THE BIOSYNTHESIS OF C\textsuperscript{13} COMPOUNDS

I. THE BIOSYNTHESIS OF C\textsuperscript{13}-LABELED STARCH\textsuperscript{*}

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INTRODUCTION

Although a rather extensive use has been made recently of the isotopes of carbon in metabolic studies of bacteria and fungi, only a few papers have appeared dealing with similar phenomena in higher plants. Ruben, Hassid, and Kamen (1), who were among the first to employ these isotopes in metabolic work, used C\textsuperscript{12} as a tracer in an attempt to determine the first product of photosynthesis in barley. Smith and Cowie (2) attempted to determine by means of C\textsuperscript{14} the relation of the dark uptake of carbon dioxide to the photosynthetic process in sunflower leaves. Belkengren (3) timed the appearance of labeled carbon in fractions of various seed plants after short periods of photosynthesis in an atmosphere containing C\textsuperscript{18}O\textsubscript{2}. Rates of translocation were measured by Rabideau and Burr (4) in the bean plant by use of C\textsuperscript{13}. No chemical fractions were isolated.

None of these investigations has had as its main objective the use of the green seed plant as a means of biosynthesis.

The purpose of this series of studies is to explore the possibilities of biosynthesis of compounds suitable for metabolic studies, using C\textsuperscript{13}O\textsubscript{2} as the starting material and the photosynthetic process in the green plant (or chemosynthesis by autotrophic bacteria) as a means of initial synthesis. While this will result in mass labeling of the compounds so produced, rather than specific radical synthesis which can be accomplished by chemical means, it offers the advantage of the production of a large variety of compounds which cannot be readily produced by the latter process.

Many problems are apparent at the outset. Among the more important of these are the following: (a) devising specific techniques to minimize or obviate dilution of the usable products by C\textsuperscript{12} compounds already present in the cells of the plant; (b) either attaining high yields of these desired compounds or limiting dilution of the non-usable fractions to such an extent that they can be incinerated and their C\textsuperscript{13} recovered for re-use, and (c) isolating the desired compounds in a purified state.

\textsuperscript{*} Reported at the meeting of the American Association for the Advancement of Science, Boston, December, 1946.
Several alternative methods of approach are possible, each possessing specific advantages as well as drawbacks restricting its usefulness. An over-all summary of some potentially promising lines of attack is presented in Fig. 1.
The most direct approach is via short-term experiments as outlined in No. I in Fig. 1. This method aims at the production of some specific compound as a relatively direct result of the photosynthetic reaction. The main body of this paper summarizes the procedure and results of an experiment of this type. An attempt was made to secure C\textsuperscript{13}-labeled starch with the minimum of dilution by preformed C\textsuperscript{12} compounds. The approach used is based on the fact that the excess carbohydrate, formed in a leaf carrying on photosynthesis at a high rate, is temporarily stored as starch in the chloroplast in which it is synthesized.

Other approaches are outlined briefly in this figure which include growing plants throughout their growing cycle (II, III, and IV) using isotopic CO\textsubscript{2} as a sole carbon source (II and III) or products from these as a source of carbon for secondary biosynthesis (IV). Suggestions for recovery and re-use of the carbon contained in non-usable products are included in the figure.

The results presented in this communication represent the initial step in a general project based upon this over-all plan.

**Experimental Procedure**

After a series of exploratory tests, leaves of *Phaseolus vulgaris*, variety Burpee's Stringless Green Pod, were selected as suitable experimental material. While satisfactory results were obtained with them, a study of other leaves is contemplated. Mature leaves were used to reduce complications introduced by growth, the leaves were subjected to a period of darkness to deplete them of the starch already present in the chloroplasts, and they were detached from the plant just before being placed in the photosynthesis chamber to obviate translocation loss after the experiment was begun.

1. **Preliminary Experiments and Their Results.**—Prior to making a final C\textsuperscript{13} run, a series of experiments was conducted with the aim of determining the following variables:

   (a) The time required by bean leaves to become completely depleted of starch. Using intact plants, it was found that it required between 70 and 90 hours in darkness, depending on the initial condition of the leaves, to insure complete starch depletion of the chloroplasts in the mesophyll.

   (b) The optimum time for the duration of the experiment. After being subjected to starch depletion, some of the leaves showed a tendency to become yellow if they were kept in the experimental chamber for a time much in excess of 48 hours. Accordingly, experiments were limited to this period of time. During these 2 days the quantity of leaves which could be readily accommodated in the chamber (about 25 gm.) was capable of utilizing practically all of the CO\textsubscript{2} in an initial gas mixture of 10 to 12 per cent CO\textsubscript{2} in air,—approximately 50 to 60 millimoles of CO\textsubscript{2}.

   (c) The respiratory rates of normal and starch-depleted leaves. The respiratory rates of detached bean leaves in both conditions were determined in a
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separate experiment in which weighed quantities of leaves were placed in closed chambers in ordinary air in the dark and the rate of CO\textsubscript{2} evolution measured by making several analyses of the enclosed gas over a period of 66 hours. The respiratory rates proved to be very constant during this time interval. The rates of CO\textsubscript{2} evolution per hour per gram of fresh weight showed no significant difference in the two lots tested, being 0.0042 mm\textsuperscript{3} for the undepleted leaves, as compared to 0.0040 mm\textsuperscript{3} for the starch-depleted series.

2. Preparation of Plant Materials.—Plants were grown in pots out of doors until 5 weeks old. They were beginning to bloom, and each plant bore several adult trifoliate leaves, which were the type used in the experiment. The potted plants were removed to an indoor darkroom to bring about starch depletion. After 90 hours of darkness, a small piece of each leaf intended for use in the experiment was removed, boiled in 70 per cent alcohol, stained in 1 per cent iodine-potassium iodide solution, and examined microscopically. All leaves were found to be entirely free of visible starch grains, except in the guard cells of the epidermis, where fairly large starch granules were still present. One leaflet from each of six different leaves was removed and stored frozen for a subsequent quantitative determination of starch and soluble sugars initially present. The leaves to be used were severed from the plants at the base of the petiole immediately before setting up the experiment. Fig. 2 is a diagram of the photosynthetic chamber employed.

The C\textsubscript{18}O\textsubscript{2} generator was prepared by placing a weighed quantity (54.01 mm\textsuperscript{3}) of BaC\textsubscript{18}O\textsubscript{3} in a porcelain evaporating dish directly beneath the tube of the dropping funnel, and introducing 100 ml. of 20 per cent lactic acid into the reservoir of the dropping funnel.

In setting up this experiment, the vases were filled with CO\textsubscript{2}-free water, and the leaves were arranged around the sides of the jar, using narrow strips of Scotch tape at the tips of the leaflets to hold them in place for maximum exposure to light. All of this and subsequent operations were carried out as rapidly as possible in subdued light. After all preparations were completed, the cover was clamped in place and the juncture between the jar rim and rubber gasket liberally painted with shellac to insure a perfect seal. CO\textsubscript{2}-free air was then flushed through the chamber for 1 hour, the chamber was evacuated to approximately 50 cm. Hg, and C\textsubscript{18}O\textsubscript{2} generated by allowing the lactic acid to flow slowly into the dish of BaC\textsubscript{18}O\textsubscript{3}. The chamber was immersed in a water bath with glass sides at approximately 24°C, and allowed to stand under reduced pressure for 1 hour to facilitate complete evolution of CO\textsubscript{2}. Finally the internal pressure was equalized to atmospheric by allowing CO\textsubscript{2}-free air to enter through one of the stopcocks.

Before removing the initial gas samples for analysis, a 100 ml. syringe was attached to the Luer stopcock, and the gas in the chamber was thoroughly mixed by 30 cycles of rapid filling and emptying of the syringe. The gas samples for analysis were removed in smaller syringes, each provided with a stopcock. In the removal of all subsequent samples, the additional provision was made before mixing, of first adjusting the chamber to atmospheric pressure by the introduction of CO\textsubscript{2}-free air.
Two banks of lights, each consisting of four 20-W fluorescent bulbs were placed outside the water bath, approximately 4 inches to either side of the chamber. These lights were turned on immediately after the removal of the initial gas samples referred to above.

Total CO₂ and O₂ content of the gas was determined in duplicate with the Scholander gas analyzer (5), using approximately a 1 ml. sample for each determination. The C¹⁴ content of the gas at the beginning and end, as well as all of the products of the experiment was determined by the mass spectrometer. Complete data on the gas analyses throughout the experiment are presented in Table I.

The experiment was terminated at the end of 48 hours. As soon as the lights were turned off, the chamber was flushed for 1 hour with CO₂-free air, and all residual CO₂ was absorbed in a train of NaOH vessels attached to the exit stopcock. The carbonate was converted to BaCO₃, filtered under CO₂-free conditions, dried, weighed, and analyzed.

The chamber was opened, and the leaves were removed and rechecked for fresh weight. The total weight was 27.8 gm. They were divided into two lots, one lot of
### TABLE I

**Gas Analysis**

**Summary Table**

*Phaseolus vulgaris* leaves (27.8 gm. wet weight) were depleted of carbohydrate and illuminated for 48 hours in a closed chamber of 11.58 liter capacity. CO₂ was generated from BaCO₃ with lactic acid in an atmosphere of CO₂-free air. CO₂ and O₂ analyzed with Scho-lander micro gas analyzer.

| Time (hrs.) | CO₂ (per cent) | O₂ (per cent) | C¹³ excess (per cent) |
|------------|----------------|---------------|-----------------------|
| 0          | 11.2           | 16.7          | 7.26                  |
| 24         | 5.45           | 22.25         | —                     |
| 41.5       | 1.5            | 26.1          | —                     |
| 47         | 0.5            | 27.0          | —                     |
| 48         | 0.1            | —             | 2.66*                 |

* This figure is too low since some contamination with atmospheric CO₂ occurred during the collection of the sample.

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* Figure for carbohydrate.
† Analyzed for Cu.
‡ Analyzed for Cu³⁺.

**Fig. 3.** Fraction of *Phaseolus vulgaris* leaves for Cu³⁺ analysis. 10 gm. aliquot of fresh leaves (dry weight, 1.709 gm.).

10 gm. and the second lot of the remaining 17.8 gm. They were placed in large test tubes, and stored in a refrigerator in a frozen state.
The water remaining in the vials was transferred to a bottle of Ba(OH)$_2$ under CO$_2$-free conditions. The precipitate was recovered by filtration, dried, weighed, and analyzed.

3. Preparation of Plant Material for Analysis.—A summary flow sheet of fractionation procedure is presented in Fig. 3. The points at which samples for carbohydrate and for C$^{14}$ analyses were removed are indicated. All of the initial steps in the procedure, including centrifugation, washing of the sediment fraction, and deproteinization of the soluble fraction were carried out in a cold room with reagents maintained at approximately 5°C. to minimize enzyme action.

The 10 gm. sample of frozen leaves was processed in a Waring blender for 5 minutes in 90 ml. of distilled water and then strained through a cloth of fine mesh. The residual tissue fragments were resuspended in 50 ml. of water and reprocessed in the blender for 5 minutes and restrained. This was repeated once more, and the entire liquid suspension combined. Microscopic examination of the coarse tissue fragments remaining indicated that they consisted principally of vascular and epidermal fragments. No intact mesophyll cells were observed, and the only visible starch-containing cells in this fraction were the guard cells in the epidermal fragments. Very few of these appeared to be destroyed by the blender treatment, and it is assumed that the major part of the epidermal tissue and therefore, the guard cell starch, remained in the coarse tissue fragment fraction. Water was used in preference to the 70 per cent alcohol usually employed because it was found that the alcohol interfered with later purification of the starch by sedimentation. The suspension contained practically all of the chloroplast starch present in the mesophyll cells of the leaves, chloroplast fragments, coagulated protoplasm, and other fine particles of insoluble material. It contained also all soluble cell constituents, including sugars, soluble proteins, organic acids, other miscellaneous organic constituents, and inorganic salts. This initial fraction was further separated by centrifugation into a total water-soluble fraction and a water-insoluble fine sediment fraction. The latter was washed and centrifuged twice with 25 ml. of water for each washing. The washings were added to the water-soluble portion.

These procedures had now separated the plant material into three fractions which are designated as the coarse tissue fragment fraction, the water-insoluble fine sediment fraction, and the water-soluble fraction.

The water-insoluble fine sediment fraction, consisting chiefly of starch, coagulated protoplasmic components, and chloroplast fragments, was subjected to repeated differential sedimentations and centrifugations which eventually resulted in the separation of a starch fraction, and fine cell fragments. These fractions were extracted with alcohol and ether, resulting in three fractions, purified starch, fine cell fragments, and the combined ether-alcohol extract. A small quantity of starch was detectable by microscopic examination in the fine cell fragments.

The total water-soluble fraction was first treated with ZnSO$_4$ to precipitate soluble proteins. The zinc protein precipitate was resuspended in water and freed from zinc with H$_2$S. The dissolved protein was purified by repeated (NH$_4$)$_2$SO$_4$ precipitation and dialysis and dried for C$^{14}$ analysis.
The deproteinized supernatant was rendered zinc-free and concentrated in a vacuum desiccator to about 80 ml. and extracted for 50 hours with ether in a reflux condenser to remove organic acids and other ether-soluble constituents for C\textsuperscript{13} analysis. Soluble sugars remained. To determine their C\textsuperscript{13} content, an aliquot portion was hydrolyzed and a phenylosazone prepared. (We are indebted to Dr. Peter Wenck of our staff for this preparation.)

Carbohydrate analyses were made on the various fractions as indicated by asterisks in Fig. 3. The general method of starch analysis consisted of grinding the tissue, washing the insoluble fraction free of soluble sugars, dispersing the starch by boiling the insoluble fraction in water, and then digesting for 1 hour with pancreatic amylase. Soluble protein was precipitated with lead acetate, and the deproteinized solution was subjected to acid hydrolysis to convert all sugars to monosaccharides. Sugar content was determined by the ceric sulfate method of Miller and Van Slyke (6). Soluble sugars in aqueous fractions were determined by the ceric sulfate method after deproteinization and acid hydrolysis.

The various fractions which were analyzed for C\textsuperscript{13} content are indicated by double daggers in Fig. 3.

RESULTS

The C\textsuperscript{13} content of the various isolated fractions is presented in Table II (see also Fig. 3 for isolation procedures). All quantities were computed on the basis of the total quantity of plant material used in the experiment (27.8 gm. fresh weight).

The C\textsuperscript{13} excess in the CO\textsubscript{2} taken up by the plants during the experiment was 3.884 mm, computed by subtracting 0.013 mm C\textsuperscript{13}O\textsubscript{2} recovered from the vase water and 0.012 mm recovered from the residual CO\textsubscript{2} from the 3.910 mm initially supplied as C\textsuperscript{13}O\textsubscript{2}.

The C\textsuperscript{13} content of the ether-soluble fraction of the aqueous extract (0.28 atom per cent C\textsuperscript{13} excess) and the soluble protein fraction (0.31 atom per cent excess) indicate that very little exchange or resynthesis occurred in these fractions during the course of the experiment. The amounts of material in these two fractions were too small to allow total carbon determinations, so no data are available as to the amount of C\textsuperscript{13} contained in them. The remaining soluble constituents had a C\textsuperscript{13} excess of 2.10 atom per cent, most of which was in the soluble sugars present in the mixture (see Table IV).

Approximately one-half of the total starch initially present in the suspension (by starch analysis) was recovered in a purified state by the fractional sedimentation technique used. Microscopic examinations showed no constituents other than starch in this fraction. The alcohol-ether-soluble fraction included the plastid pigments, as well as a mixture of lipids and other water-insoluble organic materials. Since this fraction had only a C\textsuperscript{13} content of 0.50 atom per cent excess, no further fractionations were made. The remaining fine cell fragments included some of the smaller starch grains, which probably account for the major
portion of the C\textsuperscript{13} content of this fraction (2.26 atom per cent C\textsuperscript{13} excess). It would have been of interest to have prepared a carbohydrate-free sample of this fraction in order to determine the C\textsuperscript{13} content of the chloroplast and protoplasmic fragments, but no such preparation was made.

As indicated in the discussion of methods above, the coarse tissue fragments which were retained by the fine mesh cloth used for straining consisted chiefly of vascular debris and fairly large epidermal fragments (including guard cells and their contained starch). The C\textsuperscript{14} excess present in this fraction, 0.66 atom per cent, would, in part, be accounted for by increase in starch and by a possible increase or exchange involving other cell constituents. These fragments were also extracted with alcohol and ether, thereby obtaining a plastid pigment-lipid fraction. The C\textsuperscript{13} content of this extract was 0.91 atom per cent C\textsuperscript{13} excess. It is to be noted that this is higher than the value for the comparable extract from the fine sediment. Since no attempts were made to isolate separate constituents from either of these fractions, no explanation of this difference is proposed.

A quantitative summary of the carbohydrates present in the leaves at the beginning and end of the experiment, as determined by analysis, is presented in Table III. Table IV presents a quantitative summary of the C\textsuperscript{14} excess accounted for in the carbohydrate fractions. It should be noted that the starch

**TABLE II**

\textit{C\textsuperscript{14} Excess in Fractions Isolated from Leaves of Phaseolus vulgaris}

Leaves were previously depleted of starch and reilluminated. C\textsuperscript{13} excess in the CO\textsubscript{2} taken up by the leaves during the experiment was 3.884 m\textsuperscript{M}.

| Fraction                                      | C\textsuperscript{13} excess (\text{atom per cent}) | Excess utilised (\text{per cent}) |
|-----------------------------------------------|----------------------------------------------------|-----------------------------------|
| Total suspension                              |                                                    |                                   |
| Total soluble fraction                        |                                                    |                                   |
| Ether extract                                 | 0.28                                               |                                   |
| Soluble protein                               | 0.31                                               |                                   |
| Soluble protein-free fraction                 | 2.10                                               | 0.643                             | 16.56 |
| Total sediment                                |                                                    |                                   |
| Alcohol-ether-soluble fraction                 | 0.50                                               | 0.018                             | 0.67  |
| Purified starch                               | 7.05                                               | 1.411                             | 36.30 |
| Fine cell fragments                           | 2.26                                               | 0.313                             | 8.06  |
| Coarse tissue fragments                       | 0.66                                               | 0.378                             | 9.73  |

**TABLE III**

Quantitative summary of carbohydrates present in the leaves at the beginning and end of the experiment, as determined by analysis.
TABLE III
Carbohydrate Summary
Carbohydrate gained by leaves of *Phascolus vulgaris* depleted of starch, then illuminated for 48 hours. Carbohydrate is computed as glucose.

|                | Initial | Final | Gain |
|----------------|---------|-------|------|
|                | gm.     | per cent | gm. | per cent | gm. | per cent |
| Soluble sugar  |         |         |      |          |      |          |
| Suspension     | 0.176   | 0.63   | 0.397 | 1.43     | 0.221 | 126.0    |
| Coarse tissue fragments | 0.0   | 0.0   | 0.0 | 0.0     | 0.0 | —        |
| Starch         |         |         |      |          |      |          |
| Suspension     | —*      | —*     | 1.112 | 4.00     | 1.112 | —        |
| Coarse tissue fragments | 0.115† | 0.42†  | 0.129 | 0.46     | 0.014 | 12.2     |

* Assumed to be zero since no starch was detectable in mesophyll upon microscopic examination (iodine test).
† Determined in total sample, but assumed to be in the coarse tissue fragments, since starch was visible in chloroplasts of guard cells only.

TABLE IV
*C*<sup>18</sup> Excess Summary
The amounts of starch and sugar were determined quantitatively (see Table III). All values were computed as glucose. *C*<sup>18</sup> atom per cent excess was determined on an aliquot of the starch isolated by fractional sedimentation and on a phenyllosazone prepared from the soluble sugar fraction.

*C*<sup>18</sup> excess in CO<sub>2</sub> taken up by the leaves during the experiment = 3.884 mm.

|                | Gm. | *C*<sup>18</sup> excess |
|----------------|-----|------------------------|
|                |     | mm         | Atom     | *C*<sup>18</sup> excess utilised |
|                |     | per cent   | per cent |
| Suspension     |     |            |          |                                |
| Soluble sugar  | 0.397 | 0.889     | 6.72     | 22.90                           |
| Starch         | 1.112 | 2.613     | 7.05     | 67.28                           |
| Total carbohydrate | 1.509 | 3.502 | —       | 90.18                           |
| Residue (coarse tissue fragments) | 1.50 | 0.378* | 0.66 | 9.73 |
| Total          | 1.50 | 99.91     |          |                                |

* Determined on total tissue residue fraction.

synthesized during the experiment accounts for 67.28 per cent of the total *C*<sup>18</sup> excess taken up by the leaves, while the soluble sugar accounts for 22.90 per cent making a total of 90.18 per cent of the total accounted for in these fractions. These figures were computed by combining the data obtained from
quantitative carbohydrate determinations with the C\textsuperscript{13} assay of purified aliquots of these fractions. The coarse tissue fragments removed by straining contained an additional 9.23 per cent of the total, thus accounting for 99.91 per cent of the C\textsuperscript{13} absorbed.

DISCUSSION

In this experiment, a compound which cannot be synthesized chemically has been produced with an efficiency comparable with that of a relatively simple chemical synthetic procedure. With an initial C\textsuperscript{13} content of 7.26 atom per cent excess in the CO\textsubscript{2} supplied, the starch with a C\textsuperscript{13} atom per cent excess of 7.05 represents a dilution of only 2.9 per cent; while the soluble sugar fraction, with a C\textsuperscript{13} atom per cent excess of 6.72 shows a reduction in isotope concentration of 7.4 per cent. The starch which can be isolated with little difficulty, accounts for approximately 67 per cent of the C\textsuperscript{13} absorbed by the plants. The soluble sugar fraction accounts for approximately 23 per cent. The latter fraction, however, has not yet been isolated.

A summary flow sheet tracing the probable fate of the carbon in the experimental system is proposed in Fig. 4. Assuming a respiratory rate of 0.004 mM of CO\textsubscript{2} per hour per gram of fresh weight of the leaves, as determined in a separate experiment, the 27.8 gm. used would evolve 5.66 mM of CO\textsubscript{2} during the 48 hour run. Adding this to the initial CO\textsubscript{2} supplied (53.36 mM)\textsuperscript{1} would give a total amount of 59.52 mM of CO\textsubscript{2} of which the respiratory CO\textsubscript{2} constitutes 9.5 per cent.

It is of interest to note that the net respiratory turnover during the experiment (5.66 mM of CO\textsubscript{2}) would require 0.94 mM (169 mg.) of hexose if this were the sole substrate. This quantity constitutes almost as much soluble sugar as was present in the leaves initially, and 42.6 per cent of the amount of soluble sugar present in them at the end. If this quantity is added to the amount present at the end of the experiment, the total soluble sugar involved would be 566 mg., and the quantity synthesized during the experiment would be 390 mg. On this basis, respiration would utilize 30 per cent of the gross amount. The fate of the pool of soluble carbohydrate initially present in the leaves bears an important relation to the amount of dilution which occurred in the final carbohydrate fractions. If a major portion of the soluble carbohydrate initially present remained in the cells unchanged, most of it would be accounted for as a dilution factor in the soluble carbohydrate fraction at the end of the experiment. Conversely, if this constituted the chief respiratory substrate during the course of the experiment, we should expect a considerable dilution of the

\textsuperscript{1}This figure was based on gas analysis. The slight discrepancy between it and that derived from the weight of the BaC\textsuperscript{13}O\textsubscript{3} employed is probably due to the impossibility of measuring accurately the volume of gas in the experimental chambers.
CO₂ initially provided and consequently of all products formed from it. The following computations may throw some light on these questions.

**53.86 mM CO₂**

**Photosynthesis**

**CO₂ recovered from vase-water**
- **0.309 mM CO₂**
- 4.19 atom per cent C¹³ excess
- 0.013 mM excess C¹³

**Respiratory CO₂†**
- **5.66 mM**

**Starch**
- **37.07 mM C**
- 7.05 atom per cent C¹³ excess
- 2.613 mM excess C¹³

**Pool of soluble sugar**

| Pool of soluble sugar | Initial | Final |
|-----------------------|---------|-------|
| total C               | 5.62 mM | 13.23 mM |
| C¹³ excess            | 0.0 atom per cent | 6.72 atom per cent |
| C¹³ excess            | 0.0 mM excess C¹³ | 0.889 mM excess C¹³ |

**Residual CO₂**
- **0.466 mM C**
- 2.66 atom per cent C¹³ excess
- **0.012 mM excess C¹³**

* See text for explanation.
† Computed from control sample.

**Fig. 4. CO₂ flow diagram.** Volume of chamber = 11.58 liters. CO₂ = 11.3 per cent of gas mixture (53.86 mM)*. C¹³ excess in CO₂ = 7.26 atom per cent (3.910 mM).

If the soluble carbohydrates initially present (0.977 mM as glucose, containing 5.87 mM of carbon) had been unused, and if then the soluble carbohydrate content had been built up to 2.205 mM by the addition of 1.227 mM of glucose containing 7.36 mm of carbon synthesized entirely from the CO₂ supplied (7.26 atom per cent C¹³ excess), the final concentration of C¹³ in this fraction at the end of the experiment would have been...
which may be seen to be much lower than that obtained (6.72 atom per cent C\textsuperscript{13} excess). Therefore we know that a considerable amount of the original pool of soluble carbohydrate must have disappeared from the fraction.

If, on the other hand, all of the carbon in the soluble carbohydrate initially present (5.87 mm of carbon) had remained in the carbohydrate-CO\textsubscript{2} system and become redistributed by contributing materially to the respiratory CO\textsubscript{2}, or had remained as carbohydrate per se, then the C\textsuperscript{13} concentration of the entire system should have been

\[
\frac{(5.87 \times 0 \text{ per cent}) + (53.86 \times 7.26 \text{ per cent})}{53.86 + 5.87} = 6.64 \text{ atom per cent}
\]

average C\textsuperscript{13} excess. This represents the maximum average C\textsuperscript{13} excess which would have been possible in the combined carbohydrate fractions under the conditions specified above. The figure obtained was 6.97 atom per cent excess. From these considerations we may conclude that the carbon in the initial soluble carbohydrate fraction which disappeared was not totally converted to CO\textsubscript{2} by respiration, nor did it all remain in the carbohydrate fraction.

The only apparent alternative appears to be that a portion of the carbohydrate initially present in the leaves took part during the experiment in some unknown synthetic process yielding non-carbohydrate products, and that the respiration of the leaves during the course of the experiment was supported principally by the primary product of photosynthesis. The small amount of dilution which did occur in this experiment was probably derived principally from the initial soluble carbohydrate fraction, both by respiration and retention as carbohydrate, but the importance of this dilution factor was materially less than was anticipated.

There was only a small amount of C\textsuperscript{13} present in the non-carbohydrate fractions isolated. Apparently very little resynthesis or exchange in these fractions occurred in the mature leaves used, and in the short time involved in the experiment.

**SUMMARY**

1. Starch, containing 7.05 atom per cent C\textsuperscript{13} excess has been produced in the mesophyll cells of bean leaves, starting with C\textsuperscript{13}O\textsubscript{2} containing 7.26 atom per cent C\textsuperscript{13} excess. Approximately 67 per cent of the C\textsuperscript{13} taken up by the leaves was determined in the starch fraction.

2. The soluble carbohydrate, containing 6.72 atom per cent C\textsuperscript{13} excess, accounts for approximately 23 per cent of the C\textsuperscript{13} taken up by the leaves. The remainder was principally in the coarse tissue fragments fraction (9.73 per cent of the C\textsuperscript{13} utilized).
3. The apparatus and procedures used in this experiment are described. We are indebted to the Houdry Process Corporation of Marcus Hook, Pennsylvania, and particularly to Dr. Sidney Weinhouse for the C\textsuperscript{18} analyses; also to Swarthmore College for the loan of their Scholander analyzer.

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