THE POSITION OF C^{14} IN SUNFLOWER LEAF METABOLITES AFTER EXPOSURE OF LEAVES TO SHORT PERIOD PHOTOSYNTHESIS AND DARKNESS IN AN ATMOSPHERE OF $C^{14}O_2$[1]

MARTIN GIBBS

Received January 6, 1951

The rate at which assimilated carbon dioxide is distributed in the carbon atoms of plant sugars and other plant metabolites should provide indication of the interrelationships between the sugars and these substances after exposure of the plant to carbon dioxide in the light and in the dark.

WOOD and BURR (**9**) were the first to determine the distribution of isotopic carbon in the carbohydrates of a bean plant allowed to photosynthesize in $C^{13}O_2$. They found that sucrose had a higher C^{13} isotope content than the hexoses, dextrin or starch. They also noted that there was a difference in the C^{13} content in the various carbon atoms of the sugars. CALVIN and BENSON (**2**) have reported that during short period photosynthesis in $C^{14}O_2$ sucrose was the only non-phosphorylated carbohydrate-containing label. The specific activity of carbon atoms 3,4 of the hydrolyzed sucrose was higher than carbon atoms 2,5 and 1,6. In some experiments, the specific activity of carbon atoms 2,5 was equal to carbon atoms 1,6 while in others they were unequal. Malic acid was predominantly carboxyl labeled. The distribution of label in alanine was somewhat similar to that of hexose. The carboxyl carbon having the highest specific activity, the beta carbon the least.

GIBBS (**3**) reported that with a one-hour period of photosynthesis by barley plant in $C^{14}O_2$, the distribution of label in the hexoses was the reverse of that published by the California workers (**2**), *i.e.*, carbons 1,6 had the highest specific activity. Recently VITTORIO, KROTKOV, and REED (**8**) have shown that the discrepancy between the result in the two studies was due to a dilution of carbon atoms 3,4 by $C^{12}O_2$ after the hexose was uniformly labeled.

VARNER and BURRELL (**7**) found the same distribution of label within the carbon atoms of the soluble hexoses as in the glucose from starch isolated from *Bryophyllum calycinum* leaves which had photosynthesized for 30 minutes in $C^{14}O_2$, carbon atoms 3,4 having the highest specific activity. Malic acid isolated from Bryophyllum leaves which had been exposed to $C^{14}O_2$ in the dark for 2.5 hours was predominantly carboxyl labeled.

The present work is concerned with the distribution of C^{14} in the glucose, fructose, sucrose, dextrin, starch, alanine, and malic acid of sunflower leaves

[1] Research carried out at Brookhaven National Laboratory under the auspices of the U. S. Atomic Energy Commission.

after a short period of photosynthesis in $C^{14}O_2$. The distribution of isotope in the same compounds was also determined after exposure of sunflower leaves to isotopic carbon dioxide in the dark. The sunflower leaf was selected since it synthesizes carbohydrate in light and dark at a suitable rate.

Methods

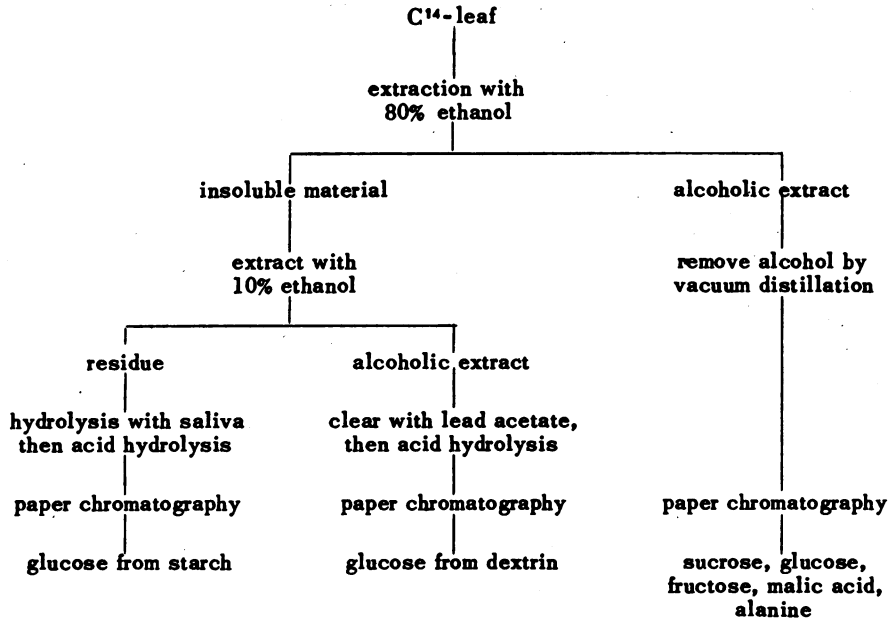
Exposure of leaves to isotopic carbon dioxide

A detached leaf of a sunflower plant was allowed to photosynthesize in air for 15 minutes in a chamber similar to that of Aronoff *et al.* (1). The leaf was then exposed to carbon dioxide containing 100 microcuries of C^{14} released from 500 mg. of barium carbonate for one and one-half, two or four minutes. The temperature was 20° C and the illumination was equivalent to 10,000 foot-candles. After exposure, the chamber was swept three minutes with CO_2-free air. The leaves were then immersed in boiling 80% alcohol for 20 minutes.

The leaves used in the dark exposure experiments were taken from plants previously kept in the dark for 40 hours. The petioles of the detached leaves were placed in a few ml. of a 7% glucose solution, then exposed at 25° C to $C^{14}O_2$ in the dark for 16 and 27 hours. The leaves were then immersed in boiling 80% alcohol for 20 minutes.

Isolation of C^{14}-labeled compounds

The isolation of the C^{14} metabolites is summarized in the following flow sheet:



The initial alcoholic extract was evaporated under vacuum at room temperature to approximately 2 ml. About 20 microliters of this solution was subjected to 2-dimensional paper chromatography. Phenol and a mixture of butyl alcohol and propionic acid were the solvents. To isolate the glucose from fructose or sucrose, the sucrose spot was cut out of the paper chromatogram, the sucrose eluted with water, hydrolyzed with invertase, and rechromatographed on paper with phenol as solvent (**6**). The alanine and malic acid were obtained for degradation by cutting out the appropriate spots from the 2-dimensional chromatogram and eluting the compounds with warm water.

The material remaining after the 80% ethanol extraction was placed in a 50-ml. round bottom centrifuge tube and dried overnight in a vacuum oven. After drying, a small amount of sharp sand was added and the sample frozen by pouring about 25 ml. of liquid nitrogen into the tube. The frozen leaf was ground to a fine powder with a thick stirring rod. The ground tissue was extracted four times with cold 10% alcohol. The 10%

TABLE I

DEGRADATION OF SYNTHETIC LACTIC ACID ACTIVITY IN MILLIMICROCURIES (1×10^{-3} MICROCURIES) PER MG. OF CARBON

	Carbon atom		
	COOH	CHOH	CH_3
Lactic acid-1-C^{14}	273.0	0.0	0.0
Lactic acid-2-C^{14}	2.3	209.0	0.0
Lactic acid-3-C^{14}	0.0	0.62	25.8

alcohol extract which contained the dextrin, was cleared with lead acetate and hydrolyzed with 1 *N* sulphuric acid. The acid was removed by addition of solid barium carbonate. The glucose from the dextrin was isolated by paper chromatography. The residue remaining after the 10% alcohol extraction which contained the starch was dried, hydrolyzed with saliva, and then treated in the same manner as the 10% alcohol extract.

Degradation procedures

The sugars were degraded by the method of WOOD, LIFSON, and LORBER (**8**). The reactions are:

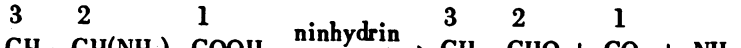
1. $\overset{1}{CHO}-\overset{2}{CHOH}-\overset{3}{CHOH}-\overset{4}{CHOH}-\overset{5}{CHOH}-\overset{6}{CH_2OH} \xrightarrow{\text{Lactobacillus casei}} \overset{1,6}{CH_3}-\overset{2,5}{CHOH}-\overset{3,4}{COOH}$

2. $CH_3-CHOH-COOH \xrightarrow{KMnO_4} \overset{1,6}{CH_3}-\overset{2,5}{CHO} + \overset{3,4}{CO_2}$

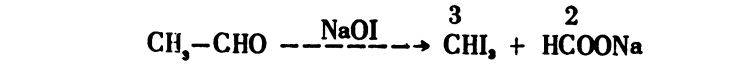
3. $CH_3-CHO \xrightarrow{NaOI} \overset{1,6}{CHI_3} + \overset{2,5}{HCOONa}$

Test of the fermentation (reaction 1) has been carried out (**4**). The iodoform resulting from reaction 3 is oxidized to CO_2 by chromic acid and the formate to CO_2 by mercuric oxide. Table I presents a test of reactions 2 and 3.

Alanine was degraded by the following reactions:



1. $\overset{3}{CH_3}-\overset{2}{CH(NH_2)}-\overset{1}{COOH} \xrightarrow{\text{ninhydrin}} \overset{3}{CH_3}-\overset{2}{CHO} + \overset{1}{CO_2} + NH_3$

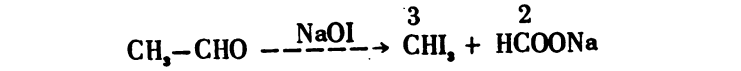


2. $CH_3-CHO \xrightarrow{NaOI} \overset{3}{CHI_3} + \overset{2}{HCOONa}$

Table III represents a test of the alanine degradation procedure.

The malic acid was degraded by the following reactions (**11**):

1. $\overset{4}{HOOC}-\overset{3}{CH_2}-\overset{2}{CHOH}-\overset{1}{COOH} \xrightarrow{KMnO_4} \overset{3}{CH_3}-\overset{2}{CHO} + \overset{1,4}{CO_2}$



2. $CH_3-CHO \xrightarrow{NaOI} \overset{3}{CHI_3} + \overset{2}{HCOONa}$

The iodoform and formate of the alanine and malic acid degradation were treated as described under sugar degradation.

TABLE II

DEGRADATION OF SYNTHETIC ALANINE-2-C^{14}

Carbon atom	mμc./mg. carbon
COOH	0.12
$CH(NH_2)$	823.0
CH_3	0.06

The C^{14} was determined as $BaCO_3$ on a sintered disk and counted with a methane flow proportional counter. The techniques of counting C^{14} and the method of oxidizing carbon compounds for total activity used in this laboratory have been described by STEELE and SFORTUNATO (**5**).

Results and discussion

Of the various carbohydrates, the free monosaccharides (glucose and fructose) contained no label. This fact is in agreement with the mechanism for photosynthesis proposed for Chlorella, barley, and Scenedesmus (**2**).

The results of the degradation of the compounds isolated from sunflower leaves exposed to strong light in the presence of $C^{14}O_2$ for short periods of time are shown in table III. The figures represent percentages of the total C^{14} in the various carbon atoms. Carbon atoms 3,4 had the highest activity in all the sugars. In the 1.5-minute and 4-minute exposures, the specific activity of carbon atoms 2,5 was practically equal to 1,6. In the 2-minute

exposure, the specific activity of position 2,5 of sucrose was greater than 1,6. Unequal (**2, 3, 4, 5**) and equal (**2**) distribution of activity in 2,5 and 1,6 has been reported. The factors leading to this peculiar distribution of label are obscure. With opposite leaves from the same plant, an equal distribution of C^{14} between 2,5 and 1,6 was found with one, an unequal distribution with the other. It seems possible that the hypothetical C_2 fragment might be formed by two pathways, one of which passes through a symmetrical molecule. The degree of labeling would then depend on the rates of the two mechanisms. The glucose and fructose derived from sucrose during the 1.5-

TABLE III

DEGRADATION OF PHOTOSYNTHETIC PRODUCTS. FIGURES ARE PERCENTAGES OF C^{14} IN VARIOUS CARBON ATOMS.

Compound	1.5 min.	2 min.	4 min.
Sucrose			
Fructose			
3,4	48	52	37
2,5	26	31	33
1,6	25	17	30
Glucose			
3,4	48	51	
2,5	25	31	
1,6	27	18	
Dextrin			
3,4	36		37
2,5	33		35
1,6	31		28
Starch			
3,4	72		61
2,5	14		17
1,6	14		22
Alanine			
COOH	47		41
$CHNH_2$	28		29
CH_3	25		30
Malic Acid			
COOH	76		76
CHOH	11		12
CH_2	13		12

and 4-minute periods of photosynthesis had a similar distribution of label which would indicate a common source.

The dextrin achieved uniform labeling the fastest of the carbohydrates. In spite of this, the starch was synthesized slowly from the newly formed carbohydrates. If the plant had been starved, the label in the starch might have been equal to the dextrin and sucrose.

The distribution of label in the alanine was the same as in sucrose. The carboxyl carbon predominated while the alpha carbon was practically equal to the beta carbon. With increasing exposure time, the distribution became more uniform. The malic acid was predominantly carboxyl labeled and did

not change with time. The specific activity of the alpha carbon was equal to the beta carbon. The 2-dimensional radiograms of the alcoholic extract which contained the alanine and malic acid were similar to those of BENSON and CALVIN (2). The alanine showed a dark spot while the malic acid showed a very faint spot. This result has been interpreted by these workers to indicate that strong light inhibits the formation of compounds of the tricarboxylic acid cycle. The degradation data would tend to substantiate this interpretation.

The results of the degradation of compounds isolated from sunflower leaves which had been exposed in the dark to an atmosphere of $C^{14}O_2$ for 16 and 27 hours are shown in table IV. The figures represent percentages

TABLE IV

DEGRADATION OF DARK FIXATION METABOLITES. FIGURES REPRESENT PERCENTAGES OF C^{14} IN VARIOUS CARBON ATOMS.

Compound degraded	16 hours	27 hours
Sucrose		
3,4	90	87
2,5	5	6
1,6	5	7
Dextrin		
3,4	90	
2,5	5	
1,6	5	
Starch		
3,4	84	96
2,5	8	1
1,6	8	3
Alanine		
COOH	98	100
$CH(NH_2)$	0	0
CH_3	2	0
Malic Acid		
COOH	92	
CHOH	4	
CH_3	4	

of C^{14} in the various carbon atoms. The dark exposure experiments were carried out to determine whether carbon dioxide fixation by a leaf in the dark was similar to fixation of carbon dioxide by non-photosynthetic organisms. As in the photosynthesis experiments, the monosaccharides contained no label. It is evident from table IV that the carbohydrates contained practically all their C^{14} in positions 3,4 while alanine and malic acid were carboxyl labeled. The presence of label in positions 1,2,5,6 of sugar was partially caused by the *L. casei* fermentation during degradation (4).

Mammalian tissues fix carbon dioxide in the 3,4 position of glucose (10) and lower organisms fix CO_2 in the carboxyl groups of organic acids. Since a similar distribution of label occurred in the sunflower metabolites, it would indicate that the carbon dioxide entered the plant metabolites by a Wood-

Werkman or similar reaction and in the case of the sugars followed by the reverse of glycolysis.

The distribution of label in the sunflower metabolites after exposure of a leaf to light in the presence of $C^{14}O_2$ in contrast to its distribution in the dark indicate that light is necessary to initiate the cycle which synthesizes the C_2 acceptor.

Summary

Detached sunflower leaves were allowed to photosynthesize in strong light for a short period in an atmosphere of $C^{14}O_2$, after which the carbohydrates (sucrose, glucose, fructose, dextrin, and starch), alanine, and malic acid were isolated and the location of the C^{14} determined. The same compounds were isolated and degraded from leaves which had been exposed to $C^{14}O_2$ in darkness.

1. In the photosynthesis experiments: (a) The monosaccharides contained no label. (b) Dextrin approached uniform distribution of carbon more rapidly than the other substances. (c) The labelling of alanine was similar to that of sucrose. (d) Malic acid remained predominantly carboxyl labeled during all exposures.

2. In the dark experiments: (a) Of the carbohydrates, glucose, fructose, and sucrose, only the sucrose contained label. When the sucrose was inverted and degraded, the invert sugar was labeled only in carbon atoms 3 and 4. (b) The alanine and malic acid were labeled only in the carboxyl carbon.

3. The data indicate that before label can occur in positions other than carbon atoms 3 and 4 of sugar and the carboxyl carbon of amino acids and organic acids, light is necessary to initiate the cycle which synthesizes the C_2 acceptor.

The author wishes to thank Dr. B. M. Tolbert, Radiation Laboratory, University of California, for the chemically synthesized C^{14}-lactic acid and C^{14}-alanine. He also wishes to thank Miss Ruth Gastel and Mrs. Frances A. Bennett for their able assistance. He also expresses appreciation to Dr. Robert Steele of this laboratory for frequent valuable suggestions made during the course of this work.

DEPARTMENT OF BIOLOGY
BROOKHAVEN NATIONAL LABORATORY
UPTON, LONG ISLAND, NEW YORK

LITERATURE CITED

1. ARONOFF, S., BENSON, A., HASSID, W. Z., and CALVIN, M. Distribution of C^{14} in photosynthesizing barley seedlings. Science **105**: 664–665. 1947.
2. BENSON, A. A. and CALVIN, M. Carbon dioxide fixation by green plants. Ann. Rev. Plant Physiol. **1**: 25–42. 1950.

3. GIBBS, M. Distribution of labeled carbon in plant sugars after a short period of photosynthesis in $C^{14}O_2$. Jour. Biol. Chem. **179**: 499–500. 1949.
4. GIBBS, M., DUMROSE, R., BENNETT, F. A., and BUBECK, M. R. On the mechanism of bacterial fermentation of glucose to lactic acid studied with C^{14}-glucose. Jour. Biol. Chem. **184**: 545–549. 1950.
5. STEELE, R. and SFORTUNATO, T. Techniques in the use of C^{14}. Brookhaven National Laboratory Technical Report T–6. 1949.
6. UDENFRIEND, S. and GIBBS, M. Preparation of C^{14} uniformly labeled fructose by means of photosynthesis and paper chromatography. Science **110**: 708–709. 1949.
7. VARNER, J. E. and BURRELL, R. C. Use of C^{14} in the study of the acid metabolism of *Bryophyllum calycinum*. Arch. Biochem. **25**: 280–287. 1950.
8. VITTORIO, P., KROTKOV, G., and REEN, G. B. Labelling in the glucose deposited as starch during photosynthesis. Proc. Soc. Exptl. Med. Biol. **74**: 775–776. 1950.
9. WOOD, H. G. and BURR, G. O. Photosynthesis with $C^{13}O_2$ and the distribution of heavy carbon in the sugars. Fed. Proc. **6**: 303–304. 1947.
10. WOOD, H. G., LIFSON, N., and LORBER, V. The position of fixed carbon in glucose from rat liver glycogen. Jour. Biol. Chem. **159**: 475–489. 1945.
11. WOOD, H. A., WERKMAN, C. H., HEMINGWAY, A., and NIER, O. A. The position of carbon dioxide carbon in succinic acid synthesized by heterotrophic bacteria. Jour. Biol. Chem. **139**: 377–381. 1941.