^{14}O_2$ was fed by acidifying 2.1 mg of 19% $BaC^{14}O_3$ with HCl in the presence of a four week old lemon grass plant in a closed glass cylinder. After 48 hours in the greenhouse, the plant was removed and the citral isolated as described above (Methods and Procedures). Results are reported in Table III.

TABLE III

Incorporation of $C^{14}O_2$ and C^{14}-acetate into citral in intact lemon grass plants.

C^{14} compound fed	Specific act	Amt. fed (mg)	CPM/5×10^{-5} mol citral
$C^{14}O_2$	19% C^{14}	0.5	2,402
1-C^{14}-NaOAc	1 mc/mM	9.0	238

In the second experiment a four week old lemon grass plant was allowed to take up 9.4 mg 1-C^{14}-NaOAc for 30 hours in the dark. The citral was isolated according to the procedure described above. The results are reported in Table III. The low orders of activities obtained in these two experiments suggest that lemon grass is a poor system for the study of terpene synthesis. The results also indicate that citral is metabolically very inactive in lemon grass. This plant was therefore abandoned and search continued for a plant in which terpene synthesis occurs at a greater rate.

Taraxacum kok saghyz.

The Russian dandelion, kok saghyz, contains rubber in the latex vessels of its fleshy roots. Considerably greater rates of incorporation of C^{14} intermediates into terpene material were found with this plant than were found with lemon grass. The plants were grown from seed. They were four months old and weighed about 11.3 gm fresh weight when used.

In an initial experiment $C^{14}O_2$ was fed to two groups of three plants each. The plants were placed in the glass cylinder used in feeding the lemon grass. $C^{14}O_2$ was obtained by acidification of 2 mg of 19% $BaC^{14}O_3$ inside the cylinder. One group was also removed from the cylinder and allowed to grow in a normal atmosphere for five more days. The rubber was extracted and degraded as described above. The data for this experiment, given in Table IV, indicate two things. First, rubber is not made sufficiently rapidly to dilute out

TABLE IV

Incorporation of $C^{14}O_2$ into Taraxacum kok saghyz rubber in intact plants.

Days fed	Days growth after feeding	CPM/10^{-4}mol levulinic 2,4-dinitrophenyl-hydrazone
1	0	1,820
1	5	2,490

to any large extent C^{14} which has already been incorporated into rubber. Second, C^{14} which does not appear in rubber immediately after fixation by photosynthesis is not incorporated to any great extent into rubber during the subsequent period in unlabeled CO_2. This suggests that rubber biosynthesis in kok saghyz does not involve intermediates which persist for any length of time before incorporation into rubber.

Acetate labeled with C^{14} in the carboxyl position was fed through the roots to kok saghyz seedlings in two separate experiments. In the second experiment 2-C^{14}-acetate and 3-C^{14}-BMC were also fed to plants from the same group. These two experiments are reported below.

In the first experiment, 12 plants about 4 cm tall were placed in a solution containing 2 mg of 1-C^{14}-NaOAc of specific activity 1 mc/mM. The plants were allowed to take up the C^{14}-acetate for 18 hours in the dark. About 60% of the C^{14}-acetate solution was taken up in this time. The rubber was then isolated and degraded as described above. The results are reported in Table II. Thirty-two and four-tenths milligrams crude rubber were obtained. This experiment was the first to suggest that the rubber tetrabromide may contain C^{14} compounds other than rubber.

In the second experiment, 120 kok saghyz plants were divided into three groups and fed C^{14}-labeled compounds through the roots as indicated in Table V. The rubber was

TABLE V

C^{14} labeled compounds fed to intact kok saghyz seedlings in the dark through the roots.

Compound fed	Amount fed (mg)	Specific activity	Duration of feeding
1-C^{14}-NaOAc	2	1 mc/m M	21 hours
2-C^{14} NaOAc	2	3.5 mc/m M	21 hours
3-C^{14} BMC (Na salt)	1	1 mc/m M	21 hours

then extracted and degraded as described above. These results are presented in Table VI. The low specific activities of these samples make them subject to considerable error.

It is interesting to note that the iodoform from levulinic acid derived from the 1-C^{14}-acetate labeled rubber was unlabeled. On the contrary, iodoform derived from levulinic acid from 2-C^{14}-acetate labeled rubber contains about one-third of the total label in the molecule. This is predicted by the scheme proposed in Figure 2. BMC is the most poorly incorporated of the substrates tried. At the time it was felt that poor incorporation was due to inability of the plant to form the CoA ester of BMC. The work on Hevea reported below, however, indicates that this is probably not the case.

Hevea brasiliensis.

Hevea seeds from Puerto Rico were germinated in the Earhart Plant Research Laboratory. When these plants were 20 cm high and weighed about 12.5 gm wet weight, they were used for C^{14}-labeling experiments.

In the first experiment, $C^{14}O_2$ was fed to one Hevea plant (12.5 gm fresh weight) to determine the relative rate of rubber synthesis as compared to that of the other plants investigated. $C^{14}O_2$ was prepared by acidifying 3.8 mg of 19% $BaC^{14}O_3$. The $C^{14}O_2$ was fed to the plant for 21 hours under natural photoperiod. The plant was then removed from the chamber and ground with ethanol in an omnimixer. The

TABLE VI

Incorporation of 1-C^{14}-acetate, 2-C^{14}-acetate and 3-C^{14}-BMC into the rubber of intact kok saghyz seedlings.

Material	CPM/10^{-4} mol rubber
1-C^{14}-acetate exp. (47 mg rubber obtained)	
Crude rubber	184
Levulinic 2,4-dinitrophenyl-hydrazone	102
Iodoform	3
2-C^{14}-acetate exp. (14.6 mg rubber obtained)	
Crude rubber	900
Levulinic 2,4-dinitrophenyl-hydrazone	150
Iodoform	61
3-C^{14}-BMC exp. (29.2 mg rubber obtained)	
Crude rubber	20

plant material was extracted four times with hot methanol. This extract contained 64 x 10^6 CPM. The rubber was next extracted and degraded according to the procedures described above. Twelve milligrams of rubber were obtained and contained 9,200 CPM/10^{-4} mol of rubber. This result shows that the rate of incorporation on a specific activity basis is considerably higher in Hevea than in kok saghyz.

In a second experiment, 10 Hevea seedlings, 134.5 gm fresh weight, were fed 24 mg. of 2-C^{14}-NaOAc (3.5 mc/mM) through the roots for 24 hours in the dark. All of the acetate solution was taken up. Rubber was isolated according to the procedures described above but with the following modification which was aimed at removal of contaminating materials. The plant material was first extracted successively with hot methanol, ethanol and acetone. The residue was then extracted with benzene as described above. Sixty milligrams of crude rubber were obtained. The degradations to levulinic acid and iodoform were carried out as described above. The results in Table VII show that the labeling in the levulinic acid obtained in this degradation is consistent with that found in kok saghyz and with the scheme proposed in Figure 2.

Dr. Walter McNutt of this laboratory carried out similar 2-C^{14}-acetate feeding and 1-C^{14}-acetate feeding experiments on the same group of Hevea plants. The results of these

TABLE VII

Incorporation of 2-C^{14}-acetate into the rubber of intact Hevea plants.

Material	CPM/10^{-4}mol rubber
Crude rubber	5,340
Levulinic acid 2,4-dinitrophonyl-hydrazone	3,190
Iodoform	1,000

C^{14}-acetate feeding experiments, summarized in Table VIII, show again that only 2-C^{14}-acetate labels the methyl carbon of rubber. The amount of labeling in the methyl group as compared to levulinic acid as a whole is approximately that which would be expected on the basis of the scheme for terpene biosynthesis presented in Figure 2. This pattern of labeling is therefore in agreement with the overall scheme initially presented in Figure 2.

In a third experiment, three groups of 10 Hevea plants each were fed 1-C^{14}-BMC, 3-C^{14}-BMC and 4-C^{14}-BMC respectively through the roots for 24 hours in the dark. The 4-C^{14}-BMC experiment was performed by Dr. Walter McNutt. He isolated the rubber and carried out the rubber degradation by essentially the same procedure described above. The amounts and specific activities of BMC fed to the plants are reported in Table IX.

Rubber was isolated from the whole plants as reported above. The rubber was degraded to levulinic acid and purified as the levulinic 2,4-dinitrophenylhydrazone. The results are reported in Table X.

It may be seen that BMC under these conditions is a very poor precursor of rubber. If 4-C^{14}-BMC is incorporated into rubber without randomization, one half the C^{14} would be expected to occur in the iodoform obtained by degradation. If complete randomization occurred, one fifth of the activity would be expected to be found in the iodoform. The results

TABLE VIII

Incorporation of 1-C^{14}-acetate and 2-C^{14}-acetate into the rubber of intact Hevea seedlings. *

Material	CPM/10^{-4}mol rubber
1-C^{14}-acetate exp.	
Crude rubber	970
Levulinic 2,4-dinitrophenyl-hydrazone	758
Iodoform	2
Succinate	785
2-C^{14}-acetate exp.	
Crude rubber	1,030
Levulinic 2,4-dinitrophenyl-hydrazone	870
Iodoform	235

* Experiment performed by Dr. Walter S. McNutt.

TABLE IX

Amounts of C^{14}-BMC fed to intact Hevea plants.

Compound fed	Specific activity (mc/mM)	Amount fed (mol)
1-C^{14}-BMC	1	2.4×10^{-4}
3-C^{14}-BMC	1	2.4×10^{-4}
4-C^{14}-BMC	6.4	0.78×10^{-4}

TABLE X

Incorporation of 1-C^{14}-BMC and 4-C^{14}-BMC into the rubber of intact Hevea seedlings.

Material	CPM/10^{-4}mol rubber
1-C^{14}-BMC exp.	
Levulinic 2,4-dinitrophenyl-hydrazone	178
3-C^{14}-BMC exp.	
Levulinic 2,4-dinitrophenyl-hydrazone	205
4-C^{14}-BMC exp.	
Levulinic 2,4-dinitrophenyl-hydrazone	317
Iodoform	63

in Table X indicate that the latter is the case.

One possible reason for the slight incorporation of BMC into rubber might be an inability of Hevea plants to activate the substance. This possibility was investigated first by chromatography of an ethanol extract of C^{14}-BMC fed plants. If the material is not activated, it should remain intact and the chromatograms should show only one C^{14}-labeled band corresponding to the free acid. The chromatogram of the ethanol extract of the 4-C^{14}-BMC fed Hevea plants reported in Table X run on Whatman #3 paper in ethanol : NH_4OH : H_2O, 40:4:8 revealed that this is not the case. Assay of the chromatogram with a Nuclear Chicago model 1620 strip counter showed that about half the BMC was converted into at least 12 other C^{14}-labeled compounds during the feeding period. The extensive labeling in plant compounds other than rubber suggest that BMC is activated in the Hevea plant, but does not act as a direct precursor of rubber. If BMC catabolism occurs in Hevea as described by Coon (22) for animal tissues, then branched chain terpene precursors (BOG) should arise before acetate. It might be expected, in this case, that BMC would be a fairly good rubber precursor. The above experiments show that BMC is actually considerably less effective than acetate as a precursor of rubber. This suggests that, in Hevea, BMC may be degraded by some process which does not give rise to rubber precursors.

The results of experiments on the incorporation of C^{14}-labeled CO_2, acetate and BMC into the rubber of intact kok saghyz and Hevea plants may be summarized as follows.

a. C^{14}-labeled acetate is a more effective precursor of rubber (when fed as the free acid) than either $C^{14}O_2$ or C^{14}-BMC.

b. The incorporation of acetate carbons is consistent with the scheme presented in Figure 2.

c. BMC is incorporated into many other carbon compounds to a much greater extent than into rubber. BMC, as the free acid at least, is not on the pathway of rubber synthesis in Hevea plants.

Enzymatic experiments.

It was reported by Teas and Bandurski (14), during the course of the present work, that 1-C^{14}-acetate is incorporated into rubber by an enzymatic system from Hevea latex. Similar experiments had been tried by the present author without success. It has, however, proved possible to, in part, repeat and to extend their work. Four C^{14} compounds, 1-C^{14}-acetate, 3-C^{14}-BMC, 3-C^{14}-BOG and 2-C^{14}-mevalonic acid, were incubated in a system similar to that used by Teas and Bandurski. Of these substrates, only mevalonic acid is rapidly incorporated into the rubber. Acetate, though not incorporated into rubber, is rapidly metabolized under anaerobic conditions and up to 10% of the total activity fed is recovered in an unidentified non-polar compound.

1. Aerobic incorporation of 1-C^{14}-acetate into Hevea latex. A repeat of the Teas and Bandurski experiment.

Hevea latex was collected as described above and added to the reaction mixture described in Table XI. This reaction mixture is identical to that used by Teas and Bandurski (14). One reaction mixture was precipitated at 0 time with ethanol. Two other mixtures were incubated for two hours and forty-five minutes at 37°C. The reaction mixtures were washed as follows: two times with 3 ml acetone, two times with 3 ml H_2O and one time with acetone : H_2O, 1:1. The rubber was dissolved in a solution of 12% TCA in benzene and then counted as the rubber tetrabromide. Results are given in Table XII.

Little, if any, acetate is incorporated into rubber by this system. The 0 time value obtained by Teas is many times higher than that obtained in the present experiment indicating that he used a poorer washing procedure. Several other efforts to repeat the Teas and Bandurski experiment with latex from Earhart grown trees were also unsuccessful.

2. Anaerobic incorporation of 1-C^{14}-acetate into Hevea latex.

Since the formation of terpene hydrocarbons from organic acids is a reductive process, several incubations were run under anaerobic conditions. It was found that the 1-C^{14}-acetate is rapidly incorporated into a volatile non-acidic, non-polar compound (but not into rubber) under anaerobic conditions. Anaerobic conditions were obtained by maintaining

TABLE XI

Reaction mixture used to study the enzymatic incorporation of 1-C^{14}-acetate into Hevea latex.

Material	Concentration	Amount in ml
ATP	M/10	.01
MgFDP	M/10	.01
CoA	1 mg/ml	.01
DPN	1 mg/ml	.01
EDTA	M/10	.01
$(K)PO_4$	M/10 (pH 7)	.01
Sucrose	1 M	.06
1-C^{14}-NaOAc	4 mg/ml (1 mc/mM)	.05
latex		.1
H_2O		.03
Total volume		0.30 ml

TABLE XII

Enzymatic incorporation of 1-C^{14}-acetate into rubber.
A repeat of the experiment described by
Teas and Bandurski (14).

Time of incubation	Ant. rubber (mg)	CPM/10^{-4}mol rubber by rubber tetrabromide
0	72	1
2^{h} 45^{m}	70	0

an argon atmosphere over the reaction mixture. Reaction mixtures were made up as shown in Table XI, except that 0.3 ml of latex was added to each reaction mixture and no water was added. Incubation was carried out for three hours at room temperature under argon. The reaction was stopped by the addition of 3 ml of ethanol. The precipitate was then extracted three times with hot ethanol. The residue was dried and dissolved in $CHCl_3$. The $CHCl_3$ solution was next filtered and washed three times with an equal volume of 10% KOH. The final extraction with 10% KOH produced no change in the specific activity of the $CHCl_3$ solution which indicates that all acidic C^{14}-labeled materials had been removed from the $CHCl_3$. The activity of this purified rubber fraction was rapidly lost when the $CHCl_3$ extract was heated to 50°C. Some material other than rubber contains the activity.

The following experiment demonstrates the volatility of the unknown compound. An aliquot from the $CHCl_3$ extract of reaction mixture 2, reported in Table XIII, was counted at intervals of about 90 minutes. The result obtained is plotted in Figure 8 and is characteristic of the several experiments done to confirm this observation.

Treatment of the unknown compound with 2,4-dinitrohydrazine gave negative results indicating the absence of carbonyl groups. The insolubility of the compound in base indicates the absence of carboxyl groups.

Table XIII gives the results of an experiment in which the

TABLE XIII

Dependence of 1-C^{14}-acetate incorporation into the non-acidic, $CHCl_3$ soluble fraction of Hevea latex on anaerobic conditions and the presence of cofactors.

Description of reaction mixture	CPM/mg $CHCl_3$ soluble material	Total CPM in $CHCl_3$ soluble fraction
1. Aerobic + cofactors	42	905
2. Anaerobic + cofactors	1,520	32,700
3. " + " - ATP	1,750	35,000
4. " + " - DPN	89	1,640
5. " + " - CoA	136	3,200

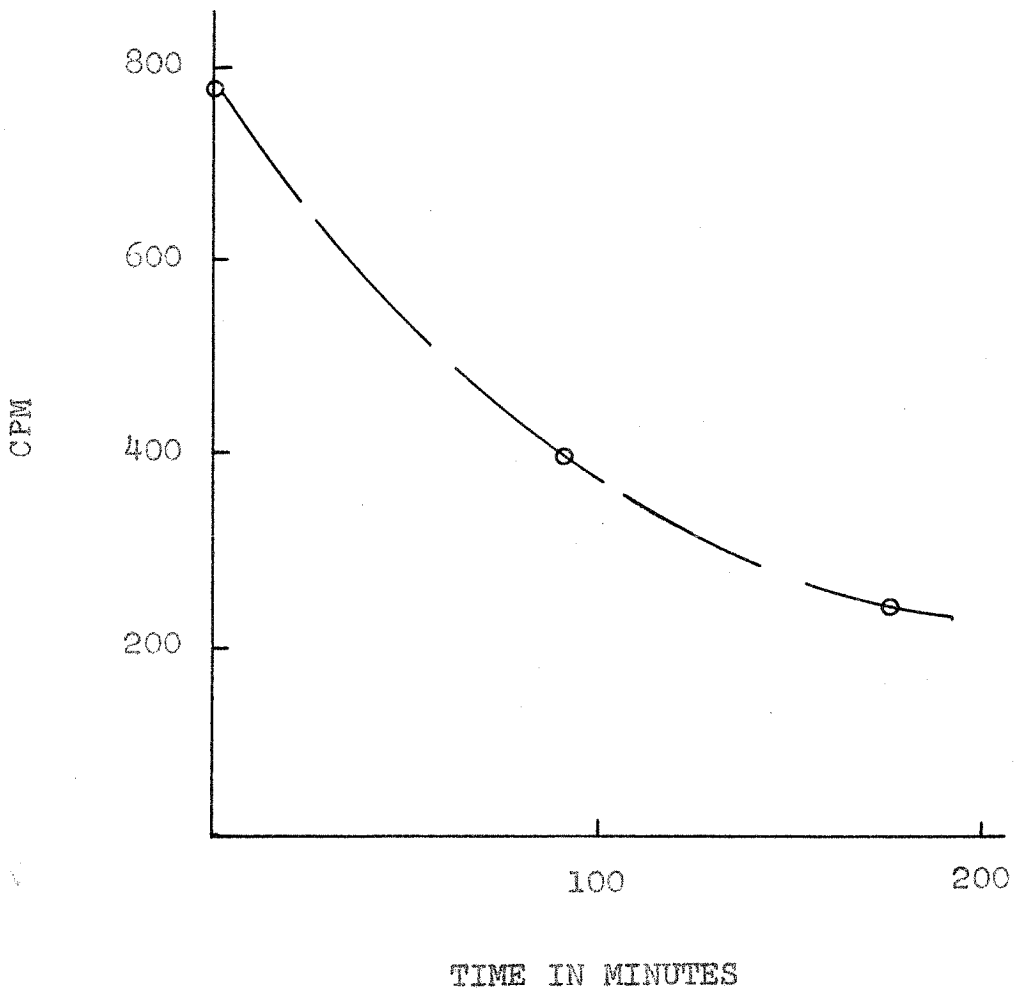


Figure 8. Loss of counts from the non-acidic, $CHCl_3$ soluble fraction of Hevea latex after incubation with 1-C^{14}-acetate under anaerobic conditions.

incorporation of 1-C^{14}-acetate into the volatile $CHCl_3$ soluble fraction was investigated for dependence upon anaerobic conditions and presence of cofactors. Apparently Hevea latex has the enzymatic machinery necessary for producing ATP endogenously. CoA and DPN, on the other hand, considerably enhance incorporation of 1-C^{14}-acetate into the volatile compound. The necessity of CoA for acetate activation and DPN for oxidation reduction reactions supports the idea that acetate is incorporated into a larger and more reduced molecule.

3. Enzymatic incorporation of 3-C^{14}-BOG into Hevea latex.

The enzymatic incorporation of BOG into rubber was also investigated. The system described in Table XI was incubated with 3-C^{14}-BOG (0.8 mc/mM) except that 0.3 ml of latex was used and no water was added. After two hours of incubation under aerobic or anaerobic conditions a small amount of activity appeared in the non-polar fraction and no label appeared in the rubber. Chromatography of an ethanol extract of the reaction mixture in ethanol : NH_4OH : H_2O, 40:4:8 showed that 95% of the label was recovered as BOG. Five percent of the label was in a compound corresponding in Rf to BMC. The identity of this compound with BMC was not further investigated.

4. Incorporation of mevalonic acid into rubber in an Hevea latex enzyme system.

Rapid enzymatic incorporation of 2-C^{14}-mevalonic acid into animal terpenes suggested that mevalonic acid might be an intermediate in plant terpene synthesis. Merck and Co., Inc. supplied the 2-C^{14}-mevalonic acid (0.1 mc/mM) used in these experiments. Two experiments were done on the enzymatic incorporation of 2-C^{14}-mevalonic acid into rubber. The reaction mixtures are indicated in Table XIV. Experiment #1 was incubated at room temperature for one hour. Experiment #2 was incubated at room temperature for three hours. The reaction mixtures were stopped by addition of 2 ml of ethanol. The reaction mixtures were then extracted twice with hot ethanol. Chromatography of the ethanol extract in ethanol : NH_4OH : H_2O, 40:4:8 indicates that no other C^{14}-labeled substances are formed during the incubation. The rubber was extracted and degraded as described above. The recrystallized 2,4-dinitrophenylhydrazone of levulinic acid was rechromatographed in n-butanol : ethanol : 0.5 N NH_4OH, 7 : 1 : 2. The eluted material melted at 202-205°C. (The pure derivative melts at 208°C.) The methyl carbon of levulinic acid was determined by conversion to iodoform as described above. The succinate contained activity, but too little was obtained to count accurately after crystallization. These results are reported in Table XV. Mevalonic acid is the only terpene precursor which has been

TABLE XIV

Reaction mixtures used to study the enzymatic incorporation of 2-C^{14}-mevalonic acid into rubber.

Material	Concentration	Exp. 1	Exp. 2
2-C^{14}-D,L mevalonic acid	100 µm/ml (0.1 mc/mM)	0.05 ml	0.1 ml
DPN	1 mg/ml	-	0.01 ml
Latex		0.2 ml	0.5 ml

TABLE XV

Enzymatic incorporation of 2-C^{14}-mevalonic acid into rubber.

Material	CPM/10^{-4} mol rubber	Total CPM in rubber
Experiment 1		
Levulinic 2,4-dinitrophenylhydrazone	740	2,720
Experiment 2		
Levulinic 2,4-dinitrophenylhydrazone	748	9,900
Iodoform	0	0

found to be rapidly incorporated into rubber. No activity appeared in the methyl carbon of levulinic acid, but activity did appear in the succinate. The results of this and the earlier experiments are considered below.

The enzymatic experiments may be summarized as follows: All efforts to incorporate C^{14}-acetate into rubber in an Hevea latex enzymatic system were unsuccessful. Acetate is rapidly incorporated into a non-acidic, non-polar fraction under anaerobic conditions. Incorporation of acetate into this fraction is greatly enhanced in the presence of CoA and DPN. Addition of ATP does not affect the rate of C^{14}-acetate incorporation into this fraction. Mevalonic acid is rapidly incorporated into rubber in an Hevea latex enzyme system. Chromatography of an alcoholic extract of the reaction mixture does not indicate the presence of other C^{14}-labeled compounds. These facts indicate that the conversion of mevalonic acid to rubber is the major path of mevalonic acid metabolism in this system.

DISCUSSION AND CONCLUSIONS

Teas and Bandurski (14) reported the incorporation of C^{14}-labeled acetate into rubber in an enzymatic system from Hevea latex. To find whether the activity obtained was actually in rubber, these workers degraded the rubber to levulinic acid. This degradation was performed by ozonizing the rubber. The ozonide was then hydrolyzed. The ozonolysis products were treated with 2,4-dinitrophenylhydrazine and the precipitate obtained was counted. Paper chromatography of this material yielded only one spot which corresponded in Rf to the 2,4-dinitrophenylhydrazone of levulinic acid. The activity of the crude precipitate was then assumed to be due to levulinic acid. Since the specific activity of the purified material was not determined (41), the conclusions drawn by these workers are probably not justified. In our experiments which were carried out in similar manner, the purified derivative of levulinic acid obtained by degradation of the incubated rubber contained no activity.

The data presented in this dissertation show that the incorporation of C^{14}-acetate, C^{14}-BMC and C^{14}-mevalonic acid into rubber in intact plants and in enzymatic systems is consistent with the scheme presented in Figure 2. The fact that the data are consistent with this scheme does not necessarily mean that the scheme is correct. Acetate, BOG and BMC interconversions have been demonstrated in plant systems. In this dissertation it is shown that mevalonic acid

is rapidly incorporated into rubber. The interconversion of BOG, mevaldic acid and mevalonic acid has not been demonstrated in a biological system. Until such a conversion is shown, there is a real possibility that the reduction of a carboxyl group may occur before mevalonic acid is finally assembled. For example, condensation of acetyl-CoA and acetoacetate may occur after acetoacetate has undergone reduction. Acetate and mevalonic acid are probably real intermediates in terpene synthesis. Clarification of the intermediate steps, especially those relating BOG and its possible reduction products, is still needed in both animal and plant systems.

REFERENCES

1. Tchen, T. T. and Bloch, K., J. Biol. Chem., 226, 921-930 (1957).

2. Arisz, W. H., Arch. Rubbercultuur, 4, 425-458 (1924).

3. Johnston, J. A., Racusen, D. W. and Bonner, J., Proc. Nat. Acad. Sci., 40, 1031-1037 (1954).

4. Langdon, R. G. and Bloch, K., J. Biol. Chem., 200, 129-134 (1953).

5. Cornforth, J. W. and Popjak, G., Biochem. J., 58, 403-407 (1954).

6. Wursch, J., Huang, R. L. and Bloch, K., J. Biol. Chem., 195, 439-446 (1952).

7. Diturl, F., Cobey, F. A., Warms, J. V. B. and Gurin, S., J. Biol. Chem., 221, 181-189 (1956).

8. Bucher, N. L. R., J. Am. Chem. Soc., 75, 498 (1953).

9. Bucher, N. L. R. and McGarrahan, K., J. Biol. Chem., 222, 1-15 (1956).

10. Rabinowitz, J. L. and Gurin, S., J. Biol. Chem., 208, 307-313 (1954).

11. Corwin, L. M., Schroeder, L. J. and McCullough, W. G., J. Am. Chem. Soc., 78, 1372-1375 (1956).

12. Bonner, J. and Arreguin, B., Arch. Biochem. and Biophys., 21, 109-124 (1949).

13. Arreguin, B., Bonner, J. and Wood, B. J., Arch. Biochem. and Biophys., 31, 234-247 (1951).

14. Teas, H. J. and Bandurski, R., J. Am. Chem. Soc., 78, 3549-3550 (1956).

15. Zabin, I., J. Biol. Chem., 226, 851-859 (1957).

16. Grob, E. C. and Bütler, R., Helv. chim. acta, 38, 1313-1316 (1955).

17. Kremers, R. E., J. Biol. Chem., 50, 31-34 (1922).

18. Prokofiev, A. A., Bull. Acad. Sci. U.S.S.R., 908-923 (1939).

19. Millerd, A. and Bonner, J., Arch. Biochem. and Biophys., 49, 343-355 (1954).

20. Patrick, A. D., Nature, 180, 37 (1957).

21. Rudney, H., J. Biol. Chem., 227, 363-377 (1957).

22. Bachhawat, B. K., Robinson, W. G. and Coon, M. J., J. Biol. Chem., 219, 539-550 (1956).

23. Adams, R. and Van Duuren, B. L., J. Am. Chem. Soc., 75, 2377-2379 (1953).

24. Klosterman, H. J. and Smith, F., J. Am. Chem. Soc., 76, 1229-1230 (1954).

25. Rudney, H., J. Am. Chem. Soc., 76, 2595-2596 (1954).

26. Clark, L. C., Harary, I., Reiss, O. and Bloch, K., Federation Proc., 13, 192-193 (1954).

27. Rudney, H., J. Am. Chem. Soc., 77, 1698-1699 (1955).

28. Rabinowitz, J. L., J. Am. Chem. Soc., 76, 3037-3038 (1954).

29. Skeggs, H. R., Wright, L. D., Cressor, E. L., MacRae, G. D. E., Hoffman, C. H., Wolf, D. E. and Folkers, K., J. Bact., 72, 519-524 (1956).

30. Wright, L. D., Cresson, E. L., Skeggs, H. R., MacRae, G. D. E., Hoffman, C. H., Wolf, D. E. and Folkers, K., J. Am. Chem. Soc., 78, 5273-5275 (1956).

31. Wolf, D. E., Hoffman, C. H., Aldrich, P. E., Skeggs, H. R., Wright, L. D. and Folkers, K., J. Am. Chem. Soc., 78, 4499 (1956).

32. Wolf, D. E., Hoffman, C. H., Aldrich, P. E., Skeggs, H. R., Wright, L. D. and Folkers, K., J. Am. Chem. Soc., 79, 1486-1487 (1957).

33. Hoffman, C. H., Wagner, A. F., Wilson, A. N., Walton, E., Shunk, C. H., Wolf, D. E., Holly, F. W. and Folkers, K., J. Am. Chem. Soc., 79, 2316-2318 (1957).

34. Tavormina, P. A., Gibbs, M. H. and Huff, J. W., J. Am. Chem. Soc., 78, 4498-4499 (1956).

35. Tavormina, P. A. and Gibbs, M. H., J. Am. Chem. Soc., 78, 6210 (1956).

36. Shunk, C. H., Linn, B. O., Huff, J. W., Gilfillan, J. L., Skeggs, H. R. and Folkers, K., J. Am. Chem. Soc., 79, 3294-3295 (1957).

37. Wright, L D., Federation Proc., 16, 274 (1957).

38. Amdur, B. H., Rilling, H. and Bloch, K., J. Am. Chem. Soc., 79, 2646-2647 (1957).

39. Diturl, F., Gurin, S. and Rabinowitz, J. L., J. Am. Chem. Soc., 79, 2650-2651 (1957).

40. Willits, C., Swain, M. and Ogg, C., Ind. Eng. Chem., Annal. Ed., 18, 439-442 (1946).

41. Teas, H. J., Personal communication.

II. FRACTIONATION OF THE STABLE CARBON ISOTOPES IN PLANTS

GENERAL INTRODUCTION

A basic objective of biology is the description of the chemical and physical phenomena which occur within cells. One useful technique for the attainment of this end is the use of artificial isotope ratios as tracers of metabolic pathways. Radioactive isotopes are especially useful in this respect since their abundance is easily determined by electronic counting techniques. The success of all such tracer experiments depends on the chemical similarity of the artificially introduced isotope to the normal isotope in question. Actually, two molecules, which differ only in the isotopic species of the atoms of which they are composed, are not chemically identical. Variation in the mass of an atom produces, in many cases, predictable effects on the thermodynamic properties of molecules which contain this atom. Such effects are, in general, pronounced in the case of small molecules composed of light elements. Light elements (H,C,N,O) are the abundant ones in organic material. It is known that measurable fractionation of these isotopes occurs in biological systems. Little is known, however, about the actual processes that cause these isotopic fractionations. It is anticipated that accurate determinations of isotopic fractionation in biological systems under controlled conditions should yield information about the nature of the processes which carry out the fractionation. This thesis concerns a

particular instance of isotope fractionation by living organisms, namely the fractionation of the stable carbon isotopes in plants. In this study, the fractionation of C^{13} and C^{12} in plants is correlated with various aspects of plant metabolism.

Considerable work has been done on the C^{13}/C^{12} ratios of natural carbons. The results of some of these investigations are reviewed below.

HISTORICAL REVIEW

Early work in this field was primarily concerned with the determination of C^{12}/C^{13} ratios of carbon in nature (42, 43). This ratio was found to equal about 90. The first instrument capable of detecting significant differences in the C^{12}/C^{13} ratios of different natural carbons was the mass spectrometer developed by Nier (44). With this instrument Nier and Gulbranson (45) measured C^{12}/C^{13} ratios to $\pm$ 0.5% of the ratio. They demonstrated for the first time that the C^{12}/C^{13} ratio of a natural carbon depends on its source. For example, the C^{12}/C^{13} ratios of all the limestones investigated were found to be smaller by about 3% than those of land plant carbon. The maximum range of the C^{12}/C^{13} ratio in nature was found to be 5% of the ratio.

During the next several years a number of investigations were carried on in Nier's laboratory at the University of Minnesota. Murphy and Nier (46), in a survey of C^{12}/C^{13} ratios in natural carbon containing compounds, found that the C^{12}/C^{13} ratios of limestone and of the dissolved CO_2 of sea water are about 3% less than the C^{12}/C^{13} ratios of land plants. Coal was found to possess a C^{12}/C^{13} ratio identical to that of present day wood. Some petroleums, on the other hand, were found to be enriched in C^{12} by about 1% of the ratio as compared to the carbon of present day plants. The C^{12}/C^{13} ratio reported for air CO_2 in the survey is similar

to that of wood and in the light of recent evidence is probably incorrect.

Murphy (47) obtained the first evidence suggesting that not all fractions within a plant have the same C^{12}/C^{13} ratio. Belkengren (48) and Rabideau (49), also working at the University of Minnesota, were the first investigators to make use of C^{13} as a tracer in plant metabolism. Belkengren noted that plant lipid, carbohydrate and cellulose possess similar C^{12}/C^{13} ratios and are enriched in C^{12} as compared to air CO_2. Plant proteins, on the other hand, were enriched in C^{13} as compared to air CO_2. Evidence presented in this dissertation suggests, however, that something is wrong with Belkengren's experiments and that his conclusions are not all correct. Belkengren, in addition, studied the translocation of C^{13} from plant leaves to plant roots and his experiments are among the first direct ones on the subject.

West (50), in 1945, reported a survey of the C^{12}/C^{13} ratios of 35 petroleums. With his mass spectrometer he obtained the average value, 94.1 ± 0.4 for 32 petroleums from the Silica and Ellinwood Fields of Kansas. He also determined the average C^{12}/C^{13} ratio of three weeds and obtained the value 92.6. This confirmed Nier's observation that petroleum is enriched in C^{12} as compared to present day plants.

The development of a more precise mass spectrometer during the past ten years has brought about modification of a

number of conclusions reached by early investigators. Development of the high resolution mass spectrometer which is used today took place from 1947 to 1950. In 1947, Nier (51) reported an improved mass spectrometer design and further improvements were reported by McKinney et al. (52) in 1950. In this machine C^{12}/C^{13} ratios are determined to $\pm$ 0.01% of the ratio. An instrument of this design was used to make the analyses reported in this dissertation.

The development of better instrumentation coupled with an understanding of the thermodynamic properties of isotopic substances initiated a new period of fruitful investigation in carbon and oxygen isotope chemistry.*

---

*In 1947, Urey (53) reviewed the thermodynamic properties of isotopic substances. He presents the isotope exchange equilibrium constants for a number of reactions. A typical reaction is of the type:

$$C^{13}O_2 + C^{12}O_3^{=} \rightleftharpoons C^{12}O_2 + C^{13}O_3^{=} \quad .$$

Urey has calculated the values

$$K = 1.0156 \text{ at } 0°C$$

$$= 1.0123 \text{ at } 25°C$$

for this particular exchange reaction. Thus, the C^{12}/C^{13} ratio of CO_2 gas in equilibrium with a carbonate solution would be expected to be larger than that of carbonate by about 1% of the ratio.

In 1952, Wickman (54) made the first extensive survey of C^{13}/C^{12} ratios of plants in nature.* This paper was soon followed by that of Craig (55) which reported C^{13}/C^{12} ratios from many sources including plants. Wickman analyzed representatives of all the major taxonomic groups of plants. His results are summarized in Figure 9. Wickman noted that desert plants are enriched in C^{13} as compared to forest plants. He also noticed that hydrophytes growing in flowing or turbulent water are enriched in C^{13} as compared to similar plants growing in stagnant water. He has proposed a model to explain both observations and his argument runs as follows: desert plants grow in an open environment which is usually windy and in which the plants are constantly exposed to a well mixed atmosphere. Forest plants, on the other hand, grow in an environment in which much of the CO_2 is derived from soil respiration. Since the respired CO_2 is derived from decaying plant material, in a steady state it must have the same C^{13}/C^{12} ratio as plants themselves. Thus, according to Wickman, forest plants photosynthesize some recycled CO_2 rather than just the heavier CO_2 of normal air. Wickman

---

*Terminology used to describe C^{13}/C^{12} ratios is discussed in Materials and Methods (Part I) under Terminology.

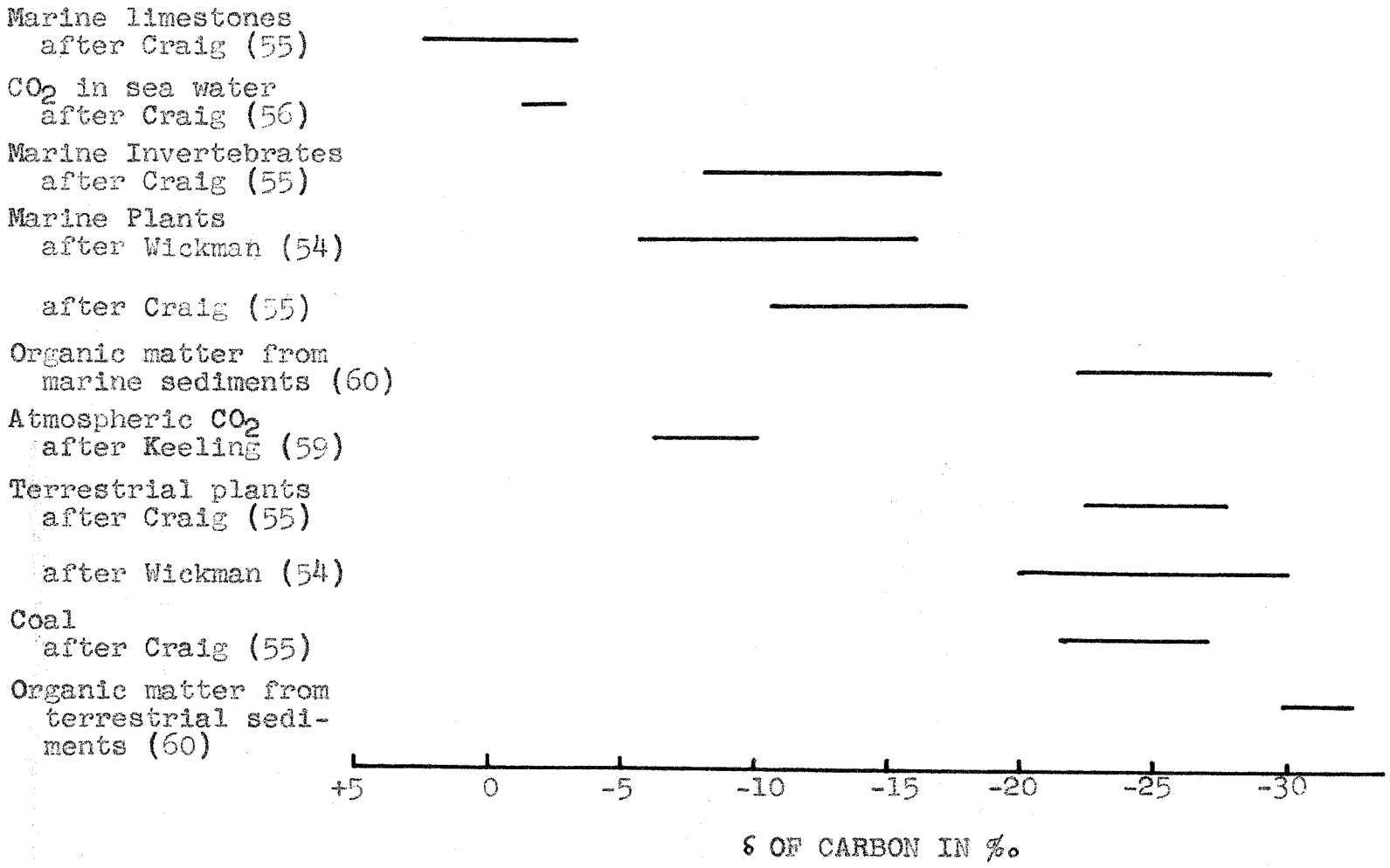


Figure 9. C^{13}/C^{12} ratios of carbon in nature.

then draws an analogy between forest and desert plants and stagnant and non-stagnant hydrophytes respectively. He concludes that desert plants and marine plants in the open sea exhibit the true fractionation factors for water and land plants. On the other hand, plants in a local atmosphere are lighter due to assimilation of recycled CO_2.

This hypothesis has received extensive criticism from Craig (56) who concludes that local environments are not important in producing C^{13}/C^{12} ratio variations in plants. Actually, there is evidence that under certain conditions the development of a local CO_2 atmosphere due to catabolism of organic substrates is very marked. Fuller (57) has made measurements of the CO_2 concentration at various heights above Illinois forest and grassland. His data are presented in Table XVI. All measurements were made at 1:00 P.M. From the data, it is seen that the CO_2 concentrations at less than 10 cm above the ground may be increased six-fold over the normal air concentration of 0.03%. Since this CO_2 is concentrated by virtue of respiration of organic substrates, it probably has a C^{13}/C^{12} ratio similar to that of plant residues in soil. Low growing plants, such as mosses and liverworts, may be influenced by such a local CO_2 atmosphere in some cases. Both Verduin (58) and Keeling (59) have observed an increase in the atmospheric CO_2 concentration during nighttime. Keeling found that the increase in CO_2 concentration during nighttime is accompanied by an

TABLE XVI *

CO_2 concentrations of the atmosphere above forest and grassland at 1 P.M.

Height above ground in cm	Woodland CO_2 in %	Grassland CO_2 in %
0-1	.101	.098
7-8	.099	.050
15-16	.050	.036
30-31	.047	.041
60-61	.036	.029
91-92	.039	.032

* From the data of Fuller (57).

enrichment in C^{12}. This suggests that plant respiration accounts for both of these effects. During daytime, the concentration and C^{13}/C^{12} ratio of CO_2 returned to that of normal air.

These data suggest that variations in C^{13}/C^{12} ratios of plants may, in part, result from development of a local CO_2 atmosphere, especially in the case of plants growing within 10 cm of fertile ground.

In 1953, Craig (55) published C^{13}/C^{12} ratios of many naturally occurring carbon compounds. His data are also plotted in Figure 9.

It is seen that land plants have a C^{13}/C^{12} ratio 18‰ less than that of atmospheric CO_2. Marine plants have a C^{13}/C^{12} ratio about 13‰ less than that of ocean carbonates. Craig also found that fossil wood and coals have the same C^{13}/C^{12} ratios as present day plants. Some petroleums, on the other hand, have a C^{13}/C^{12} ratio about 5‰ less than that of present day land plants. Other petroleums have a C^{13}/C^{12} ratio about equal to that of land plants. This situation has been much clarified by Silverman and Epstein (60), who show that the C^{13}/C^{12} ratios of a petroleum depend on whether it was formed in a marine or in a terrestrial environment. This paper is further considered below.

Craig (56) proposes an explanation different from that of Wickman for the variations in the C^{13}/C^{12} ratio of plants. Craig suggests that there is more than one fractionation

process in the plant and that the compounding of these fractionation processes results in the total fractionation. Environmental effects on the several processes could account for isotopic variations.

The data which have been presented pose a problem. What process or processes bring about C^{12} enrichment during photosynthesis and what can such processes tell us about the mechanisms of photosynthesis? Craig (56) considers the C^{12} enrichment that would be expected to occur by diffusion of CO_2 to a leaf through a stagnant air layer. He concludes that this effect is not large enough to account for the observed enrichment, and therefore suggests that other unspecified processes within the leaf are responsible for fractionation. Baertchi (61) has performed two experiments which bear on the mechanism of fractionation. His first observation has to do with the fractionation of carbon isotopes during the absorption of CO_2 by a basic solution. When CO_2 is absorbed by a basic solution, either from pure CO_2 or from a CO_2, nitrogen mixture, the absorbed carbon is enriched in C^{12}, as compared to the overlying CO_2, by a factor of 1.014. Craig (55) obtained the value 1.015 in a similar experiment. Baertchi and Craig conclude that diffusion gas to the absorbing surface in the experiment is not the rate controlling step in absorption of CO_2. This has also been assumed to be true for plants, in which leaves are the absorbing surface. Baertchi's (62) next experiment involved analysis of the CO_2 respired by germinating bean

seeds. The C^{13}/C^{12} ratio of respired CO_2 was only 0.5‰ greater than that of the initial isotopic composition of the seed. It was concluded that no fractionation occurs during respiration. Baertchi then grew two bean plants for 16 days (from which the cotyledons had been removed) in 0.5% CO_2 of known isotopic composition. By measuring the initial and final weights as well as the C^{13}/C^{12} ratios of the plant carbon and with knowledge of the C^{13}/C^{12} ratios of the atmospheric CO_2 and respired CO_2, it is possible to calculate the C^{12} enrichment factor for photosynthesis. Baertchi found this to be 1.026 ± .001. These data are, however, difficult to interpret since the initial carbon of the plant constituted one-half of that present at the end of the 16 day photosynthetic period. The extent to which the initial plant material remained intact or was respired is unknown. Interpretation of the respired CO_2 value for this experiment is complicated for the same reason. Nevertheless, the experiments of Baertchi provide the most unambiguous information to date on the extent of carbon isotope fractionation in photosynthesis.

The use of C^{14} as a tracer of carbon pathways in photosynthesis has stimulated a number of experiments on the magnitude of C^{14} isotope effects in plant systems. Some of these experiments involve simultaneous measurement of C^{13} discrimination in photosynthesis and are therefore of interest here. Weigl and Calvin, in two papers (63,64), have noted

that algae assimilate $C^{12}O_2$ about 20% faster than $C^{14}O_2$. The assimilation by barley of $C^{14}O_2$ was found to be 17% slower than assimilation of $C^{12}O_2$. In an aquarium in which 70% of the total available carbon had been assimilated by algae, the assimilation rates of $C^{13}O_2$ and $C^{14}O_2$ were 5% and 20% slower respectively than the assimilation rate of $C^{12}O_2$. In 1952, Van Norman and Brown (65) performed an experiment in which the C^{12}, C^{13}, and C^{14} content of CO_2, supposedly in equilibrium with an aquarium of growing algae, was continuously measured. $C^{12}O_2$, $C^{13}O_2$, and $C^{14}O_2$ were assimilated at rates which are in the ratio of 1.00 : 0.96 : 0.85 respectively.

The aquarium fractionation experiments of both Weigl (63) and of Van Norman (65) are susceptible to many interpretations. Several fractionation factors may be involved. These include kinetic effects during CO_2 absorption on the water surface and cell membrane specificity as to the passage of CO_2 or bicarbonate ions. The term fractionation as it has applied to algal assimilation is actually a complex of a number of separate fractionations. In the absence of information as to the relative contribution of each in the various experiments, the data must be regarded as qualitative.

That carbon isotope fractionation in an ecological community may be demonstrated experimentally was shown by Buchanan et al. (66) in 1953. In these experiments an aquarium containing $C^{14}O_2$ and a population of plants, fish

and snails was sealed for three years. At the end of this time the distribution of C^{14} was determined. C^{14} was found to be most concentrated in the carbonates and least concentrated in the plants. Some of these data are given in Table XVII. In a second experiment plants alone were grown in a sealed aquarium in the presence of $C^{12}O_2$, $C^{13}O_2$ and $C^{14}O_2$. The ratio of the C^{14} isotope fractionation to the C^{13} fractionation was determined to be 2.39 ± 0.04.

The C^{13}/C^{12} ratio of fossil carbons is of interest since it constitutes a property which may be used in identification of the origin of such carbon. Wickman (67), in 1956, reported a series of C^{13}/C^{12} ratios for coal and petroleums. He observed that petroleums are generally enriched in C^{12} as compared to coal. Silverman and Epstein (60) have pointed out that C^{13}/C^{12} ratios of petroleums of marine origin are about 10‰ greater than those of petroleums of non-marine origin. The C^{13}/C^{12} ratios of both petroleums are about 10‰ less than those of marine and non-marine plants respectively. Data from this dissertation are used by Silverman and Epstein to show that the C^{12} enrichment characteristic of petroleums, although greater than that of the plants from which they are presumably derived, is similar to the C^{12} enrichment of the lipid fraction of such plants.

Carbon isotope effects have been studied in a number of non-enzyme mediated organic reactions. Bigeleisen (68) and Bigeleisen and Mayer (69) have reported methods for

TABLE XVII

Specific activity of specimens in an aquarium after prolonged exposure to $C^{14}O_2$

Sample	Carbon activity CPM/mM x 10^{-4}
CO_2 dissolved in the water	20.4
Plants	19.2-19.7
Snail shell carbonate	20.9
Snail organic matter	19.6

From the data of Buchanan et al. (66).

the calculation of free energy, entropy, enthalpy, heat capacity and activation energies of isotopic species. Such thermodynamic calculations involve knowledge of the mass, symmetry number, vibrational frequencies and temperature of the species in question. The theory predicts that during decarboxylation, for example, the rate of carbon bond rupture will be greater for the species $C^{12}-C^{12}$ than for the species $C^{12}-C^{14}$. In general, the rates for rupture of C-C bonds during decarboxylation will vary in the order $C^{12}-C^{12} > C^{12}-C^{13} > C^{12}-C^{14}$ and by the factors 1.00 : 1.02 : 1.04. A large amount of experimental evidence has been accumulated which generally supports these predictions (70,71,72,73, 74,75,76,77).

STATEMENT OF THE PROBLEM

The survey of the literature has shown that present knowledge of C^{13}/C^{12} fractionation by plants may be summarized as follows. The C^{13}/C^{12} ratios of land plants in nature are 18 $\pm$ 4‰ smaller than that of the atmosphere in which they grow. The C^{13}/C^{12} ratios of marine plants are 13 $\pm$ 6‰ smaller than that of ocean carbonates. To what extent these variations are due to local variations in the C^{13}/C^{12} ratio of local CO_2 is not known. The respiratory processes of plants have been shown experimentally to be non-fractionating, although Craig believes that they should exhibit fractionation. Whether chemical fractions within a single plant are of the same or different isotopic composition is unknown except for the early experiments of Murphy and Belkengren which were performed with less precise instrumentation. Most important of all, there have been no experiments designed to elucidate the mechanism of photosynthetic C^{12} enrichment by marine and land plants.

EXPERIMENTAL APPROACH

The experimental work is divided into two parts. In the first part, experiments are described in which the total fractionation factor of photosynthesis is experimentally determined for the tomato. Respiratory fractionation of carbon is also studied. The C^{13}/C^{12} ratios characteristic of different parts of the plant and of the various chemical constituents of the plant are investigated. In the second part, experiments are summarized which give an indication that fractionation is a multistep process. This is shown experimentally first, by the analysis of the CO_2 dissolved within the tomato during photosynthesis or respiration, and second, by the determination of the fractionation involved in the enzymatic step by which dissolved CO_2 is converted to sugar.

PART I

CARBON ISOTOPE DISCRIMINATION DURING THE METABOLISM OF INTACT TOMATO PLANTS

Materials and Methods.

1. Method of growing plants.

Tomato seeds (var. Extra Early) were germinated on moist vermiculite at 26°C for five days in the dark. At the end of this time the etiolated seedlings were removed from the dark, planted in individual cups and placed in the temperature controlled leucite booth shown in Figure 10. Booth temperature was maintained at 26°C during the day and at 22°C during the night, both constant to ± 1°C. A bank of fluorescent bulbs over the booth gave a light intensity of 1,000 fc. at the level of the plants, and was controlled by a central time clock in the Earhart Plant Research Laboratory to give a 12 hour photoperiod. In several experiments sunlight was used as the light source.

CO_2 of known C^{13}/C^{12} ratio was admitted to the booth by acidification of $CaCO_3$ with HCl. CO_2 derived in this way was generated at a rate 200 times faster than the photosynthetic fixation capacity of the plants at maximum size. The CO_2 was diluted to the desired concentration by addition of CO_2 free air. Such air was prepared from Earhart Laboratory compressed air which was first dried with anhydrous $CaCl_2$. It was then passed through tubes 1.5 cm in diameter and 80 cm

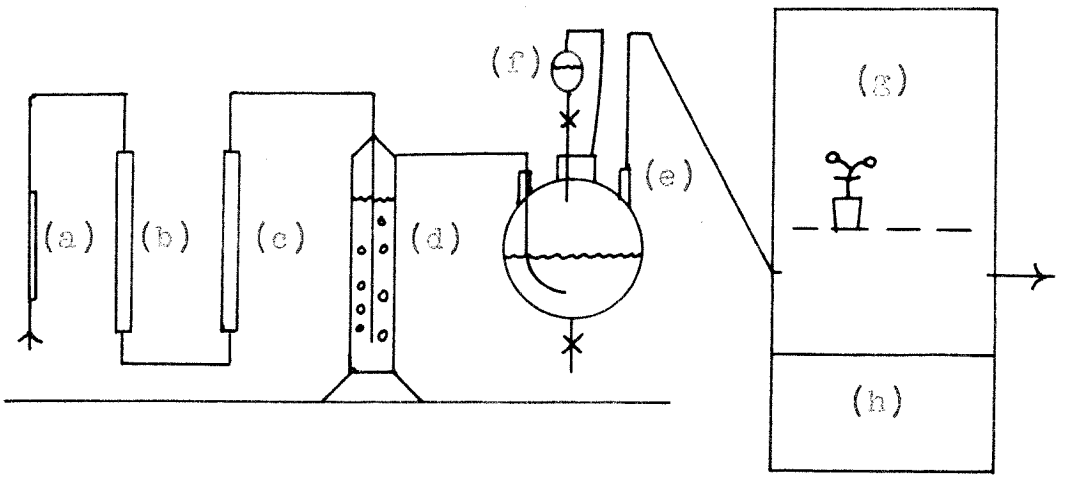


Figure 10. Temperature controlled booth and CO_2 generating apparatus. (a) Flowmeter. (b) Anhydrous $CaCl_2$ + ascarite tube. (c) Ascarite tube. (d) $Ba(OH)_2$ trap. (e) Flask containing $CaCO_3$ slurry. (f) HCl supply. (g) Booth containing growing plants. (h) Temperature controlling mechanism and fan.

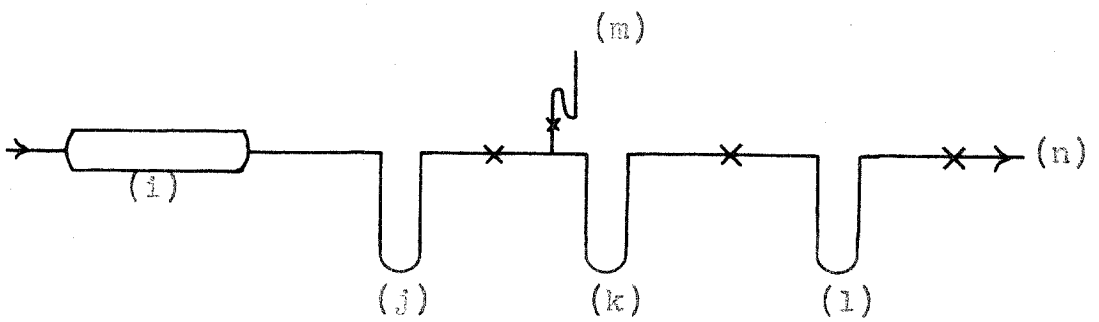


Figure 11. Apparatus for collection of respired CO_2. (i) Respiratory chamber. This chamber is preceded by (a), (b) and (c) shown in Figure 10. (j) Dry ice trap. (k), (l) Liquid nitrogen traps. (m) Manometer. (n) Vacuum pump preceded by liquid nitrogen trap.

in length containing NaOH coated asbestos (Ascarite) and finally through a $Ba(OH)_2$ trap. Air washed in this way contains no CO_2 when assayed with an infrared CO_2 analyzer or when passed through a series of liquid nitrogen traps at 1 cm of mercury pressure and the volume of trapped gas measured manometrically. The ability of ascarite to remove CO_2 under these conditions was also demonstrated by the fact that the $Ba(OH)_2$ solution never became cloudy. Variations in airflow rate and acidification rate of $CaCO_3$ gave a $\pm$ 10% error in the concentration of CO_2 being given to the plants during an experiment as determined by an infrared CO_2 analyzer.

Dark germinated seedlings were placed in the booth during the evening which allowed 12 hours for the cage to flush with CO_2 of known isotopic composition and concentration before the lights were turned on. During this time the booth was flushed with seven times its own volume of new atmosphere. $CaCO_3$ and HCl were added as necessary to the CO_2 generation chamber. The ascarite, $CaCl_2$ tube was replaced every 12 hours though the white zone indicating Na_2CO_3 had travelled only a few cm down the tube.

The plants were watered with nutrient solution once per day by removing the rubber cork containing the air outlet tube and inserting a metal hose. This operation took less than one minute and was performed in the evening so that any contamination with atmospheric CO_2 would be flushed

from the booth before the lights were turned on.

At the end of 13 days, the young plants were removed from the booth and dried in a vacuum desiccator over H_2SO_4. Any loss of volatile compounds from the plants under these conditions was not sufficient to change the isotopic composition as was shown by analyzing plants directly without the drying process. These results are given in Table XVIII.

2. Respiratory CO_2 collection.

After removal from the booth, some plants were used for the collection of respired CO_2. The apparatus used for this study is shown in Figure 11. The plants were placed in the 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 this air by the criteria described in the previous section. All stopcocks were then closed and the plants allowed to respire for 12 hours during the dark period. After 12 hours the dry ice and liquid nitrogen traps were placed on the line. Stopcocks were adjusted so that 70 cc of CO_2 free air swept through the system each minute. 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. After sweeping the system for two hours, the CO_2 was transferred to the second trap and then to the mass spectrometer sample tube. The sample volumes were determined manometrically.

TABLE XVIII

The effect of the drying method on the C^{13}/C^{12} ratio of tomato leaves.

CO_2 source	δ of frozen leaves in ‰.	δ of dried leaves in ‰.
Air	-29.9	-30.1
$CaCO_3$	-25.6	-26.0

In later experiments the line was swept continuously with CO_2 free air and respired CO_2 collected at desired intervals.

3. Preparation of the lipid fraction.

The dried plant was first ground up with a mortar and pestle and then subjected to the fractionation scheme shown in Figure 12. The plant was first extracted with ethanol in order to allow more complete subsequent extraction with chloroform. 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. At the time intervals shown in Table XIX 2 cc of the solvent were removed from the extraction 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 results in Table XIX indicates that no measurable exchange of carbon between solvent and solute took place during the extractions.

4. Fractionation of the 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 13. This method for plant cell wall fractionation is described by Ordin, Cleland and Bonner (78) with the exception of the lignin and cellulose preparation

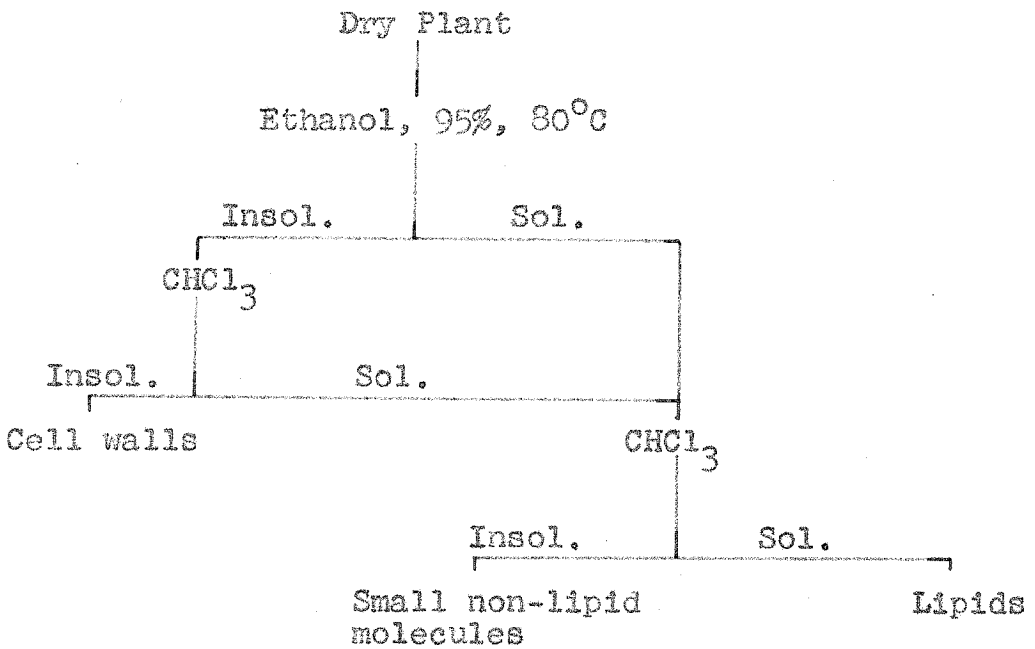


Figure 12. Fractionation scheme for isolation of lipid fraction from plants.

TABLE XIX

Exchange of carbon isotopes between the solvent and lipid extract.

Elapsed time in hours	δ of ethanol soluble material in ‰	δ of $CHCl_3$ soluble material in ‰
6	-21.4	-22.5
24	-21.3	-22.5
30	-21.4	-22.4

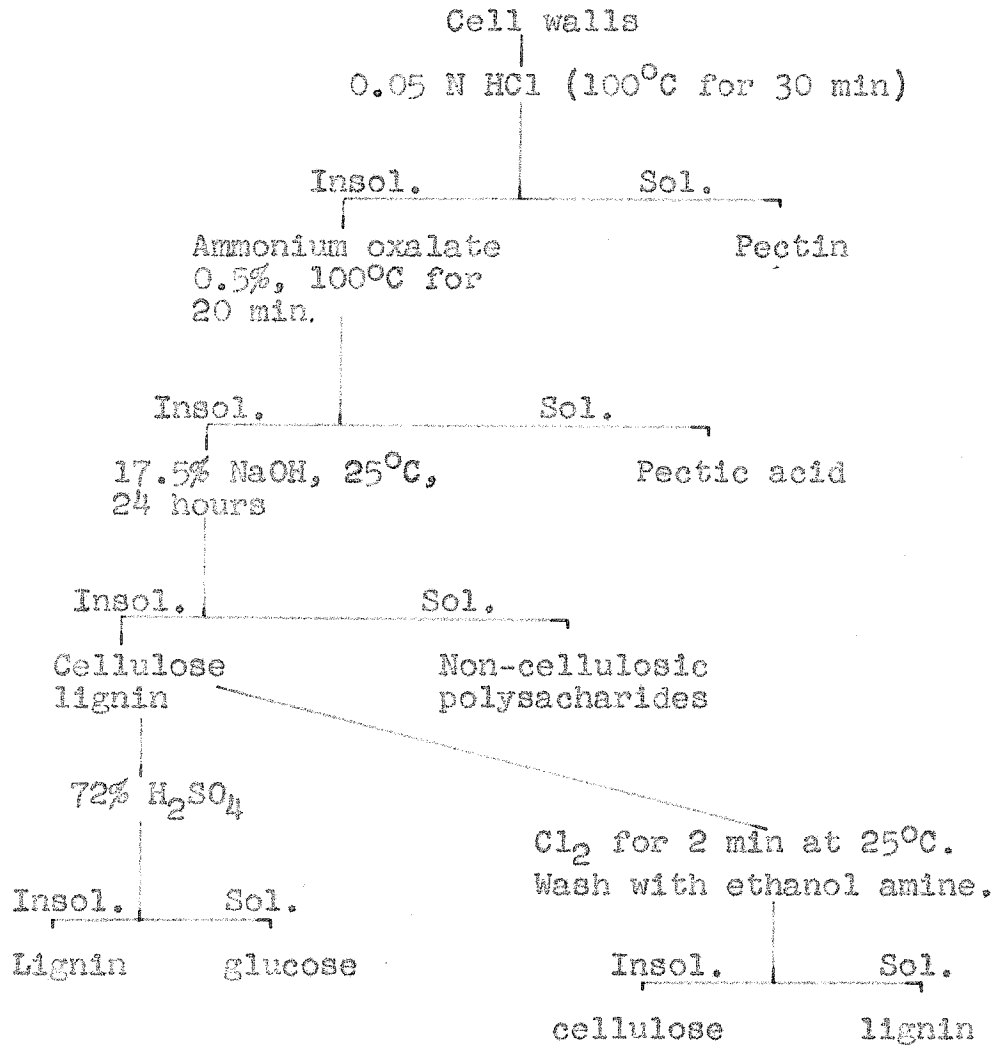


Figure 13. Fractionation of cell wall material.

which were prepared according to the methods cited by Bonner (79,80). The HCl and NaOH fractions were reduced to small volume in a desiccator and precipitated with 95% ethanol. The fractions were then converted to CO_2 and the isotopic composition determined.

5. Combustion of Samples.

Plant materials were converted to CO_2 according to the procedure described by Craig (55). In this procedure samples are combusted over copper oxide in an oxygen atmosphere at 800-900°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 is distilled into a sample tube at liquid nitrogen temperature. Water and most other contaminants remain in the trap at dry ice temperature.

6. Preparation of CO_2 from Calcium Carbonate

CO_2 was prepared from calcium carbonate according to the procedure described by McCrea (81). Concentrated phosphoric acid is placed in the side arm of a reaction vessel and the calcium carbonate sample is placed in the central portion of the vessel. After evacuation, the acid is tipped into the calcium carbonate and is allowed to react for one hour. The volume of CO_2 is then measured manometrically and transferred into a sample tube.

7. Mass Spectrometer Analysis

The CO_2 samples were analyzed in a Nier sixty-degree sector type mass spectrometer which incorporates the improvements described by McKinney et al. (52) for determining C^{13}/C^{12} and O^{18}/O^{16} ratios to ± 0.1‰. . All values obtained were corrected for mixing of the standard and sample gas, and for the O^{17} contribution to the mass 45 peak. The corrections are described in detail by Craig (82).

8. Terminology.

Two methods have been used for reporting stable carbon isotope ratios. Early work and that of Wickman are reported in absolute C^{12}/C^{13} ratios. This has the disadvantage of presenting the data as small differences between large numbers.

A more useful method for reporting of isotope ratios is that used by Urey's laboratory and others, in which results are presented as per mil deviations of the C^{13}/C^{12} ratio of the sample from an arbitrary standard. This method has been adopted in the present work. The standard used is CO_2 prepared from a cretaceous belemnite, Belemnitella americana, from the Peedee formation of South Carolina. This standard is the one used in Urey's laboratory at the University of Chicago. This scale is called the PDB scale.

The isotopic composition (δ) of a sample is then given as follows:

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

Thus, samples enriched in C^{12} as compared to the standard assume negative values while samples enriched in C^{13} as compared to the standard assume positive values. The precision of the data is $\pm$ 0.1‰. .

The enrichment factor or fractionation factor between two compounds, A and B, is defined as:

$$\varepsilon = \frac{R_A}{R_B}$$

where R_A and R_B are the respective C^{13}/C^{12} ratios of compounds A and B.

δ in this dissertation will refer to C^{13}/C^{12} ratios on the PDB scale unless otherwise stated.

Results.

It is the purpose of these experiments to determine the extent of carbon isotope fractionation during the photosynthetic fixation of CO_2 by higher plants. Tomato plants were germinated and grown according to the procedures described above (Materials and Methods). In the initial experiments, 1.5% CO_2 (by volume) of known isotopic composition was used as the carbon supply for photosynthesis. These conditions do not duplicate natural ones since the CO_2 content of normal air is only 0.03-0.04%. Further experiments were therefore run using CO_2 concentrations approximating those which are present in nature. Experiments to determine the effects of light intensity on photosynthetic fractionation were also carried out.

The $CaCO_3$ which was used as a source of CO_2 was analyzed according to the procedure described above. The δ value for this carbon was found to be

$$\delta(CO_2 \text{ from } CaCO_3) = +2.6‰.$$

1. Tomatoes grown in an atmosphere containing 1.5% CO_2 of isotopic composition $\delta = +2.6‰$.

Nineteen plants were grown for 13 days and prepared for analysis as described above (Methods). The initial etiolated seedlings averaged 1.7 mg dry weight and had an isotopic composition of = -24.9‰. The final plants weighed (dry) 42 to 68 mg. Because of the comparatively large increase

in weight of the plants during the experimental period and because of the similarity in isotopic composition, correction for the carbon contribution of the seedlings is unimportant. The leaves, stems and roots (excluding the cotyledons) from three plants were analyzed separately. These results are given in Table XX and are plotted in Figure 14.

The C^{13}/C^{12} ratio of the stems may be used as the average value for that of the plant as a whole. The enrichment factor, as defined in the section entitled Terminology in Materials and Methods, may be calculated for these plants:

$$\varepsilon = \frac{R_{CO_2}}{R_{plant}} = 1.0279 \ .$$

2. Tomatoes grown from 1.5% CO_2 of isotopic composition $\delta = +2.6‰$. A repetition of the previous experiment.

Results of experiment 2, a repeat of experiment 1, are given in Table XXI and are plotted in Figure 14. Again taking the value for the stems as the average value, the enrichment factor may be calculated:

$$\varepsilon = \frac{R_{CO_2}}{R_{plant}} = 1.0285 \ .$$

As mentioned above, the CO_2 concentrations used in the first two experiments (1.5% by volume) are larger than those of normal air, which range between 0.03 and 0.04%. Two experiments bracketing the air CO_2 concentration range were

TABLE XX

C^{13}/C^{12} ratios of tomato plants grown in 1.5% CO_2 (δ = 2.6‰).

Plant no.	Dry wt. (mg)	δ leaves in ‰	δ stems in ‰	δ roots in ‰
AP-2	67.5	-25.3	-25.0	-24.7
AP-4	41.8	-25.0	-24.7	-24.7
AP-5	54.1	-24.5	-24.2	-24.2
Average		-24.9	-24.6	-24.5

TABLE XXI

C^{13}/C^{12} ratios of tomato plants grown in 1.5% CO_2 (δ = 2.6‰). Repeat experiment.

Plant no.	Dry wt. (mg)	δ leaves in ‰	δ stems in ‰	δ roots in ‰
BP-2	50.0	-26.1	-25.1	-24.6
BP-3	47.4	-25.8	-25.4	-24.4
BP-4	37.0	-26.1	-25.2	-24.3
Average		-26.0	-25.2	-24.4

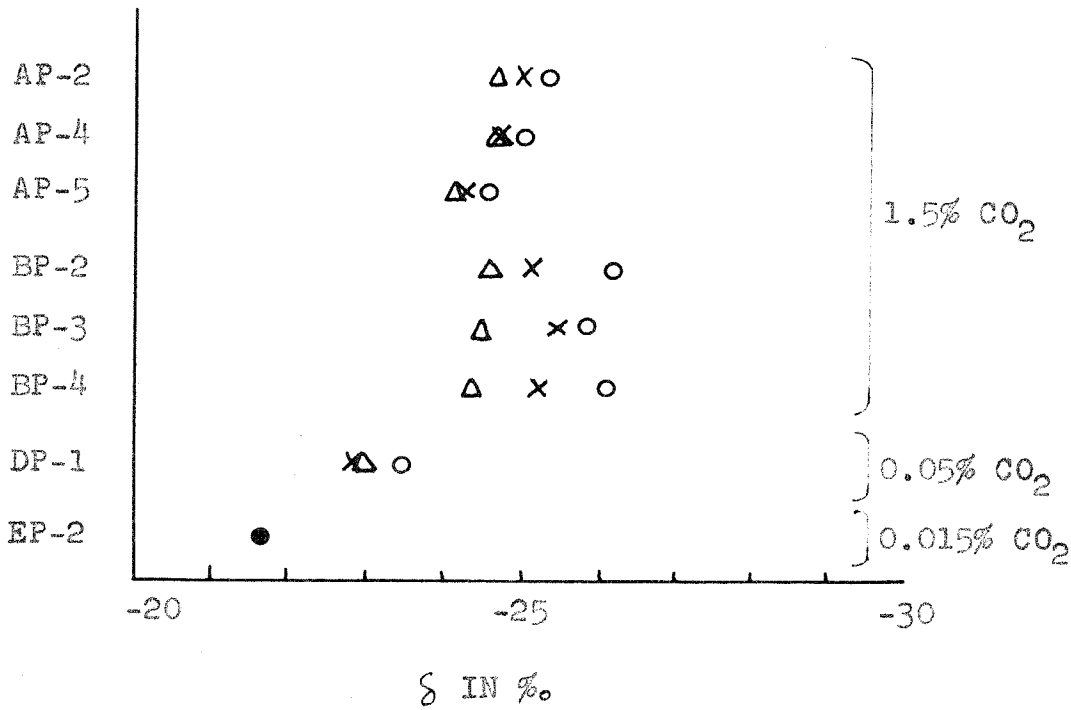


Figure 14. C^{13}/C^{12} ratios of tomato plants grown in different CO_2 concentrations.

○- leaves
×- stems
Δ- roots

therefore performed. Due to the limited facilities for production of CO_2 free air, only one plant could be grown in each of the following two experiments.

3. Tomatoes grown in 0.05% CO_2 of isotopic composition $\delta = +2.6‰$.

This plant was grown at the same temperatures, light intensity and for the same length of time as those grown in 1.5% CO_2. The results are given in Table XXII and the isotopic compositions plotted in Figure 14.

A weighted average for the plant as a whole is $\delta = -23.2‰$. The assumption that all of the seedling carbon resides in the plant when analyzed gives a correction of +0.1‰. Thus the isotopic composition of the plant as a whole may be considered as $\delta = -23.1‰$.

The enrichment factor may be calculated:

$$\varepsilon = \frac{R_{CO_2}}{R_{plant}} = 1.0263 \ .$$

4. Tomatoes grown in 0.015% CO_2 of isotopic composition $\delta = +2.6‰$.

The plant in this experiment was grown under conditions identical to those of the previous experiments except for the concentration of CO_2. In this case the dry weight of the plant was so small that separate isotopic analyses of the leaves, stem and root were not possible. This plant weighed 9.3 mg. The C^{13}/C^{12} ratio was -22.3‰. Correction for the carbon

TABLE XXII

C^{13}/C^{12} ratio of a tomato plant grown in 0.05% CO_2 (δ = +2.6‰).

Plant no.	Dry wt. (mg)	δleaves in ‰	δstems in ‰	δroots in ‰	δcotyledons in ‰
DP-1	35.1	-23.4	-22.8	-23.1	-22.8

contributed by the seedling to the final plant, assuming that all of the seedling carbon is contributed to the final plant, gives the value $\delta = -21.6‰$. This result is plotted in Figure 14. The enrichment factor may then be calculated:

$$\varepsilon = \frac{R_{CO_2}}{R_{plant}} = 1.0247 \ .$$

5. Air CO_2 as a carbon source for tomato plants under experimental conditions.

In this experiment, two tomato plants were grown under conditions identical to those of the previous experiments except that the CO_2 was supplied by (greenhouse) air. Air was pumped through the booth at a rate of 10 l/minute. The results of this experiment are given in Table XXIII. Taking -30.25‰ as an average isotopic composition and applying the correction for carbon contribution of the seedling, $\delta = -30.8‰$. Taking -7‰ as a representative value for the isotopic composition of air CO_2, the enrichment factor may be calculated for tomato plants grown in air as:

$$\varepsilon = \frac{R_{CO_2}}{R_{plant}} = 1.0246 \ .$$

6. Effect of high light intensities on the fractionation factor of photosynthesis.

This experiment was designed to determine whether light intensities greater than 10^3 foot-candles influence the fractionation factor of tomato plants grown in 1.5% CO_2 derived

TABLE XXIII

C^{13}/C^{12} ratios of tomato plants grown in air CO_2 under experimental conditions.

Plant no.	Dry wt. (mg)	δ of whole plant in ‰
FP-1	19.3	-30.0
FP-2	19.3	-30.5

from $CaCO_3$, $\delta = +2.6‰$. Higher light intensities were obtained by moving the booth and carbon dioxide generating unit into a greenhouse exposed to natural sunlight. Under these conditions a light intensity of 2,000 foot-candles was recorded inside the booth during mid-day. The experiment was run during the late April and early May so that the day length exceeded 12 hours. The experiment was run for 13 days. Results are given in Table XXIV. By taking the stems as an average value, the fractionation factor may be computed:

$$\varepsilon = \frac{R_{CO_2}}{R_{plant}} = 1.0277 \quad .$$

The data of the foregoing experiments are summarized in Table XXV. It is seen that greater C^{12} enrichment occurs at high CO_2 concentrations. Also, light intensity is not important over the intensity range studied.

7. Respired CO_2 collected over long intervals.

The 19 plants of the second 1.5% CO_2 experiment (BP series) were removed from the booth after 13 days and transferred, after 30 minutes exposure to normal air in the dark, to the respiration apparatus described above. The respired CO_2 was collected in separate lots during a series of time periods. After the first 14-hour period 14 plants were removed for isotopic analysis and five plants were left in the respiratory chamber. These plants ultimately began to decay.

TABLE XXIV

C^{13}/C^{12} ratio of a tomato plant grown at high light intensity in 1.5% CO_2 (δ = + 2.6‰).

Plant no.	Dry wt. (mg)	δ leaves in ‰	δ stems in ‰	δ roots in ‰
HP-1	48.4	-24.7	-24.4	-24.1

TABLE XXV

Summary of fractionation factors for tomato plants grown in different CO_2 concentrations.

CO_2 conc. in %	Av. plant wt. (mg)	Fractionation factor ± 0.0001
1.5 ± .15	50.1	1.0279
1.5 ± .15	42.1	1.0285
0.05 ± .005	35.1	1.0263
0.015 ± .002	9.3	1.0247
Air CO_2 *	17.8	1.0246
1.5 ± .15 (High light intensity)	48.4	1.0277

* C^{13}/C^{12} ratio of air assumed to be -7‰.

Later data probably represent CO_2 derived from bacterial decomposition of the plants. The data from this experiment are summarized in Table XXVI and plotted in Figure 15. 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 XXVII.

The inconsistencies in this table are within the error imposed by uncertainty in the elementary composition of the fractions respired. 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 respiration residue.

8. Respired CO_2 collected over shorter time 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 3.5 week old tomato plants were placed in the respiratory chamber at 5:30 P.M. The chamber was darkened and rapidly swept with CO_2 free air for four minutes. CO_2 was then collected at the intervals given in Table XXVIII. These data are plotted in Figure 16.

9. Distribution of labeling within plant compounds.

In the two experiments in which tomatoes were grown in 1.5% CO_2, a portion of the plants were fractionated according

TABLE XXVI

Time course of CO_2 respired from tomato plants over long time intervals.

No. of plants	Elapsed time from start of exp. in hours	µmols CO_2	Δ of respired CO_2 in %.
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

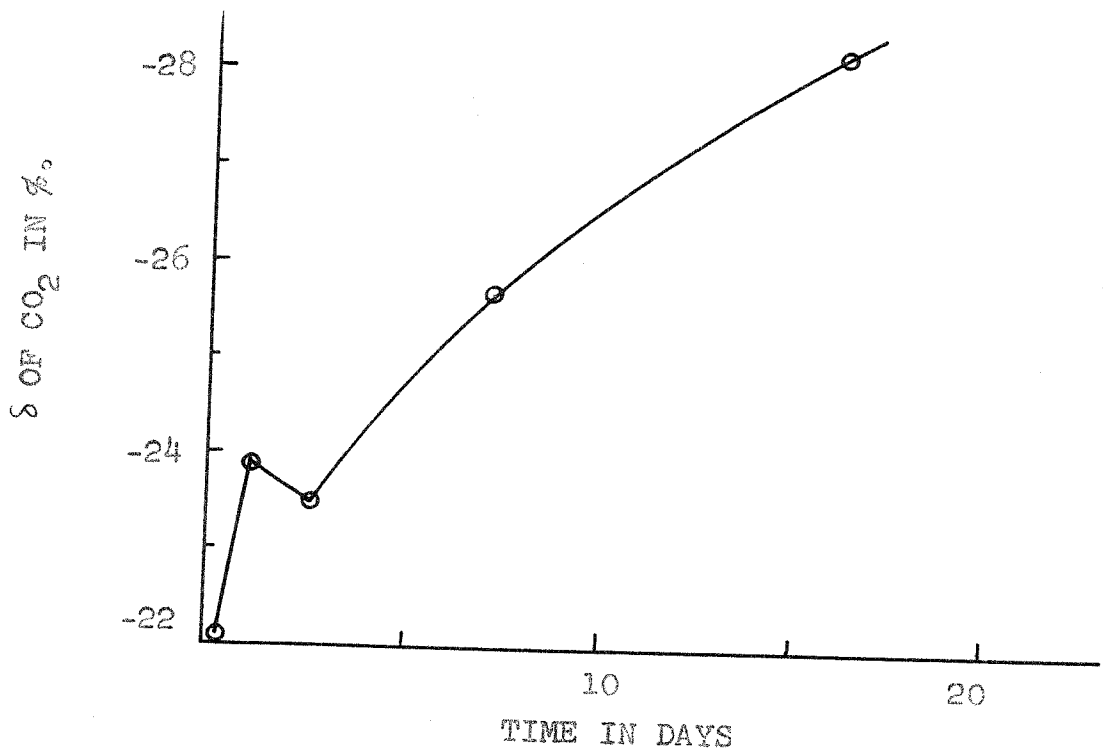


Figure 15. C^{13}/C^{12} ratios of CO_2 evolved by tomato plants in which the fixed carbon has a C^{13}/C^{12} ratio $\delta = -25.2‰$.

TABLE XXVII

Material balance for respired CO_2 and residue as compared to the initial plants.

Material (calculated on basis of 1 plant)	Amount of C in mM	δ of CO_2 in ‰
1. Intact plant (42.1 mg) Assume C = 43.6% dry wt. (83)	1.53	-25.2
2. Respired CO_2	0.93	-26.2
3. Residue Assume C = 43.6% dry wt. (83)	0.73	-24.7
4. Sum of lines 2 and 3	1.66	-25.6

TABLE XXVIII

Respired CO_2 over short time intervals from tomatoes in which δ = -32.4‰ for fixed carbon.

Sample no.	Elapsed time from start of exp.	μmols CO_2	δ in ‰
1	10 min.	51	-24.3
2	2 hours	488	-25.8
3	4 "	373	-27.2
4	6 "	174	-27.6
5	16 "	1,100	-28.2
6	40 "	2,116	-30.0

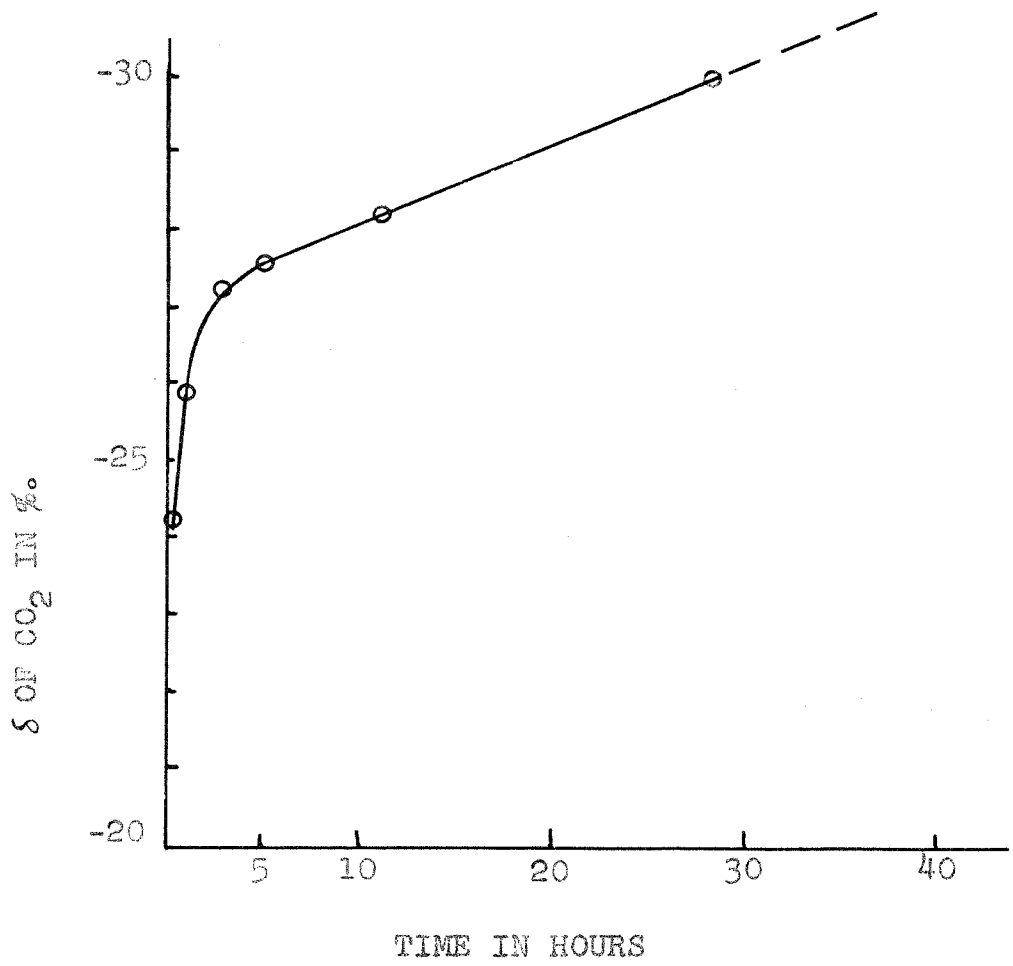


Figure 16. C^{13}/C^{12} ratios of CO_2 respired by tomatoes in which the fixed carbon has the C^{13}/C^{12} ratio $\delta = -32‰$.

to the scheme described above. These fractions were subjected to isotopic analysis with the results given in Table XXIX. The only fractions which differ greatly in isotopic composition from that of the plant as a whole are the $CHCl_3$ soluble or non-polar lipid fractions. These fractions are enriched in C^{12}.

To establish the generality of C^{12} enrichment in lipid fractions of various plant taxonomic groups, a number of other plants were analyzed with respect to the isotopic composition of the plant lipids as compared to that of the plant as a whole. These results are given in Table XXX. In all plants investigated the lipid fraction was enriched in C^{12} as compared to the plant as a whole.

Discussion.

The C^{12} enrichment factor of the experimental plants, as compared to the CO_2 from which they were derived, varied from 1.025 to 1.028. This indicates that the environmental conditions to which the plants were exposed in these experiments produced only relatively small changes in the total C^{12} enrichment of the plant. These environmental influences are now considered separately to determine whether there is a correlation between the environment and the observed C^{12} enrichment factors.

Plant dry weight over a given period may be taken as a measurement of the plant growth rate during the period. The

TABLE XXIX

C^{13}/C^{12} ratios of chemical fractions within the tomato plants grown in 1.5% CO_2.

Fraction	δ in ‰ for AP series	δ in ‰ 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 XXX

C^{13}/C^{12} ratios of plants and their respective lipid fractions as found in nature.

Plant	δ of carbon in ‰				Lipid in % dry wt.
	Whole plant	Lipid	Residue	Water soluble	
Ulva sp. (green alga)	-15.7	-23.5	-15.4	-	4.0
Gelidium sp. (red alga)	-20.5	-24.3	-19.9	-	9.4
Macrocystis pyrifera (brown alga)	-12.6	-21.0	-12.7	-	1.7
Mixed Phyto-plankton *	-9.2	-14.2	-8.3	-10.4	6.8
Asplenium bulbi-forum (fern)	-29.2	-37.9	-30.7	-30.5	3.8
Triticum vulgare (wheat)	-28.5	-31.1	-27.6	-28.3	11.6
Redwood needles	-26.4	-28.3	-25.2	-24.8	6.4

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

C^{13}/C^{12} ratios of plant stems grown in 1.5% CO_2 are plotted as a function of the plant dry weight in Figure 17. These data indicate that there is no direct relation between the fractionation factor and plant growth rate under these conditions.

It is interesting to note that the carbon of leaves is invariably the lightest carbon of the plant. Craig (55) made the observation that the C^{13}/C^{12} ratio of tree leaves is usually about 1‰ less than that of the wood of the tree and has suggested from the early data of Murphy (47) that this might be due to the presence of isotopically light waxes in the leaf. It has been shown above (Results) that the C^{13}/C^{12} ratio of the lipid fraction of tomato is 5.6‰ less than that of the plant as a whole. In a mature tomato plant the leaves, stem and root were found to contain 17.1%, 6.0% and 10.6% lipid respectively on a dry weight basis. The high proportion of lipid in tomato leaves as compared to stem and root appears to account in part for the consistent enrichment of C^{12} in tomato leaves as compared to stems and roots. The reason for a greater difference in the C^{13}/C^{12} ratios of leaves, stems and roots in the second 1.5% CO_2 experiment is not yet clear.

Plant growth rate was markedly reduced at low CO_2 concentrations. It is clear that low CO_2 concentration is a limiting factor in photosynthesis and hence in dry weight production by these plants. It is known from the work of

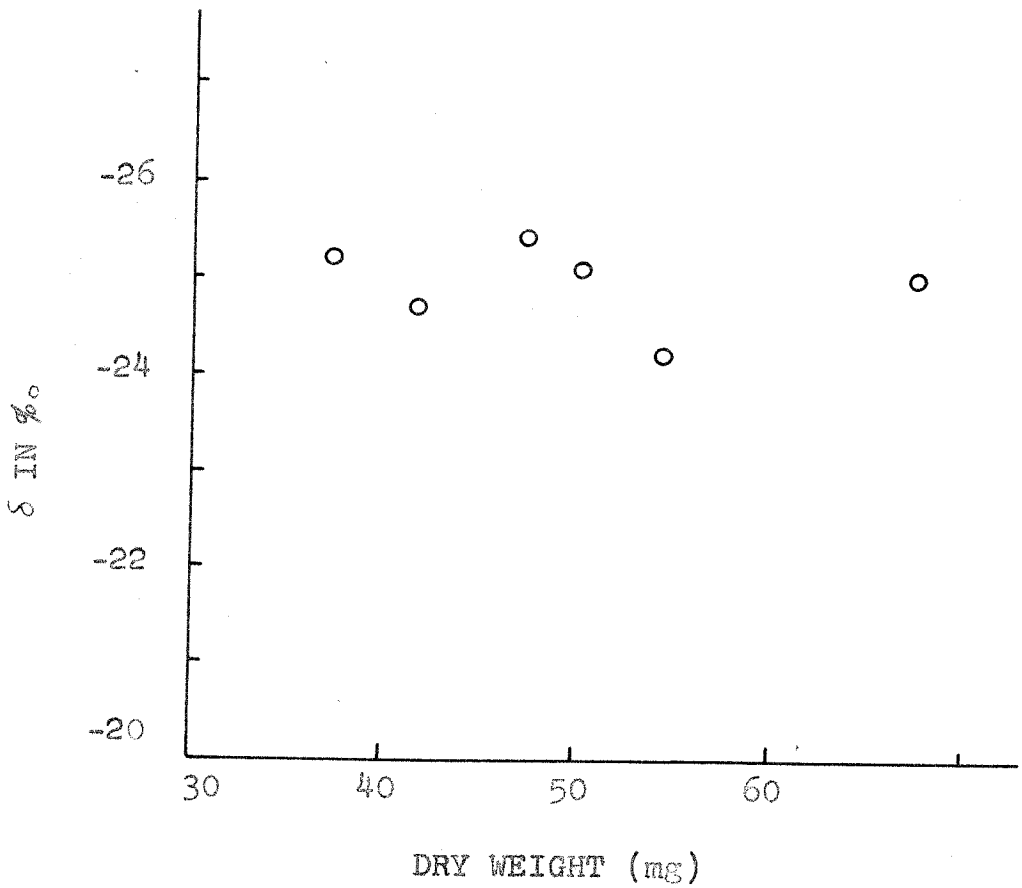


Figure 17. C^{13}/C^{12} ratios of tomato plants grown in 1.5% CO_2 as a function of plant dry weight.

many plant physiologists (84) that photosynthetic rate as a function of CO_2 concentration follows a curve similar to that in Figure 18. At high light intensities photosynthetic rate is proportional to the CO_2 concentration until the system becomes saturated with CO_2. The C^{13}/C^{12} ratios of plants grown at these CO_2 concentrations are also plotted in Figure 18. It is clear that a parallelism exists between dry weight and the fractionation factor as CO_2 concentration is increased. Fractionation is less when CO_2 is a limiting factor in photosynthetic rate as measured by plant dry weight. These data can be explained by assuming that a mechanism exists within a plant whereby the dissolved CO_2 is kept at some steady state C^{13}/C^{12} ratio. If the rate of CO_2 fixation is rapid as compared to the mechanism for maintenance of the C^{13}/C^{12} ratio of dissolved CO_2, the dissolved CO_2 would be expected to become enriched in C^{13} due to the preferential fixation of C^{12} by the enzymatic fixation of CO_2 as described in Part II. If this model is correct, it explains why C^{13} enrichment of dissolved CO_2, and consequently of the plant carbon, is more likely to occur at low CO_2 concentrations. This idea is given further support in Part II.

Table XXXI gives a comparison of the fractionation factor for tomatoes grown in the greenhouse as compared with the fractionation factor for plants grown from air CO_2 in the experimental booth. The larger fractionation observed in the booth must be due to some difference in the experi-

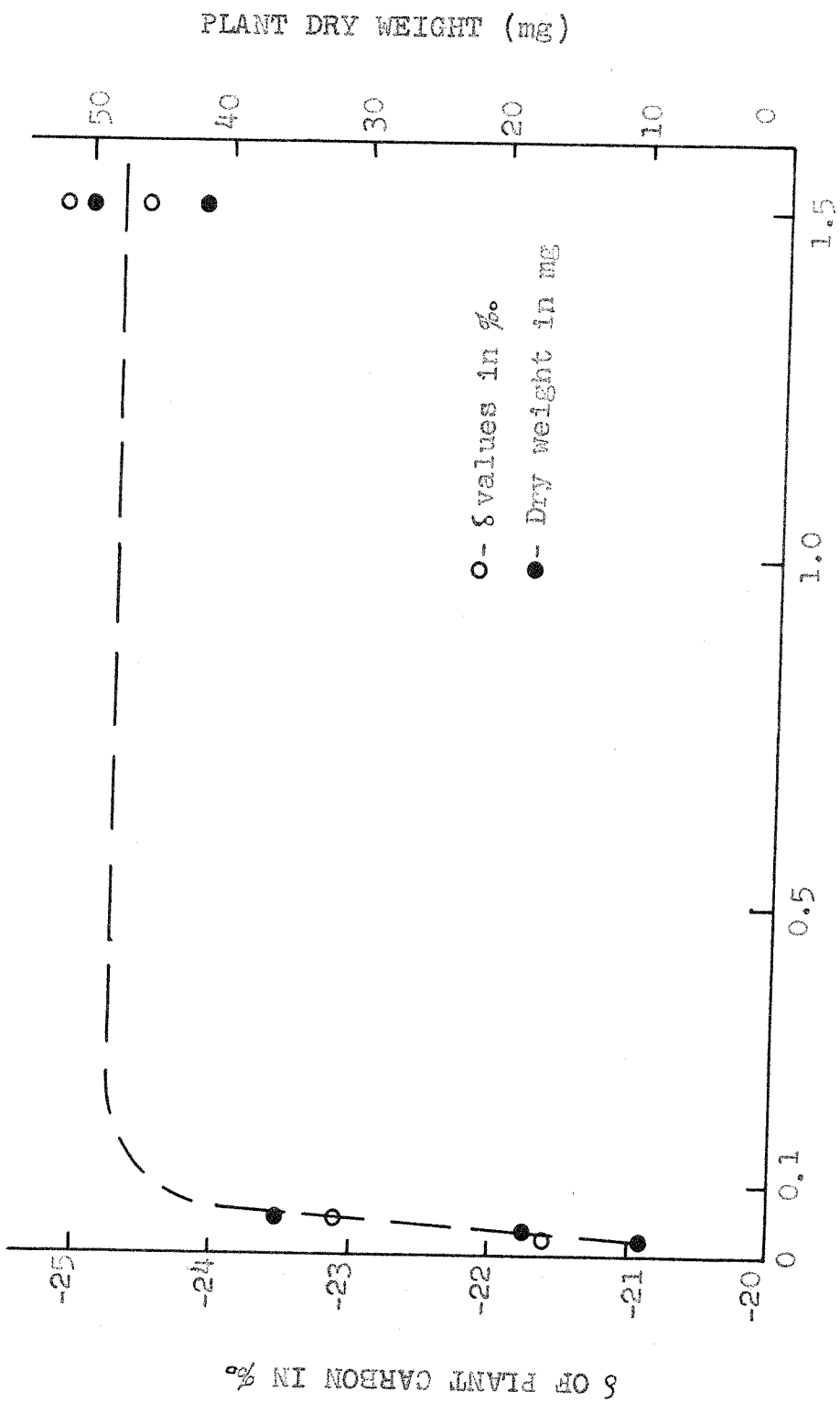


Figure 18. Plant dry weight and c^{13}/c^{12} ratio as a function of CO_2 concentration.

TABLE XXXI

Fractionation factors for greenhouse tomatoes as compared to tomatoes grown from CO_2 of known isotopic composition.

Plants	Fractionation factor
Grown in 0.05% CO_2 (ε = +2.6‰)	1.0263
Grown in 0.015% CO_2 (δ = +2.6‰)	1.0247
Heaviest greenhouse plant	1.0211 *
Lightest greenhouse plant	1.0262 *

* Air CO_2 assumed to be -7‰.

mental conditions under which the plants were grown. Light intensity was shown to be unimportant in determining the fractionation factor provided the intensity is above 10^3 fc. Temperature is not important since similar day and night temperatures were chosen for each experiment. The difference may be more closely related to the extent to which a local C^{13} enriched atmosphere was allowed to develop around the plants. In the booth new CO_2 is constantly added in great excess of that used in photosynthesis. Mixing of the CO_2 is rapid. Linear air speed over the leaves is about 150-200 cm per minute. In the greenhouse, on the other hand, air is not circulated nearly so rapidly and a C^{13} enriched atmosphere has a greater opportunity to develop in the vicinity of the leaf. Such an atmosphere would produce relatively C^{13} enriched leaves. It is interesting to note that the seeds from which these plants grew are -24.9‰. These seeds were grown under field conditions where air movement was probably slower than that in the greenhouse. Fairly rapid air speeds may be necessary to eliminate the local C^{13} enriched atmosphere over a leaf surface if a maximum fractionation factor is to be observed.

As mentioned above (Review), Keeling (59) has interpreted CO_2 concentration increase and C^{12} enrichment in the atmosphere under natural night conditions as due to the addition to the atmosphere of respired CO_2 of -22‰ by plants. The average C^{13}/C^{12} ratio of land plants is given

by Craig as -24.6‰. This suggests that CO_2 respired by plants may be similar in isotopic composition to the plant as a whole. Baertchi (62) determined that CO_2 respired by germinating beans possessed the same C^{13}/C^{12} ratio as the whole seed. In the light of the data presented for tomatoes this is not surprising, if it is assumed that a material balance exists between C^{12} enrichment in the lipid fraction and C^{13} enrichment in the respired CO_2 of tomatoes. This suggests that when a plant system is not manufacturing lipid, the C^{13}/C^{12} ratio of respired CO_2 is that of the sugars and lipid being respired as a whole. This is probably the case in germinating seeds where lipids are not being formed, but are being oxidized along with carbohydrate as an energy source. In Figure 16 it is seen that C^{13} enriched CO_2 is respired by plants only during the early portions of the respiration experiment. This can be interpreted as the period during which enough sugar is present from the photosynthetic period so that lipid production still occurs. As long as C^{12} enriched lipid is produced in the system, C^{13} enriched CO_2 will be respired. When lipid is not produced, respired CO_2 will have a C^{13}/C^{12} ratio closer to that of the plant as a whole. C^{12} enrichment in CO_2 evolved during decomposition of the plant may result from preferential decomposition of the lipids, fractionation during bacterial respiration or a combination of these two phenomena. Respiration and lipid formation are closely related biochemical systems. The

same two-carbon fragment, acetate, is used both for lipid synthesis, and respiration in the tricarboxylic acid cycle. Isotope selection at the level of the two carbon unit provides an explanation for the data obtained so far. Figure 19 is an outline of the biochemical pathways leading to lipid and respired CO_2 formation. That isotope selection actually does occur at this point is indicated in Figure 20 in which enrichment of the lipids in C^{12} for plants grown in nature as compared to the plant residue after extraction is plotted as a function of the % dry weight of lipid in 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 notion that C^{12} enriched C_2 units are selected for lipid synthesis. It is also interesting to note the wide taxonomic group of plants which seem to obey this relation.

The mechanism of this selection is not clear. C_2 units do not exist in a free form in plants, but are bound into large molecules such as acetyl-CoA. Some chemical effect and not diffusion must be responsible for the selection of C_2 units.

Figure 21 shows that the C^{13}/C^{12} ratio of marine organisms is about 10‰ greater than that of non-marine organisms. A similar relation exists between petroleums of marine and non-marine origin according to Silverman and Epstein (60). Both petroleums are enriched in C^{12} as compared to their

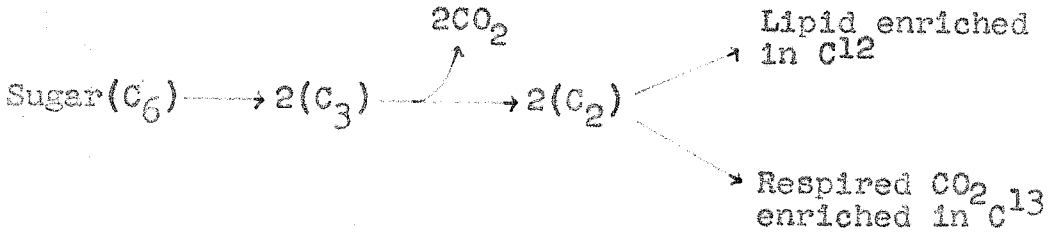


Figure 19. The biochemical pathway leading from sugars to two carbon units and their subsequent metabolism.

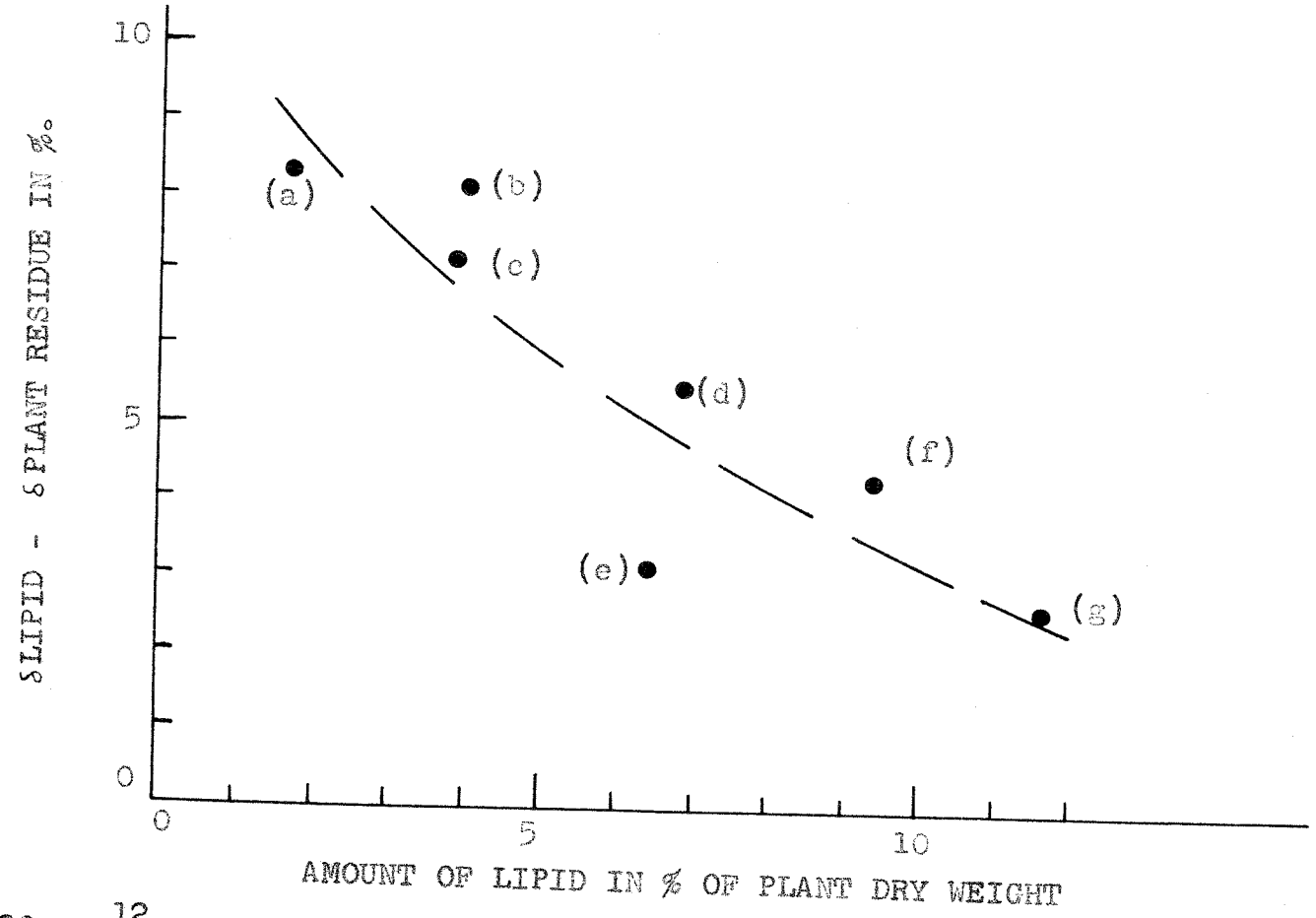


Figure 20. C^{12} enrichment in lipid fraction as compared to the plant residue as a function of the amount of lipid in % of plant dry weight. (a) brown alga (b) green alga (c) fern (d) phytoplankton (e) conifer (f) red alga (g) grass.

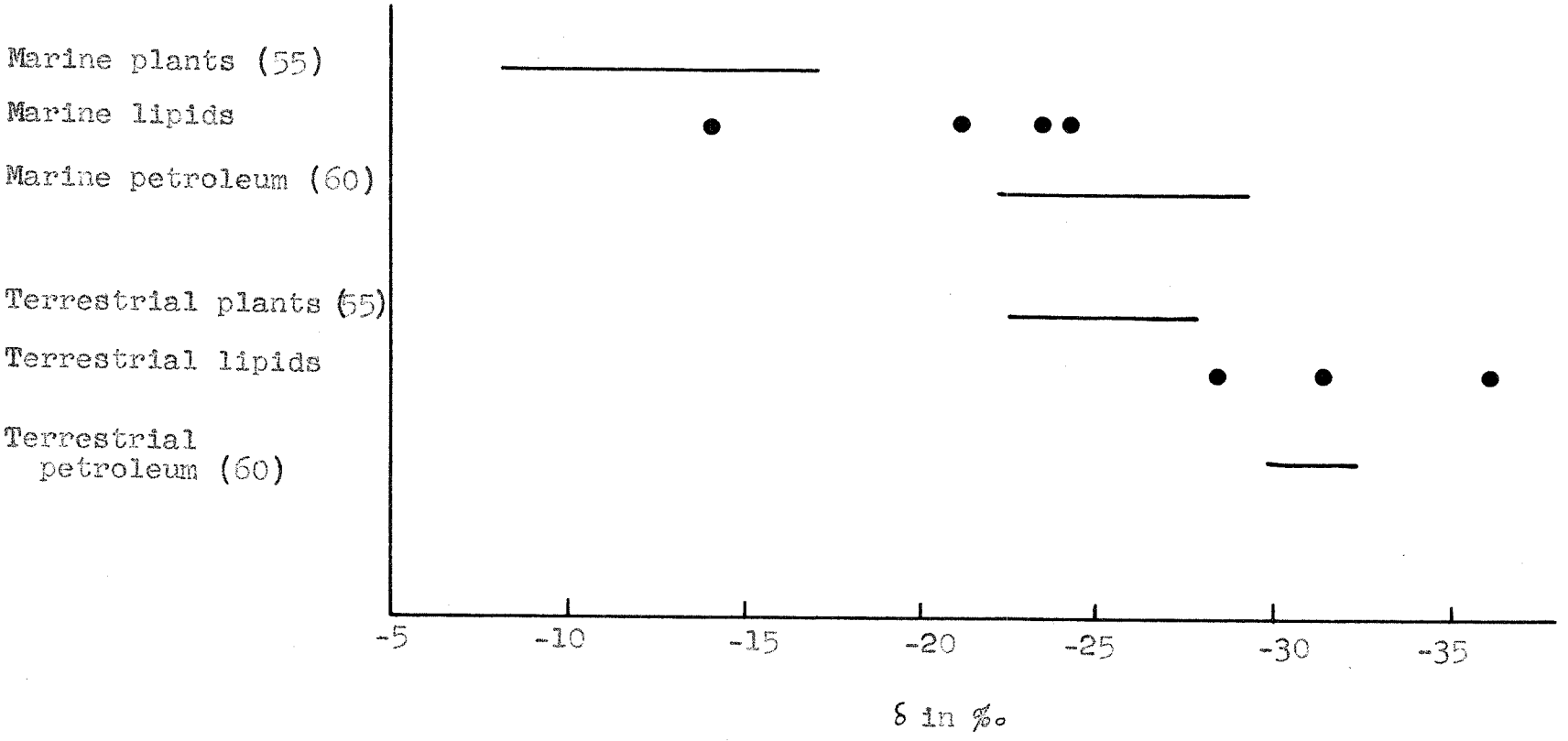


Figure 21. C^{13}/C^{12} ratios of terrestrial and marine plants and petroleums as compared to the lipid fractions of marine and terrestrial plants.

biological sources. Figure 21 also shows the C^{13}/C^{12} ratios of the lipids of the various plants reported above. The phytoplankton value may be a special case since these organisms were collected from a plankton bloom. The data just presented indicate that the isotopic compositions of petroleum correspond roughly to those of the lipids of their plant sources.

If the whole plant is preserved, petroleum formation must involve a discrimination against C^{13}. On the other hand, if only the lipid fractions of plants are preserved, fractionation is not an obligatory process in petroleum formation. In an attempt to ascertain whether the process of petroleum formation involves preservation of lipid substances without carbon isotope fractionation or whether petroleum formation also involves transformation of non-lipid materials, Silverman and Epstein (60) analyzed the non-polar extractable organic matter from three ocean cores. In a Florida core the amount of non-polar extractable organic matter increased with depth while the C^{13}/C^{12} ratios over the same interval decreased from -20.3‰ to -26.0‰. These data indicate that transformation of non-lipid substances to hydrocarbons does occur. Such a transformation might be carried out by bacteria during production of bacterial lipid from non-lipid organic matter. A core from Santa Barbara basin, on the other hand, contained an amount of extractable organic matter which was constant with depth and all of

isotopic composition -23.3‰ . This core indicates that lipid fractions of marine plants are preserved and are not diluted by transformation of other materials into lipid. Unfortunately, data of this kind are not yet plentiful enough to determine the relative importance of the two processes.

Since the C^{12} enrichment of the lipid fraction of a plant is inversely related to the amount of lipid found, C^{13}/C^{12} ratio variations in sedimentary non-polar organic matter might also be associated with variations in the species present in the plant populations which contribute the organic matter to the sediments. These are intriguing and interesting problems in which carbon isotope analysis of marine organic matter will be a powerful investigational technique.

Summary of Part I

Tomato plants grown from CO_2 of known isotopic composition are enriched in C^{12} by a fractionation factor of $1.025 \pm .001$ at normal CO_2 concentrations. The fractionation factor is somewhat greater at high CO_2 concentrations. Isotopic analyses of fractions from several plant phyla show that only the lipid fraction differs markedly from the C^{13}/C^{12} ratio of the plant as a whole. The C^{12} enrichment in the lipid fraction is inversely related to the amount of lipid in the plant. Respired CO_2 is initially heavier than the plant as a whole, eventually attaining C^{13}/C^{12} ratios equal to that of the plant. C^{13} enrichment in respired CO_2 is probably closely related to C^{12} enrichment in the lipid fractions of plants.

PART II

PHOTOSYNTHETIC FRACTIONATION IN HIGHER PLANTS AS A MULTISTEP PROCESS

CO_2 in the atmosphere is not the immediate source of CO_2 for photosynthesis. Rather the dissolved CO_2, HCO_3^- system of the cell cytoplasm constitutes the immediate reservoir from which a plant draws its CO_2. The cytoplasm of a plant cell has a pH of about 7. This means that about 85% of the CO_2 present in the cytoplasm will be in the form HCO_3^-. At equilibrium, the C^{13}/C^{12} ratio of the CO_2 in the reservoir will be expected to be 6‰ less than that of the bicarbonate. Craig (56) has shown that this is the fractionation factor between air CO_2 and ocean bicarbonate. This system is generally assumed to be in equilibrium. Carbonic anhydrase undoubtedly hastens the attainment of equilibrium in plants in many cases. Factors which affect the C^{13}/C^{12} ratio of this internal CO_2 reservoir will necessarily affect the C^{13}/C^{12} ratio of the final plant. Such factors are rates of CO_2 exchange between cytoplasm and atmosphere, photosynthetic rate, respiratory rate, C^{13}/C^{12} ratio of CO_2 in the atmosphere and transport of dissolved CO_2 from cells during translocation. The importance of these factors will be considered below (Discussion). The above consideration suggests that photosynthetic fractionation in higher plants can, as a first approximation, be considered as a two step

process. The first step involves fractionation between atmospheric CO_2 and CO_2 dissolved within the plant. The second step involves fractionation during the fixation of CO_2 into sugars. This system is pictured more quantitatively in equation 1.

$$\frac{R_{CO_2}}{R_{\text{total plant}}} = \frac{R_{CO_2}}{R_{\text{dissolved } CO_2}} \times \frac{R_{\text{dissolved } CO_2}}{R_{\text{total plant}}} \qquad (1)$$

where R is the C^{13}/C^{12} ratio of the fraction described in the subscript. The expression of equation 1 is, however, not yet complete. A factor must be included to take into account the C^{12} enrichment caused by the respiratory loss of C^{13} enriched CO_2. The total expression then takes the form:

$$\frac{R_{CO_2}}{R_{\text{total plant}}} = \frac{R_{CO_2}}{R_{\text{dissolved } CO_2}} \times \frac{R_{\text{dissolved } CO_2}}{R_{\text{plant carbohydrate}}}$$

$$\times \frac{R_{\text{plant carbohydrate}}}{R_{\text{total plant}}}$$

where the lipid contribution to the fractionation factor is taken as a measure of C^{13} enrichment in respired CO_2. This factor is only about 1.0005 and therefore makes only a small contribution to the total fractionation.

R_{CO_2}/R_{plant} was determined for greenhouse tomatoes in Part I (see Table XXXI) and shown to equal 1.0211 to 1.0262. This section is concerned with the experimental determination of $R_{CO_2}/R_{dissolved\ CO_2}$ for greenhouse tomatoes and $R_{dissolved\ CO_2}/R_{plant}$ for the fixation of CO_2 by the enzyme which catalyzes this process in photosynthesis. It is shown that when the experimentally determined values for the individual fractionation steps are substituted into equation 1 they yield a value for R_{CO_2}/R_{plant} which is, within the experimental error, the same as that determined experimentally in Part I. Further consequences of this fractionation model are considered below (Discussion).

The experiments in which $R_{CO_2}/R_{dissolved\ CO_2}$ for tomato plants is determined are described first. In these experiments dissolved CO_2 was isolated directly from tomato leaves which had been trapped in the photosynthetic state by freezing. The C^{13}/C^{12} ratio of the dissolved CO_2 was then compared with that of atmospheric CO_2, and $R_{CO_2}/R_{dissolved\ CO_2}$ calculated.

Materials and Methods.

The general procedure used to isolate the dissolved CO_2 of leaves in steady state photosynthesis was the following. Leaves were removed from a plant in bright light and rapidly frozen. The best freezing method consisted of plunging the leaves under liquid nitrogen within one second after their

removal from the plant. The leaves were then transferred to a precooled flask (-78°C) which was evacuated at dry ice temperature. Pumping was continued for about 10 minutes. The leaves were then thawed. Ten milliliters of 3% H_2SO_4 were tipped in from a side arm. The CO_2 freed from the pulpy residue of the leaves was collected and analyzed. This method involves a number of experimental pitfalls which are now taken up separately.

1. Trapping leaves in the photosynthetic state.

Initial experiments were performed by placing the leaves in a flask which was then placed in a dry ice bath. This method is a very poor one for several reasons. First, many of the leaves are in darkness when frozen. In this case respired CO_2 of a very different C^{13}/C^{12} ratio enters the flask and greatly complicates the picture. Second, extreme precautions are necessary to prevent contamination of the unfrozen leaves with dry ice CO_2. Dry ice CO_2 is greatly enriched in C^{12} as compared to most natural carbons. When dry ice contamination did occur the yields of dissolved CO_2 from leaves were as much as five times too high and the C^{13}/C^{12} ratios considerably less than the plant as a whole. Two such values are given in Table XXXII. In this case the leaves were frozen in a dry ice bath without stoppering the flask neck. Values and yields in which greater precautions were taken are given in Table XXXV.

The dry ice method of freezing was abandoned for the

TABLE XXXII

Dissolved CO_2 from tomato leaves contaminated with dry ice CO_2

Sample no.	Volume (cm^3) at 25°C	δ in ‰
KD-1	6	-38.1
LD-1	6.4	-37.5

reasons given above and preference given to the liquid nitrogen freezing method.

2. Contamination of leaves at -196°C by CO_2 condensation from the atmosphere.

After tomato leaves are frozen under liquid nitrogen, it is necessary to transfer the leaves to a cold flask. During this exposure to air, CO_2 could condense on the cold surface of the leaf and not be completely removed by evacuation of the flask at dry ice temperature for 10 minutes. To test this possibility, three frozen tomato leaf samples were prepared and 200 mg of dry ice added to each sample. Each sample was then pumped for varying lengths of time and the yields and C^{13}/C^{12} ratios compared as given in Table XXXIII. These data show that three hours pumping is necessary to remove 200 mg of solidified CO_2 from an evacuated flask at dry ice temperature. The value obtained after this time is very close to that obtained in most of the samples run. Ten to fifteen minutes pumping is apparently enough for samples prepared by freezing in liquid N_2. This experiment also shows that little or no exchange of CO_2 occurs between the leaf and the surrounding atmosphere when the leaf is frozen. This means that exposure of a leaf to air CO_2 after initial freezing in liquid N_2 will not result in measurable contamination.

TABLE XXXIII

Contamination of CO_2 dissolved in tomato leaves by dry ice purposely added to the system.

Sample no.	Time pumped at -78°C (hours)	Amt. CO_2 (cm^3)	δCO_2 in ‰
1	1	1.0	-33.3
2	3	0.45	-13.3
3	12	0.66	-11.3

3. Air CO_2 trapped within the leaf mesophyll during freezing.

If we assume that the entire volume of an experimental leaf is air, we can calculate the amount of CO_2 contained in this volume:

Volume % CO_2 in air = 0.03

Volume leaves per experiment = 50 cc max.

$0.03 \times 10^{-2} \times 50 = 0.015$ cc CO_2

$\therefore$ 0.015 cc CO_2 equals maximum contamination.

Any air trapped within the leaf during freezing would contribute less than 1% of the total volume of CO_2 obtained from the acidified leaves. This introduces a negligible correction in terms of C^{13}/C^{12} ratios.

4. Release of CO_2 from organic compounds by treatment with 3% H_2SO_4.

A major source of contamination of dissolved CO_2 might be the decarboxylation of keto acids by 3% H_2SO_4. After dilution with the aqueous phase of the plants, the concentration of H_2SO_4 is reduced to about 0.5 N. A number of α-keto acids such as α-keto glutarate and oxalacetate exist in plant tissues. Fairly rigorous conditions are needed to decarboxylate an α-keto acid. For this reason CO_2 from α-keto acids is probably not an important contaminant. β-keto acids exist in small quantities in plants and, in addition, yield CO_2 under very mild conditions. Virtanen, cited by Thimann and Bonner,(85) reports 30-40 mg oxalacetic acid/100 gm of pea plant. This concentration of β-keto acid

in tomato leaves would produce about 0.2 cc of CO_2. This approximate calculation suggests, therefore, that the true values for dissolved CO_2 may be somewhat heavier than those actually determined in these experiments.

A method used to check for contamination due to decarboxylation of organic acids was to analyze the dissolved CO_2 of respiring plants and compare this value with the C^{13}/C^{12} ratio of respired CO_2. The plants were swept with CO_2 free air in the respiration line so that no requilibration with air could occur. After 12 hours the respiring plants were frozen in darkness on a dry ice bath and the dissolved CO_2 isolated as described above (Methods). Data from such experiments are given in Table XXXIV.

The C^{13}/C^{12} ratio of dissolved CO_2 in respiring tomato leaves is about 10% less than that of the dissolved CO_2 in photosynthesizing tomato leaves. This indicates that the CO_2 obtained by acidification must be largely an expression of the CO_2 dissolved in a leaf and not of decarboxylation of organic substrates.

Results.

C^{13}/C^{12} ratios of the dissolved CO_2 of photosynthesizing tomato leaves are reported in Table XXXV. $\delta = -13.6‰$ is the average value obtained for the C^{13}/C^{12} ratio of CO_2 dissolved in tomato leaves during photosynthesis. This represents a minimum C^{13}/C^{12} ratio due to possible contamination by

TABLE XXXIV

CO_2 dissolved in respiring tomato plants.

Plant no.	δ Dissolved CO_2 in ‰.	δ Respired CO_2 in ‰.
1	-25.8	-29.3
2	-25.9	-20.8
3	-20.4	-20.8
4	-21.3	-28.5
Average	-23.4	-25.8

TABLE XXXV

CO_2 dissolved in photosynthesizing tomato leaves.

Sample no.	δ in ‰ of CO_2 dissolved in leaves during photo-synthesis
LD-19	-15.4
LD-22	-17.5
LD-31*	-12.3
LD-32*	-12.0
LD-33*	-12.3
LD-36	-15.2
LD-37	-9.6
LD-71	-14.1
Average	-13.6

* Samples collected from the same group of plants at the same time.

CO_2 from organic substrates. Assuming that air CO_2 is -7‰, the enrichment factor $R_{CO_2}/R_{\text{dissolved } CO_2}$ from tomatoes may be calculated:

$$\varepsilon = \frac{R_{CO_2}}{R_{\text{dissolved } CO_2}} = 1.0067 .$$

Experiments are now described in which $R_{\text{dissolved } CO_2}/R_{\text{plant}}$ is determined enzymatically.

Calvin (86) has shown that the principal CO_2 fixation reaction of photosynthesis is the carboxylation of ribulose 1,5-diphosphate to yield two 3-phosphoglyceric acids as shown in Figure 22.

Both Ochoa (87) and Horecker (88) have purified the enzyme which catalyzes this reaction. Horecker's system carries out a stochiometric conversion of RuDP to PGA. The enzyme has been purified 15 times from spinach leaves. This system was chosen for investigation of carbon isotope discrimination during the enzymatic fixation of dissolved CO_2 into sugars during photosynthesis.

Methods and materials.

1. Enzyme preparation.

Ribulosecarboxylase was prepared essentially according to the method described by Horecker (88). Six hundred gm of fresh spinach leaves were homogenized for three minutes at 5°C in 2,000 ml of 10^{-2} M potassium phosphate buffer at

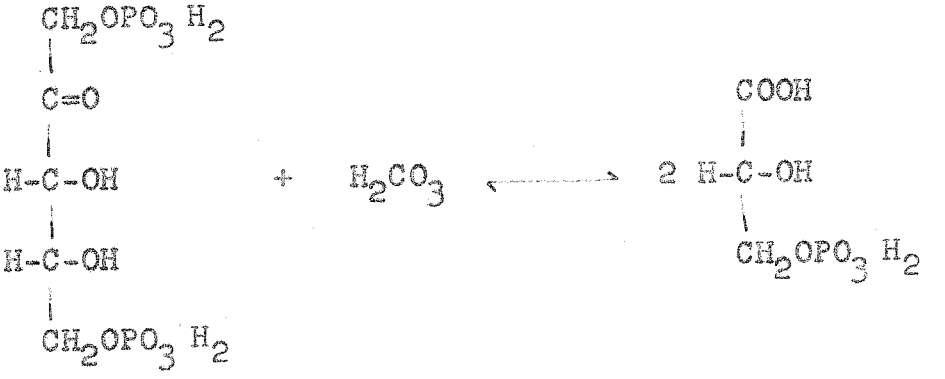


Figure 22. The CO_2 fixation reaction of photosynthesis.

pH 7.4 which contained EDTA 10^{-4} M. The homogenate was filtered through Whatman #1 filter paper and adjusted to pH 7 with 1 N NH_4OH. Then 475 gm of $(NH_4)_2SO_4$ were added and the precipitate discarded. The supernatant was treated with 210 gm of $(NH_4)_2SO_4$ and the precipitate dissolved in 100 ml 0.1 M phosphate buffer pH 7.4 which contained EDTA 5×10^{-5} M. This preparation constitutes the first purification and is called fraction A.S. I.

A.S. I was diluted one-fold with distilled water. Ninety-two and one-half milliliters of saturated $(NH_4)_2SO_4$ solution were added and the precipitate discarded. The supernatant was treated with 30.3 ml of saturated $(NH_4)_2SO_4$ solution and the precipitate collected and dissolved in 30 ml of 0.1 M carbonate buffer, pH 7.4. This preparation constituted the purified enzyme used in the following experiments and was stored at -16°C.

2. Enzymatic assay.

The enzyme was assayed for its ability to catalyze incorporation of $C^{14}O_2$ into RuDP by incubation with the reaction mixture shown in Table XXXVI for 20 minutes at room temperature. The reaction was stopped by addition of six drops of 2 N HCl. The reaction mixture was heated for four minutes at 100°C. Unreacted CO_2 liberated from the reaction mixture was trapped in ascarite by sweeping with air for ten minutes. Precipitated protein was centrifuged down, washed two times with water and discarded. The supernatant

TABLE XXXVI

Reaction mixture for assay of phosphoribulocarboxylase.

Material	Amount in µmol
$MgCl_2$	5
GSH	1.5
EDTA	0.3
$NaHCO_3$	10
$Na_2C^{14}O_3$	0.2 (8×10^4 CPM)
RuDP	1
Enzyme	0.1 ml (100γ protein)

Total volume equals 0.40 ml. Total CO_2 in reaction mixture equals 20.2 µmol. Specific activity of dissolved CO_2 equals 3,950 CPM/µmol.

was brought to pH 6.5 with freshly prepared NaOH. Saturated $BaCl_2$ solution was added dropwise to the supernatant until no more precipitate was formed. The precipitate was washed twice with ethanol:water, 1:1 and counted on a Nuclear micromil gas flow scaler, model 181.

3. Ribulose 1,5-diphosphate.

Ribulose 1,5-diphosphate was obtained as the barium salt from B. L. Horecker at the National Institute of Health. This material had been prepared by treating ribulose-5-phosphate with ATP in the presence of phosphoribulokinase purified from spinach. The product was 60% pure, the bulk of the impurity consisting of $BaPO_4$, and the remainder (probably) of ribose-5-phosphate and ribulose-5-phosphate. The barium salt was prepared for experiments by treatment with analytical grade Dowex 50 in the hydrogen form to exchange the barium for hydrogen ions.

4. Isolation of PGA from the reaction mixture.

The reaction mixture was brought to pH 1.5 with 2 N HCl and heated on a steam bath for four minutes. After the denatured protein had been centrifuged off, the supernatant was brought to pH 6.5 by dropwise addition of freshly prepared NaOH. Saturated $BaCl_2$ solution was next added dropwise until no more precipitate was formed. An equal volume of ethanol was then added. The precipitate was washed 3 times with ethanol:water, 1:1. Next the precipitate was treated

with analytical grade Dowex 50 in the hydrogen form. The supernatant was evaporated to a small volume and placed on Whatman #3 filter paper which had previously been washed with methanol:formic acid:water, 80:15:5. Authentic PGA was used for reference spots. The chromatogram was then run at 2°C. in methanol:formic acid:water, 80:15:5 according to the method of Bandurski and Axelrod (89). The paper was dried in a stream of warm air for three hours and the reference strips removed and assayed for phosphorous with molybdate reagent. The portion of the chromatogram corresponding to PGA was cut out and eluted with distilled water. The eluate was brought to pH 6.5 with fresh NaOH solution and $BaCl_2$ was added. The precipitate was washed two times with distilled water and the sample was dried and combusted for analysis.

5. The C^{13}/C^{12} ratio of the total dissolved CO_2 in reaction mixture.

The stoppered reaction mixture was frozen at the end of the experiment. The frozen reaction mixture was then evacuated at dry ice temperature and CO_2 isolated according to the procedure described above (Methods and Materials, Part I).

Results.

1. Enzyme assay with $C^{14}O_2$.

Two reaction mixtures were prepared as described in

Table XXXVI except that one reaction mixture contained no RuDP. The mixtures were incubated 20 minutes at 25°C and analyzed as described above. Results are given in Table XXXVII. Chromatography of the labeled material in methanol: formic acid:water, 80:15:5 gave an rf value for the labeled material identical to PGA. The amount of label in the -RuDP reaction mixture indicates that sweeping of the acidified reaction mixture may have been insufficient in this experiment.

2. Carbon isotope discrimination during the enzymatic fixation of dissolved CO_2 into sugars during photosynthesis.

Two reaction mixtures were prepared as described in Table XXXVIII and incubated with reagent grade $NaHCO_3$ as a CO_2 source. The reaction mixtures were stoppered and incubated for 60 minutes at 25°C. Reaction mixture #1 was frozen for dissolved CO_2 analysis and PGA was isolated from mixture #2 as described above. Results are presented in Table XXXIX.

Only 1/6 of the carbon atoms in the isolated PGA were derived from dissolved CO_2 in solution. The remaining 5/6 of the carbon atoms were derived from RuDP. The isotopic composition of the fixed carbons is given by the expression:

$$5/6\ (-13.7‰) + 1/6(x) = -15.2‰$$

where x = the value of the carbon fixed into sugar.

$$x = -22.8‰$$

TABLE XXXVII

Enzyme assay with $C^{14}O_2$

	Flask 1	Flask 2
RuDP	-	+
CPM in barium ppt.	46	2,900
CPM expected for 100% yield	3,950	3,950
% yield	1	74

TABLE XXXVIII

Reaction mixture used in large scale CO_2 fixation experiment.

Material	Flask 1	Flask 2
$MgCl_2$	110 µmol	110 µmol
GSH	33 µmol	33 µmol
EDTA	6.6 µmol	6.6 µmol
$NaHCO_3$	1,000 µmol	1,000 µmol
RuDP	-	33 µmol
Enzyme	2.2 ml	2.2 ml

Total volume equals 9.0 ml.

pH equals 7.8 - 8.0

TABLE XXXIX

C^{13}/C^{12} ratios of the reactants and products of the enzymatic CO_2 fixation reaction of photosynthesis.

Material	δ Value of carbon in ‰
Ribulose 1,5-diphosphate	-13.7
Total CO_2 dissolved in reaction mixture	-5.4
3-phosphoglyceric acid	-15.2

The difference in per mil (Δ) of the CO_2 in solution and that fixed during carboxylation is then given by:

$$\Delta = -(22.8) - (-5.4) = -17.4‰ \quad .$$

The errors compounded during this calculation give a value:

$$\frac{R_{\text{dissolved } CO_2}}{R_{\text{sugar}}} = 1.018 \pm .001 \quad .$$

Calculation of R_{CO_2}/R_{plant} from the experimentally determined values for $R_{CO_2}/R_{\text{dissolved } CO_2}$ and $R_{\text{dissolved } CO_2}/R_{plant}$.

R_{CO_2}/R_{plant} can now be calculated from the relation of equation 1.

$$\frac{R_{CO_2}}{R_{plant}} = \frac{R_{CO_2}}{R_{\text{dissolved } CO_2}} \times \frac{R_{\text{dissolved } CO_2}}{R_{plant}} \qquad (1)$$

By substituting into this equation the experimentally determined values, R_{CO_2}/R_{plant} may be calculated to be:

$$(1.007 \pm .004)(1.018 \pm .001) = 1.025 \pm .005.$$

When this result is compared with the experimentally determined values for R_{CO_2}/R_{plant} from Part I, these two values agree remarkably well. The implications of these findings are considered below.

Discussion.

A model to satisfactorily account for a fractionation process must provide a material balance for the fractionated species. This is true of C^{13}/C^{12} fractionation in plants. The C^{12} enrichment of a land plant as compared to atmospheric CO_2 determines the amount of C^{13} which must be rejected at the plant surface. A very simple model for carbon fractionation in plants would envisage a one step process in which some kinetic effect, such as collision frequency, on the plant surface, would enrich C^{12} in the dissolved CO_2 of the plant. If this effect were about 20‰, then no further fractionation would need take place during the carboxylation process of photosynthesis. Two experiments have indicated that a simple model of this type is not applicable to higher plants.

First, the CO_2 dissolved in tomato plants during photosynthesis is intermediate in C^{13}/C^{12} ratio between atmospheric CO_2 and the fixed carbon in the plant. Second, the enzymatic fixation of CO_2 from dissolved bicarbonate is shown to be a fractionating process. Apparently the fractionation process in higher plants, such as tomato, involves at least two stages. The first stage, which is undoubtedly made up of several steps, involves the maintainance of a steady state isotopic composition of dissolved CO_2 within the plant during photosynthesis. The second stage, which probably also comprises several steps, brings about discrimination between dissolved CO_2 in the plant and the carbon fixed into sugars.

These two postulated fractionating steps will now be considered separately. The second step, fractionation between dissolved CO_2 and sugars, is described first since it is more easily understood in the light of present knowledge of plant metabolism.

There is much argument in the biological literature as to whether CO_2 or HCO_3^- is the reactive form of carbon in photosynthesis. The experimental approach to this problem usually consists of the observation of growth rates of algae in media buffered at varying pH with phosphate (90). This sort of experiment is greatly complicated since the investigator never knows whether the conversion of HCO_3^- to CO_2 is the rate limiting reaction in the growth of an alga. In fact, many algae contain the enzyme carbonic anhydrase which catalyzes the equilibration of CO_2 and bicarbonate. A second difficulty with this type of experiment is that pH affects not only the ratio of HCO_3^- to CO_2, but probably also affects many other variables such as membrane permeability. Nevertheless, most investigators have concluded that CO_2 is the reactive form of carbon in photosynthesis. Recent evidence on the mechanism of CO_2 fixation from the laboratory of Calvin supports this conclusion. It was initially thought that CO_2 is fixed during photosynthesis according to the reaction in Figure 22. This reaction was thought to be a one step process in which carboxylation and hydrolysis took place simultaneously. It has recently been found in Calvin's

laboratory that there is, however, a labile intermediate in the reaction and that at least two steps are therefore involved. The first is the production of the labile intermediate, supposedly a carboxylated RuDP for which several structures have been suggested. In the second step, a hydrolysis, this intermediate is broken down to PGA. In this mechanism, carboxylation would appear to be necessarily carried out by CO_2 itself, hydrolysis taking place in a separate step.

If CO_2 is the reactive form of dissolved CO_2 in photosynthesis, one can make the following prediction. The C^{13}/C^{12} ratio of CO_2 in equilibrium with HCO_3^- is expected to be less than that of bicarbonate by 6‰. Thus, in the enzymatic system, at pH 7.8, if only a small portion of the CO_2 in the system is used, and if CO_2 is the reacting species, one would expect the fixed CO_2 to be at least 6‰ lighter than the total bicarbonate in the system. This naturally assumes that the CO_2 in the system is in equilibrium with the bicarbonate. That this is a reasonably good assumption is shown by the following experiment. One reaction mixture, similar to those used in the enzymatic carboxylation experiment, except that no RuDP was added, was incubated for one hour. The reaction mixture was divided into two parts and frozen. One portion of the reaction mixture was evacuated and acidified. The total CO_2 was collected and analyzed. The other portion was evacuated and allowed to thaw without

acidification. After five minutes at room temperature the flask was placed under vacuum and the CO_2 released from the solution was collected and anlyzed. The results are presented in Table XL.

This experiment indicates that, to a first approximation, the CO_2 in the system is in equilibrium with bicarbonate.

The results of the enzymatic experiment show that C^{12} enrichment during the enzymatic fixation of CO_2 into sugars exceeds that which would be expected on the basis of equilibrium considerations alone. The cause of this excess fractionation is a matter of conjecture. Diffusion of CO_2 to the active carboxylation site on the enzyme might bring about such a further C^{12} enrichment.

A number of factors will influence the amount of C^{12} enrichment which occurs during enzymatic fixation of CO_2 into RuDP in an intact plant. The degree to which CO_2 and bicarbonate are in equilibrium within plant cytoplasm is one important consideration. Carbonic anhydrase undoubtedly hastens the equilibration. Still, the degree to which equilibration is reached within the cytoplasm of a photosynthesizing plant is unknown. The similarity between the enzymatic fractionation factor and the fractionation factor between dissolved CO_2 and fixed carbon in tomatoes suggests that the degree of equilibration in the two systems may be somewhat similar. The isotope equilibrium exchange constant for CO_2 and $CO_3^=$ is temperature dependent. According to

TABLE XL

CO_2 in reaction mixture obtained by acidification and pumping.

Substance	Volume (cm^3)	δ in ‰
Total CO_2 by acidification	8.4	-3.2
Partial CO_2 by pumping without acidification	1.2	-11.0
Difference		7.8‰

Urey's (53) calculations, between 0° and 25°C this effect should amount to 3‰. Thus, over the range of temperatures in which tomatoes grow, the temperature effect is comparatively small.

In summary, the 17‰ fractionation favoring C^{12} observed in the enzymatic fixation of CO_2 into sugar is postulated to occur in two steps. The first step involves a 6‰ fractionation between bicarbonate and CO_2. The second step may involve some diffusion process.

The C^{13}/C^{12} ratio of CO_2 dissolved in photosynthesizing tomatoes as determined by the above experiments is about 7‰ less than that of atmospheric CO_2. A satisfactory model must account for the steady state maintenance of this ratio. A model which satisfies this requirement is shown in Figure 23.

First, consider the case in which air CO_2 is in equilibrium with the CO_2 dissolved in the plant. In this case the dissolved CO_2 will bear the same relation to atmospheric CO_2 as do ocean carbonates, namely the C^{13}/C^{12} ratio of dissolved CO_2 will be about 0‰. The data for dissolved CO_2 in photosynthesizing tomatoes show that this is not the case. Air CO_2 is apparently not in equilibrium with the dissolved CO_2 in the tomato plants. Obviously, some kinetic, non-equilibrium processes must determine the fractionation. Craig has shown that diffusion of CO_2 to the absorbing surface is usually not a rate limiting process. Still,

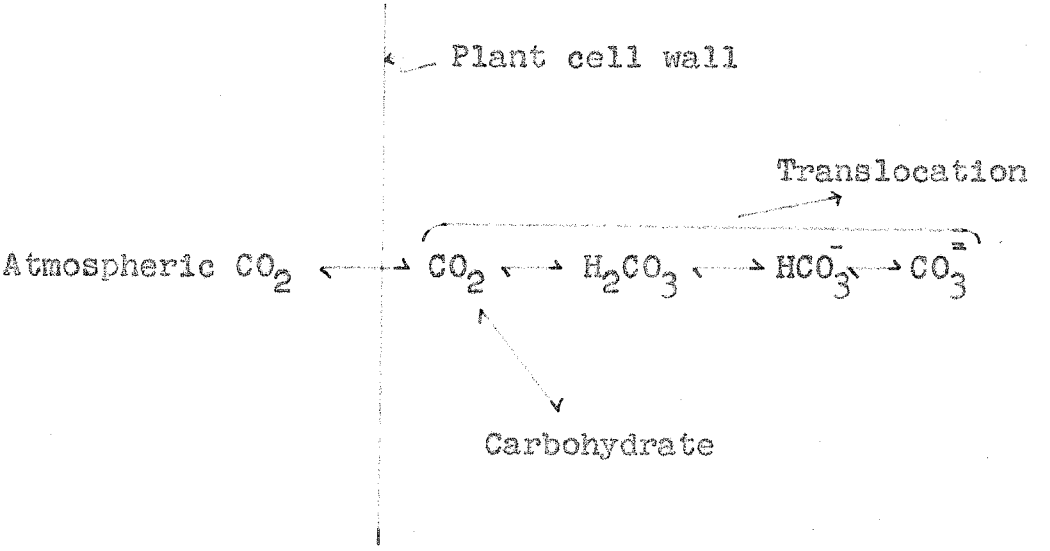


Figure 23. A model for C^{13}/C^{12} fractionation as a multi-step process.

barium hydroxide solutions preferentially remove C^{12} from atmospheric CO_2 under non-equilibrium conditions. This suggests that collision frequency may be an important factor in C^{12} enrichment of CO_2 absorbing solutions. The ratio of collision frequencies of mass 44 and 45 molecules at a given temperature against a surface is given by the following expression:

$$\frac{\sqrt{45}}{\sqrt{44}} = 1.011 \quad .$$

Thus, the CO_2 dissolved in the plant could, in principle, be as light as -18‰, assuming that the absorption rate of a molecule by the solution is directly proportional to its collision frequency on the absorbing surface. On the basis of this model, the dissolved CO_2 in a plant may be expected to vary between 0‰ and -18‰ under most conditions.

The most serious problem still remains. First stage discrimination against C^{13} at the plant surface on a collision frequency basis is not sufficient to fulfill the basic requirement of a fractionation model. Namely, a material balance between C^{12} enrichment in the fixed carbon of the plant and rejection of C^{13} at the plant surface must be maintained. The fact that C^{12} is selected during the carboxylation process necessitates C^{13} enrichment in the dissolved CO_2 of the plant. C^{13} must then be excluded from dissolved CO_2 in the plant by some other process. Initial

investigations indicate that translocation of solutes from a plant cell may be a basic aspect of this process. It is known that water is continuously supplied to photosynthesizing leaves for two purposes. The first purpose is to replace water lost from the leaves during transpiration. The second purpose is to transport from the photosynthetic cells the substances manufactured by photosynthesis to other portions of the plant. Burrows (91) has shown that in one hour, one-half the sugar in a bean leaf, made during a brief photosynthetic period, is transported out through the petiole. Several lines of investigation indicate that transport of sugar in the plant occurs by the process designated as mass flow. By mass flow is understood the transport of sugars and other solutes within a vascular plant occurring by simultaneous movement of solvent and solute in response to a pressure gradient. This means that the half time for translocation of sugar from a leaf to other portions of a plant is also the half time of water renewal in the leaf due to translocation. It is possible therefore that translocation may provide a method, in addition to collision frequency discrimination on the plant surface, for C^{13} elimination from the plant. Constant renewal of water in the cell and removal of C^{13} enriched bicarbonate by translocation makes a multi-step fractionation within a higher plant a definite possibility. An experiment is now described which lends support to this idea.

In this experiment, the leaves of a plant are allowed to photosynthesize. The stem is jacketed in a box and CO_2 evolved by this stem collected. If C^{13} enriched CO_2 and carbonate are being swept from the leaf during photosynthesis it should be possible to recover such material from the stem. A bean plant in the two leaf stage was used as experimental material. The stem of the plant was sealed into a gas tight container with silicone grease, the leaves remaining outside the box. The tightness of the seals is attested by the C^{13}/C^{12} ratios for respiration obtained during the dark period. (Air CO_2 contamination would have made those values much lower.) CO_2 free air washed through water was continuously swept through the box. During collection of samples, the CO_2 free air was not washed with water so that a large negative pressure would not develop within the box. After sample collection the water washed CO_2 free air stream was again admitted to prevent desiccation of the plant. The samples were trapped in the same apparatus described above (Materials and Methods, Part I) for collection of respired CO_2. CO_2 was first collected from the box during a light period when the CO_2 evolved might be expected to be enriched in C^{13}. The CO_2 was then collected during the following dark period when the CO_2 evolved from the stem regions would be expected to again become enriched in C^{12}. Table XLI presents the results of this experiment.

TABLE XLI

Evolution of CO_2 from bean plant stems in light and dark.

Sample no.	Elapsed time (hours)	Lights	Volume CO_2/ hour (cm^3)	δCO_2 in ‰
LR-86	0	on	1.4	-18.9
LR-87	1.5	on	2.4	-17.7
LR-88	3	on off at 3.5 hours	1.6	-19.2
LR-89	22	off	1.9	-15.9
LR-90	94	off	1.0	-23.5

It is clear that the CO_2 collected from the stem when the leaves are photosynthesizing is in fact heavier than that collected when the leaves are not photosynthesizing. There are however some complications. Thus, the isotopic composition of the CO_2 collected from the stem does not alter immediately after the plants are placed in darkness. Burrows (91) reports, however, that transport of water and solutes from bean leaves continues for 24 hours in darkness following a light period. The data are probably also complicated by diurnal rhythms in plant respiration. Nevertheless it lends support to the notion that C^{13} enriched CO_2 is transported away from photosynthetic cells of the leaf during translocation.

A valid objection to this hypothesis remains. Both liverworts and mosses show C^{12} enrichment equivalent to that shown by higher plants (54). These groups have no vascular system to facilitate the removal of C^{13} enriched CO_2 from photosynthesizing cells. Since almost nothing is known of transport mechanisms in these plants and since they grow within 10 cm of the ground and may be exposed to a local C^{12} enriched CO_2 environment, it is probably premature to conclude that their C^{13}/C^{12} ratios either support or disprove the translocation theory just proposed.

One interesting piece of evidence which supports the translocation hypothesis is the C^{13}/C^{12} ratio of "spanish moss," Ramalina sp., samples of which were obtained from a

coastal forest near Santa Barbara, California. Ramalina, a lichen which has no vascular transport system whatsoever, has a C^{13}/C^{12} ratio of -18.0‰. A heavy value would be expected since there is no mass transport of water in the lichen to remove $C^{13}O_2$ from the photosynthetic cells.

In summary, the mechanism of C^{12} enrichment in higher plants probably involves a multistage process. The data suggest that two main steps are involved. The first is fractionation during the absorption of CO_2 from the air. The second fractionation occurs between the dissolved CO_2 in the cell and fixed carbon in sugars. These two factors have been measured independently and shown to account for the total fractionation in tomatoes. $C^{13}O_2$ may be eliminated from photosynthetic cells by the process of translocation. These experiments need much confirmatory support before their implications can be regarded as definitely established.

Conclusions.

Tomato plants grown under controlled conditions from CO_2 of known C^{13}/C^{12} ratio at air concentrations are enriched in C^{12} as compared to the CO_2 gas by the fractionation factor $1.025 \pm .001$. This factor is greater by about 1.002 than the average fractionation factor observed for tomato plants in nature. Increased C^{12} enrichment in the experimental plants may result from absence of development of a local C^{13} enriched atmosphere under experimental conditions. Chemical

fractions within the plant do not all have the same C^{13}/C^{12} ratio. The C^{13}/C^{12} ratio of lipid material is as much as 8‰ less than that of the plant as a whole. The C^{12} enrichment in lipids is similar to that observed for petroleums as compared to present day plants. Respired CO_2 from tomatoes is initially enriched in C^{13} as compared to the plant as a whole. After several hours the C^{13}/C^{12} ratio of respired CO_2 approaches that of the plant. During the initial stages of decomposition, the CO_2 evolved from tomato plants becomes enriched in C^{12} as compared to the total plant carbon.

The C^{13}/C^{12} ratio of CO_2 dissolved in photosynthesizing tomato plants is about 7‰ less than that of air. The enzymatic CO_2 fixation reaction of photosynthesis involves a 17‰ fractionation in favor of C^{12} in the newly formed sugar as compared to the bicarbonate from which the CO_2 used in fixation is derived. Both these observations suggest strongly that photosynthetic C^{12} enrichment in plants is not a one stage fractionation, but involves several steps. It is suggested that the first fractionation step occurs during the absorption of air CO_2 by the cell. The second fractionation occurs during fixation of dissolved CO_2 in the cells into sugar. Translocation of CO_2 and carbonates from the leaf provides a mechanism for removal from this tissue of the C^{13} which has been rejected by the carboxylation system of photosynthesis.

It may eventually be possible to interpret variations

in the C^{13}/C^{12} ratios of plants in nature in terms of the ecological conditions under which each plant grows. A much better understanding of the mechanisms of C^{12} enrichment by plants will probably be necessary before such correlations can be made with accuracy.

C^{13}/C^{12} ratio analysis is applicable not only to the topics discussed in this dissertation but to a wide range of problems of biology. In particular, systems which involve CO_2 and HCO_3^- can now be characterized by one more parameter. The fact that CO_2 and HCO_3^- at equilibrium differ in C^{13}/C^{12} ratio suggests many interesting experiments as to membrane specificity and permeability. This dissertation shows that carbon isotope fractionation in biological systems can provide useful information which at present is unobtainable by other tracer techniques. The methods used here are applicable to many other problems in biology.

REFERENCES

42. Vaughan, A. L., Williams, J. H. and Tate, J. T., Phys. Rev., 46, 327 (1934).

43. Brosi, A. R. and Harkinds, W. D., Phys. Rev., 52, 472-474 (1937).

44. Nier, A. O., Phys. Rev., 52, 933-937 (1937).

45. Nier, A. O. and Gulbranson, E. A., J. Am. Chem. Soc., 61, 697-698 (1939).

46. Murphy, B. F. and Nier, A. O., Phys. Rev., 59, 771-772 (1941).

47. Murphy, B. F., M. A. Thesis, University of Minnesota, Minneapolis (1941).

48. Belkengren, R. O., Ph.D. Thesis, University of Minnesota, Minneapolis (1941).

49. Rabideau, G. S., Ph.D. Thesis, University of Minnesota, Minneapolis (1943).

50. West, S. S., Geophysics, 10, 406-420 (1945).

51. Nier, A. O., Rev. Sci. Instrum., 18, 398-411 (1947).

52. McKinney, C. R., McCrea, J. M., Epstein, S., Allen, H. A. and Urey, H. C., Rev. Sci. Instrum., 21, 724-730 (1950).

53. Urey, H. C., J. Chem. Soc., 562-581 (1947).

54. Wickman, F. E., Geochim. et Cosmochim. Acta, 2, 243-254 (1952).

55. Craig, H., Geochim. et Cosmochim. Acta, 3, 53-92 (1953).

56. Craig, H., The Journal of Geology, 62, 115-149 (1954).

57. Fuller, H. J., Am. Midland Naturalist, 39, 247-249 (1948).

58. Verduin, J. and Loomis, W. E., Plant Physiol., 19, 278-291 (1944).

59. Keeling, C. D., Geochim. et Cosmochim. Acta (in press).

60. Silverman, S. R. and Epstein, S., Bull. Amer. Assoc. Petrol. Geol. (in press).

61. Baertschi, P., Helv. chim. Acta, 35, 1030-1036 (1952).

62. Baertschi, P., Helv. chim. Acta, 36, 773-781 (1953).

63. Weigl, J. W. and Calvin, M., J. Chem. Phys., 17, 210 (1949).

64. Weigl, J. W., Warrington, P. M. and Calvin, M., J. Am. Chem. Soc., 73, 5058-5063 (1951).

65. van Norman, R. W. and Brown, A. H., Plant Physiol., 27, 691-709 (1952).

66. Buchanan, D. L., Nakao, A. and Edwards, G., Science, 117, 541-545 (1953).

67. Wickman, F. E., Geochim. et Cosmochim. Acta, 9, 136-153 (1956).

68. Bigeleisen, J., J. Chem. Phys., 21, 1333-1339 (1953).

69. Bigeleisen, J. and Mayer, M. G., J. Chem. Phys., 15, 261-267 (1947).

70. Beeck, O., Otvos, J. W., Stevenson, D. P. and Wagner, C. D., J. Chem. Phys., 16, 255-256 (1948).

71. Pitzer, K. S., J. Chem. Phys., 17, 1341-1342 (1949).

72. Yankwich, P. E. and Calvin, M., J. Chem. Phys., 17, 109-110 (1949).

73. Lindsay, J. G., McElcheran, D. E. and Thode, H. G., J. Chem. Phys., 17, 589 (1949).

74. Bigeleisen, J. and Allen, T. L., J. Chem. Phys., 19, 760-764 (1951).

75. Bothner-By, A. A. and Bigeleisen, J., J. Chem. Phys., 19, 755-759 (1951).

76. Lindsay, J. G., Bourns, A. N. and Thode, H. G., Can. J. Chem., 30, 163-165 (1952).

77. Yankwich, P. E., Stivers, E. C. and Nystrom, R. F., J. Chem. Phys., 20, 344 (1952).

78. Ordin, L., Cleland, R. and Bonner, J., Proc. Nat. Acad. Sci., 41, 1023-1029 (1955).

79. Bonner, J., Plant Biochemistry, Academic Press Inc., New York 10, N.Y., 122 (1950).

80. Bonner, J., Plant Biochemistry, Academic Press Inc., New York 10, N.Y., 133 (1950).

81. McCrea, J. M., J. Chem. Phys., 18, 849-857 (1950).

82. Craig, H., Geochim. et Cosmochim. Acta, 12, 133-149 (1957).

83. Latshaw, W. L. and Miller, E. C., Jour. Agric. Res., 27, 845-859 (1924).

84. Rabinowitch, E. I., Photosynthesis, Vol. II, Interscience Publishers Inc., New York, 858-864 (1951).

85. Thimann, K. V. and Bonner, W. D., Ann. Rev. Plant Physiol., 1, 75-108 (1950).

86. Quayle, J. R., Fuller, R. C., Benson, A. A. and Calvin, M., J. Am. Chem. Soc., 76, 3610-3611 (1954).

87. Jakoby, W. B., Brummond, D. O. and Ochoa, S., J. Biol. Chem., 218, 811-822 (1956).

88. Horecker, B. L., Hurwitz, J. and Weissbach, A., J. Biol. Chem., 218, 795-810 (1956).

89. Bandurski, R. and Axelrod, B., J. Biol. Chem., 193, 405-410 (1951).

90. Emerson, R. and Green, L., Plant Physiol., 13, 157-168 (1938).

91. Burrows, V. D., Personal Communication.