Photosynthesis by Sugar-cane Leaves

A NEW CARBOXYLATION REACTION AND THE PATHWAY OF SUGAR FORMATION

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1. Radioactive products in detached leaf segments were examined after periods of steady-state photosynthesis in $^{14}CO_2$. 2. After exposure to $^{14}CO_2$ for approx. 1 sec. more than 93% of the fixed radioactivity was located in malate, aspartate and oxaloacetate. After longer periods large proportions of the radioactivity appeared in 3-phosphoglycerate, hexose monophosphates and sucrose. Similar results were obtained with leaves still attached to the plant. 3. Radioactivity appeared first in C-4 of the dicarboxylic acids and C-1 of 3-phosphoglycerate. The labelling pattern in hexoses was consistent with their formation from 3-phosphoglycerate. 4. The reaction giving rise to C_4 dicarboxylic acid appears to be the only quantitatively significant carboxylation reaction. 5. Evidence is provided that the radioactivity incorporated into the C_4 dicarboxylic acid pool is transferred to sugars via 3-phosphoglycerate. A scheme is proposed to account for these observations.

Several observations which appear to be inconsistent with the scheme for photosynthesis proposed by Calvin and co-workers (Calvin & Bassham, 1962) have been considered in recent reviews by Stiller (1962) and Bassham (1964). The low activity of ribulose 1,5-diphosphate carboxylase in some tissues relative to the rate of photosynthesis, and the high concentrations of bicarbonate required by the enzyme for maximum activity, raise doubts about its quantitative importance in photosynthetic fixation of carbon dioxide (Racker, 1957; Peterkofsky & Racker, 1961; Stiller, 1962). Recently Kortschak, Hartt, & Burr (1965) reported that malate and aspartate were the major labelled products formed in sugar-cane leaves during short periods of photosynthesis in $^{14}CO_2$. The present studies confirm this observation with another sugar-cane variety and provide information about the nature of the primary carboxylation reaction and the pathway of sugar formation.

MATERIALS

3-Phosphoglyceric acid (tricyclohexylammonium salt) and wheat-germ acid phosphatase were purchased from Sigma Chemical Co. (St Louis, Mo., U.S.A.). Yeast invertase was obtained from Difco Laboratories (Detroit, Mich., U.S.A.). $Ba^{14}CO_3$ (26·2mc/m-mole), L-[U-^{14}C]malic acid (15·1mc/m-mole) and L-[U-^{14}C]aspartic acid (6·1mc/m-mole) were obtained from The Radiochemical Centre (Amersham, Bucks.).

Leaf tissue. Field-grown sugar cane (hybrid variety, Pindar) was used. Sections (approx. 18cm. long, 1·5cm. wide) containing no midrib tissue were cut from the youngest fully expanded leaf. All cuts were made under water to prevent the entry of air into the vascular tissue.

METHODS

Photosynthesis in $^{14}CO_2$. A rectangular Perspex box (19cm. × 16cm. × 9cm.), sufficiently large to accommodate six leaf segments, served as a photosynthesis chamber. The top of the chamber was detachable and split in half lengthwise. Leaf segments were clamped vertically between the halves with about 1cm. protruding from the chamber and the bases of the segments dipping into water. Soft rubber on the adjoining edges of the top provided a seal around the leaf segments but allowed them to be withdrawn from the chamber. Air, saturated with water vapour, was pumped through the chamber and the air in the chamber was circulated by a high-speed fan. Before the addition of $^{14}CO_2$ the segments were illuminated for at least 45min. by a 400w Phillips HPL lamp. The light-intensity at the leaf surface, measured with a selenium cell, was 8200ft.-candles. From a calibration curve of the selenium cell against a Kipp thermopile this intensity was equivalent to 0·39cal./cm.^{2}/min.

Immediately before the introduction of $^{14}CO_2$ the air supply to the chamber was removed and exit holes were sealed with tape; $^{14}CO_2$ (0·86mc) was injected from a syringe bringing the CO_2 concentration to 0·055%. At intervals individual leaf segments were transferred to 50ml. of boiling 80% (v/v) ethanol and kept at this temperature for 3min. In certain experiments the segments were transferred to methanol–chloroform–2·0M-formic acid (12:5:3, by vol.) at −80°, then held at −15° for 24hr. (Bieleski & Young, 1963).

A second procedure allowed both shorter treatments with

$^{14}CO_2$ and the killing of leaves while still exposed to $^{14}CO_2$ and light. After pre-illumination in the chamber leaf segments were placed in a large test tube (vol. 60ml.). $^{14}CO_2$ (45μc) was injected and then boiling 80% (v/v) ethanol was poured into the tube either simultaneously or after 1–2sec.

Extraction of tissue. Segments killed in the methanol–chloroform–formic acid mixture were extracted as described by Bieleski & Young (1963). Segments killed in boiling ethanol were homogenized and the insoluble material was re-extracted in sequence with 20ml. of boiling 80% (v/v) ethanol, and twice with 15ml. of boiling water. Water and ethanol extracts were combined. The remaining insoluble material was washed with a large volume of water followed by ethanol and then dried at 70° to a constant weight. Hereafter this material will be referred to as 'residue'. Radioactivity incorporated into segments is expressed as counts/min./100mg. of the residue.

Counting procedures. Samples of 0·1ml. or less of the combined extracts containing at least 1000 counts/min. were counted in a liquid-scintillating counter (model N664A; Ekco Electronics). A toluene–phosphor–ethanol solution (Ziegler, Chleck & Brinkerhoff, 1957) was used for these aqueous samples. The counting efficiency was 55% and quenching was not significant.

Weighed samples (approx. 10mg.) of the dried residue were counted in steel planchets with a Geiger-Müller tube. In the range used counts were proportional to the weight of the sample. The counting efficiency of this method was determined by liquid-scintillation counting of similar residue samples suspended in toluene containing phosphor and 2% (w/v) thixin.

Identification and estimation of radioactivity in individual compounds

Chromatography solvents and counting procedure. For identifying individual components and determining the amount of the radioactivity in each compound samples of extracts were chromatographed on paper with one or more of the following solvent systems: A, butan-1-ol–propionic acid–water (10:5:7, by vol.) (Benson *et al.* 1950); B, pentan-1-ol saturated with 5M-formic acid (Aronoff, 1956, p. 122); C, propan-1-ol–aq. NH_3 (sp.gr. 0·90)–water (6:3:1, by vol.) (Bieleski & Young, 1963); D, ethyl acetate–pyridine–water (8:2:1, by vol.) (White & Secor, 1953); E, phenol saturated with water (Benson *et al.* 1950). For subsequent references to these solvents the letter prefixing each will be used. Whatman no. 1 paper was used and for solvents A, C and E the paper was previously washed with oxalic acid.

The location and the amount of radioactivity in individual compounds or groups of compounds was determined with a chromatogram strip-counter and the area of individual peaks measured with a planimeter. When quantities of individual compounds were required samples of extracts were chromatographed as a band and eluted from developed chromatograms.

Marker compounds on chromatograms were detected as follows: organic acids and amino acids with ninhydrin (Aronoff, 1956, p. 120), phosphorylated compounds with ammonium molybdate (Bandurski & Axelrod, 1951) and sugars with *p*-anisidine.

Malic acid. When extracts were chromatographed in solvents A and B a peak was obtained which co-chromatographed with L-malate. With solvent B this was the only radioactive compound which moved a significant distance from the origin. The radioactivity eluted from chromatograms developed in solvent B co-chromatographed with L-malate in solvents A, C and E. When samples of this compound were treated with a fumarate hydratase preparation from wheat-germ 18–20% of the radioactivity was located in fumaric acid at equilibrium. Similar values were obtained with L-[U-^{14}C]malate.

Aspartic acid. A compound which ran as a separate peak corresponding to L-aspartate was detected with solvent A. The eluted radioactivity co-chromatographed with L-aspartate in solvents B and E, and the compound was completely degraded by treatment with ninhydrin (Greenberg & Rothstein, 1957).

Phosphorylated compounds. With solvent A a separate area of radioactivity was obtained which corresponded in mobility to 3-phosphoglycerate and hexose monophosphates. Occasionally a very small separate peak was observed with a mobility corresponding to fructose 1,6-diphosphate and ribulose 1,5-diphosphate.

When the major peak was eluted and co-chromatographed with marker compounds in solvent C the radioactivity corresponded with 3-phosphoglycerate, glucose 6-phosphate and fructose 6-phosphate. After treatment with acid phosphatase all the radioactivity was recovered in compounds which co-chromatographed with glyceric acid, glucose and fructose in solvents B, C and D. In some experiments traces of glyceric acid were detected when the original extracts were chromatographed in solvents A and B.

Sucrose. After longer periods of exposure to $^{14}CO_2$ a labelled compound appeared which chromatographed with sucrose in solvent A. This compound co-chromatographed with sucrose in solvent D and after treatment with invertase the radioactivity was located equally between glucose and fructose.

Proportions of radioactivity in individual compounds. The proportion of the total radioactivity in malate was calculated from chromatograms developed in solvent B and checked with solvent A and the proportions of aspartate and sucrose were obtained with solvent A. After determining the proportion for the total phosphorylated compounds with solvent A the amount of radioactivity in 3-phosphoglycerate and glucose 6-phosphate plus fructose 6-phosphate was calculated from chromatograms run in solvents B or D after treatment of this fraction with phosphatase.

Oxaloacetic acid. With the standard procedure employed radioactive oxaloacetate was not detected. Hence the following procedure was used. Leaves were exposed to $^{14}CO_2$ in a test tube as previously described and after approx. 1sec. photosynthesis was stopped by pouring one of the following solutions into the tube: (1) 80% (v/v) ethanol containing 10mg. of 2,4-dinitrophenylhydrazine and 0·2N-HCl at −80°, (2) the same solution at 82°, (3) the same solution at 82° but without 2,4-dinitrophenylhydrazine. The subsequent procedure was the same as already described except that the concentrated extracts were extracted with chloroform to obtain phenylhydrazones (Aronoff, 1956, p. 133). The radioactivity in the chloroform extract was determined and samples were chromatographed with authentic oxaloacetate 2,4-dinitrophenylhydrazone in the following solvents: butan-1-ol–water–ethanol (5:4:1, by vol.), butan-1-ol saturated with M-$NaHCO_3$, 0·1M-potassium

glycinate, pH 8·4, and butan-1-ol–ethanol–0·5N-NH_3 (7:1:2, by vol.) described by Block, Durram & Zweig (1958). With the chloroform extract from the extract of leaf killed at −80° at least 90% of the radioactivity co-chromatographed with the marker in all solvents. When a portion of the chloroform extract was mixed with authentic 2,4-dinitrophenylhydrazone of oxaloacetate and recrystallized twice the specific activity of the derivative remained constant.

Insoluble compounds. The insoluble residue was treated with 72% (v/v) H_2SO_4 to hydrolyse cellulose (Jermyn, 1955). Essentially all the radioactivity was recovered in a compound which co-chromatographed with glucose in solvents C and D. When the material was hydrolysed with 6N-HCl (Pirie, 1955) no radioactivity was detected in amino acids.

Degradation of labelled products

Malic acid. Radioactivity in the C-1 plus C-4 and the C-2 plus C-3 of malate was determined by MnO_2 oxidation (Friedemann & Kendall, 1928). Samples of the CO_2 and acetaldehyde, trapped in sodium hydroxide and sodium bisulphite respectively, were counted in the liquid-scintillation counter by using the procedure described for aqueous samples. The C-1 of malate was determined by a modification of the Von Peckmann reaction described by Racusen & Aronoff (1953). Trials with L-[U-^{14}C]malate established the exact conditions for the complete release of the C-1 as CO. Samples of malate containing at least 35,000 disintegrations/min. were dried in a combustion flask with L-malate carrier and heated at 80° for 45min. with 5ml. of 100% H_2SO_4 in the combustion apparatus for a Nuclear–Chicago Dynacon electrometer. The CO released was then flushed into a 250ml. ion chamber and counted. Similar samples were subjected to combustion with Van Slyke reagent to determine the total radioactivity.

Aspartic acid. Radioactivity in the C-1 plus C-4 and the C-2 plus C-3 of aspartate was determined by treatment with ninhydrin (Greenberg & Rothstein, 1957).

Glyceric acid. Glyceric acid was isolated from 3-phosphoglycerate by chromatography in solvent B after treatment with acid phosphatase. The glycerate was decarboxylated by oxidation with ceric sulphate (Zelitch, 1965). Radioactivity in the CO_2, trapped in N-NaOH, and in the residual acetic acid was determined with a liquid-scintillation counter.

Hexoses. After treatment of sucrose with invertase and hexose monophosphates with acid phosphatase the resulting glucose and fructose were isolated from chromatograms developed in solvent D and converted into their common osazone. This derivative was degraded to give the C-1, C-2 and C-3 as the mesoxaldehyde osazone, the C-4 and C-5 as formic acid and the C-6 as formaldehyde (Aronoff, 1956, p. 110).

RESULTS

Incorporation of radioactivity from $^{14}CO_2$ as a function of time. Experimental conditions were chosen to produce as nearly as possible a steady state of photosynthesis under physiological conditions of concentration of carbon dioxide and light-intensity. Studies by K. T. Glasziou & J. C. Waldron in our Laboratory have shown that the rate of photosynthesis of leaf segments, prepared as described in the Methods section, is low when first exposed to light. The rate increases to a steady value after about 30min. and is maintained for several hours. In our experiments leaves were exposed to light in a humidified stream of air for at least 45min. before the addition of $^{14}CO_2$. The intensity of visible light at the leaf surface was approx. 40% of that in direct sunlight.

The total fixation of $^{14}CO_2$ (Fig. 1) and the radioactivity in individual compounds (Figs. 2 and 3) were determined for leaves exposed to $^{14}CO_2$ for periods of up to 150sec. Over this period the rate of $^{14}CO_2$ fixation remained linear. The changing proportions of radioactivity in individual compounds is shown in Fig. 2. Three trends are apparent, the continued fall of the first-labelled products, malate and aspartate, the rise then fall of 3-phosphoglycerate and hexose monophosphates, and the steady increase of the percentage of the total radioactivity in the end products sucrose and a glucan. The plot of total radioactivity in individual compounds (Fig. 3) shows the asymptotic trend of the early products and intermediates and the exponential rise in the radioactivity associated with end products. When a similar experiment was carried out in the dark the rate of $^{14}CO_2$ fixation was only 0·4% of that in the light. Even after 5min. in $^{14}CO_2$ radioactivity was located almost entirely in aspartate and malate whereas no radioactivity was detected in 3-phosphoglycerate or hexose monophosphates. Our conditions of leaf pretreatment and light-intensity differed from those used by Kortschak *et al.* (1965). However, our

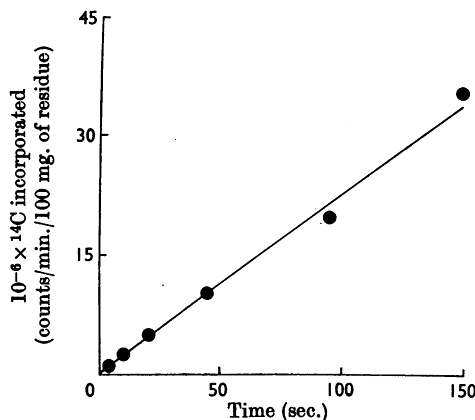


Fig. 1. Total ^{14}C incorporated into leaf segments as a function of time. The radioactivity in the residue is included and the counts are for a 55% counting efficiency. Other details are described in the Methods section.

results with the variety Pindar were qualitatively similar to those obtained by these workers with the variety H37–1933.

The effect of different killing procedures on the distribution of radioactivity in individual compounds was examined (Table 1). Results were very similar when leaves were killed either in boiling 80% (v/v) ethanol or in the methanol–chloroform–formic acid mixture at −80°. In these experiments leaf segments were transferred from the chamber to the killing mixture. To determine if any modification of the labelling pattern occurred during transfer, leaf segments were exposed to $^{14}CO_2$ in test tubes and

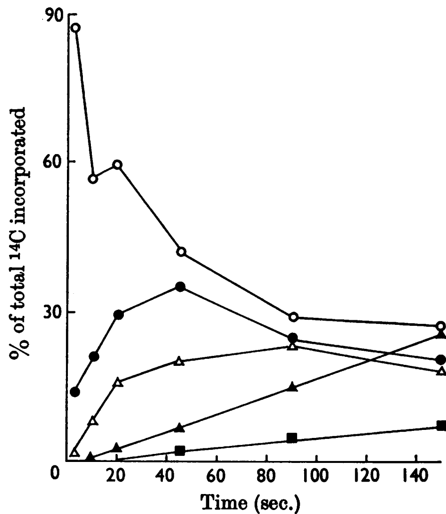


Fig. 2. Proportion of the total radioactivity in individual compounds for periods in $^{14}CO_2$ up to 150sec. Data are from the same experiment as described in Fig. 1. Details of procedures for indentifying and counting individual compounds are described in the Methods section. ○, Malate+aspartate; ●, 3-phosphoglycerate; △, hexose monophosphates; ▲, sucrose; ■, glucan.

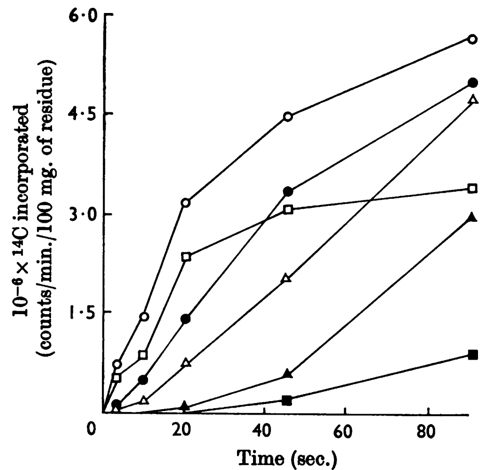


Fig. 3. Time-course for the total radioactivity incorporated into individual compounds. Data are from the same experiment as described in Figs. 1 and 2. ○, Malate+aspartate; □, malate; ●, 3-phosphoglycerate; △, hexose monophosphates; ▲, sucrose; ■, glucan.

Table 1. *Effect of different killing procedures on the distribution of radioactivity in individual compounds*

In Expt. 1 leaf segments were supplied with $^{14}CO_2$ in the chamber, and at the times shown segments were removed and placed in boiling 80% (v/v) ethanol or methanol–chloroform–formic acid (MCF) at −80°. In Expt. 2 leaves were supplied with $^{14}CO_2$ in large test-tubes and after 4 sec. one was killed by adding boiling 80 % (v/v) ethanol and the other was transferred to another tube containing the same mixture. Other details are described in the Methods section.

Expt. no.	Time in $^{14}CO_2$ (sec.)	10^{-6} × Total ^{14}C incorporated (counts/min./100mg. of residue)	Killing method	Percentage of total ^{14}C in individual compounds					
				Malate	Aspartate	3-Phosphoglycerate	Hexose monophosphates	Sucrose	Glucan
1	30	8·9	Ethanol	30	15	36	14·5	2·5	1·0
	30	10·1	MCF	32	16	34	15	2·0	1·0
	60	19·9	Ethanol	26	8·5	27	25	8·5	4·0
	60	19·3	MCF	27	10	30	23	6·0	4·5
2	4	2·6	Ethanol added to tube	53	25	15	7	0	0
	4	2·3	Leaf removed t ethanool	56	25	14	5	0	0

then killed either by the direct addition of boiling 80% (v/v) ethanol or by transferring them to the same mixture. The two procedures gave almost identical results (Table 1).

To confirm that the results obtained were not due to an alteration of the normal photosynthetic process caused by detaching and cutting the leaves experiments were conducted with leaves on the plant. The apical 25cm. of the youngest fully-expanded leaf of plants was exposed to $^{14}CO_2$ in a sealed chamber or, for short-term experiments, an open test tube (see the Methods section). A time-course study over a period from 4sec. to 90sec. in $^{14}CO_2$ gave results which were almost identical with those obtained with leaf sections. When an attached leaf was exposed to $^{14}CO_2$ for 2sec. and killed by the direct addition of boiling 80% (v/v) ethanol the proportions of the incorporated radioactivity in malate, aspartate, 3-phosphoglycerate and hexose monophosphates were 54%, 37%, 7% and 2% respectively.

We established that oxaloacetate would be completely destroyed during our isolation and chromatography procedures. Hence attempts were made to identify radioactive oxaloacetate in leaf extracts as its 2,4-dinitrophenylhydrazone. Only killing at −80° in the presence of the reagent gave reproducible results (Table 2). A much smaller proportion of the total radioactivity was recovered in the derivative when leaves were killed in boiling 80% (v/v) ethanol containing 2,4-dinitrophenylhydrazine.

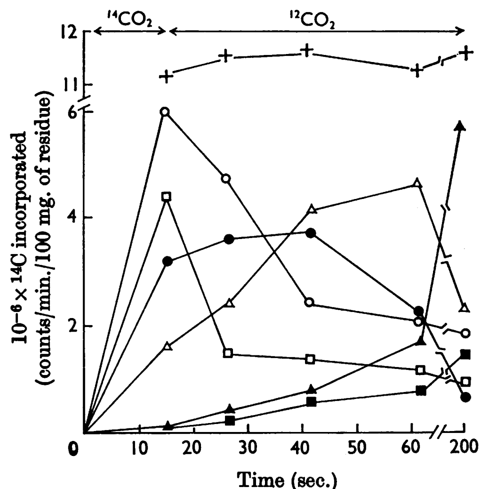


Fig. 4. Changes in the distribution of radioactivity after transfer of leaf segments from $^{14}CO_2$ to carbon dioxide. After 15sec. in $^{14}CO_2$ leaf segments were removed simultaneously from the chamber to a stream of air without changing the distance from the light source. At intervals individual segments were killed and analysed as described in the Methods section. ○, Malate+aspartate; □, malate; ●, 3-phosphoglycerate; △, hexose monophosphates; ▲, sucrose; ■, glucan; +, total radioactivity.

Table 2. *Radioactivity in oxaloacetate after different killing procedures*

Leaves were exposed to $^{14}CO_2$ for approx. 1sec. and then killed either at 82° or −80° in the presence of 2,4-dinitrophenylhydrazine or without the reagent (see the Methods section). Oxaloacetate was isolated and counted as its 2,4-dinitrophenylhydrazone.

	Percentage of total ^{14}C		
Compound	With reagent at −80°	With reagent at 82°	Without reagent at 82°
Malate	54	57	47
Aspartate	36	32	42
Oxaloacetate	3·4	0·6	0
3-Phosphoglycerate	5·2	8·0	6·0
Other compounds	3·0	2·4	5·0

Table 3. *Changes in the distribution of radioactivity in leaf segments after transfer from $^{14}CO_2$ in the light to carbon dioxide in the dark*

Illuminated segments were exposed to $^{14}CO_2$ for 9sec. At 9sec. the chamber was darkened with a shutter and humidified air was flushed through at a rate of 1l./sec. After 3sec. the first leaf segment was killed. Radioactive compounds were extracted and analysed as described in the Methods section.

	Percentage of total ^{14}C in individual compounds						
Time in the dark and carbon dioxide (sec.)	Malate	Aspartate	3-Phosphoglycerate	Hexose monophosphates	Sucrose	Alanine	Glucan
3	35	26	29	9	0	0	0·6
20	37	24	24	12	1	2	0·7
37	35	38	16	8	1	2·5	0·5
83	34	42	10	8	0	6	0·6

Changes in the distribution of radioactivity after transfer from $^{14}CO_2$ to air. When leaf segments were transferred from $^{14}CO_2$ to air but maintained at the same light-intensity there was no loss of radioactivity during the following 200sec. (Fig. 4). During the first 40sec. most of the radioactivity in malate and aspartate was transferred to the hexose monophosphate pool whereas the label in 3-phosphoglycerate increased only slightly. Subsequently radioactivity appeared in sucrose and a glucan at the expense of hexose monophosphates and 3-phosphoglycerate. The relatively constant amount of radioactivity associated with malate and aspartate during the period from 40sec. to 200sec. was probably due to label in the C-1, C-2 and C-3 (see Table 4). To further test the possibility that $^{14}CO_2$ may be released and then re-fixed during these transformations a period in $^{14}CO_2$ was followed by a period in 5% (v/v) unlabelled carbon dioxide in air. Transformations similar to those shown in Fig. 4 were observed and there was no significant loss of radioactivity.

When leaves were transferred from $^{14}CO_2$ in the light to unlabelled carbon dioxide in the dark there was little change in distribution of radioactivity in individual compounds during the first 20sec. (Table 3). Between 20sec. and 83sec. the proportion of radioactivity in 3-phosphoglycerate declined from 24% to 10%. Accompanying this fall was an increase in the radioactivity in aspartate and alanine. The total radioactivity in the leaf segments remained constant during the period in unlabelled carbon dioxide.

Radioactivity in individual carbon atoms of photosynthetic products. The primary labelling of malic acid occurred in the C-4 position (Table 4). After 20sec. the total radioactivity in this carbon became relatively constant. At this time C-4 still contained 80% of the total radioactivity, but label was not detected in the C-2 and C-3 positions until 45sec. The distribution of radioactivity in the C-1 plus C-4 and the C-2 plus C-3 of aspartate was almost identical with that of malate. At 3sec. nearly all the radioactivity in 3-phosphoglycerate was located in the carboxyl carbon atom whereas the compound was almost uniformly labelled at 150sec. It is noteworthy that, with respect to the C-1, the C-2 plus C-3 of 3-phosphoglycerate became labelled much more rapidly than the C-2 plus C-3 of malate.

Glucose and fructose from hexose monophosphates and sucrose were converted into their common osazone and then partially degraded (Table 5). Almost half of the total radioactivity was located in the C-1, C-2 plus C-3 positions after 20sec. and 45sec. The C-6 contained only a small part of the radioactivity in C-4, C-5 plus C-6 at 20sec. but this proportion doubled by 45sec.

Table 4. *Radioactivity in individual carbon atoms of aspartate, malate and 3-phosphoglycerate*

Compounds used for these analyses were from the same experiment as described in Figs. 1, 2 and 3. Procedures for isolating and degrading the compounds are described in the Methods section. Counts/min. are expressed per 100mg. of residue.

Period in $^{14}CO_2$ (sec.)	Aspartic acid: C-1 plus C-4, % of total ^{14}C	Aspartic acid: C-1 plus C-4, $10^{-4}\times$ counts/min.	Malic acid: C-1, % of total ^{14}C	Malic acid: C-1, $10^{-4}\times$ counts/min.	Malic acid: C-2 plus C-3, % of total ^{14}C	Malic acid: C-2 plus C-3, % of C-1, C-2 plus C-3	Malic acid: C-2 plus C-3, $10^{-4}\times$ counts/min.	Malic acid: C-4, % of total ^{14}C	Malic acid: C-4, $10^{-4}\times$ counts/min.	Glyceric acid: C-1, % of total ^{14}C	Glyceric acid: C-1, $10^{-4}\times$ counts/min.	Glyceric acid: C-2 plus C-3, % of total ^{14}C	Glyceric acid: C-2 plus C-3, $10^{-4}\times$ counts/min.
3	98	21·8	10	4·7	0	0	0	90	42·5	98	11·5	2	0·24
10	98	49·5	16	15·1	0	0	0	84	76·0	96	49·4	4	2·0
20	97	83·0	20	46·0	0	0	0	80	185	83	120	17	24·6
45	96	132	29	91·0	4	14	14·1	67	208	61	212	39	135
90	89	198	27	97·0	15	35	51·0	58	200	52	258	48	240
150	77	276	33	200	25	43	152	42	253	45	333	55	415

Table 5. *Distribution of radioactivity in the carbon atoms of hexoses derived from sucrose and hexose monophosphates*

Compounds were obtained from the same extracts used for the studies described in Figs. 1, 2 and 3 and Table 4. Procedures used to isolate and degrade the hexoses are described in the Methods section.

Period in $^{14}CO_2$ (sec.)	Origin of hexose	C-1, C-2, C-3 (as % of total ^{14}C)	C-6 (as % of ^{14}C in C-4, C-5, C-6)	C-4 plus C-5 (as % of ^{14}C in C-4, C-5, C-6)
20	Sucrose	52	11	89
20	Hexose monophosphate	46	10	90
45	Sucrose	45	18	82
45	Hexose monophosphate	45	20	80

DISCUSSION

Our aim was to examine the radioactive products formed from $^{14}CO_2$ under steady-state conditions for photosynthesis and at a light-intensity and concentration of carbon dioxide as close to physiological conditions as possible. Under these conditions we assumed that only physiologically significant carboxylation reactions were operative. For determining the sequence of chemical events a number of other assumptions are necessary. The major assumptions are that pool sizes remain constant and that the absolute pool size of intermediates is sufficiently large to allow the radioactivity to be detected. Other factors which must be considered are labelling of compounds by side reactions from the main path, secondary labelling of intermediates by cycling, and labelling of independent second pools.

With these points in mind we interpret the time-studies to indicate that the first product of the major carboxylation reaction is either malate or oxaloacetate and that aspartate is probably formed by a side reaction from these products. The ^{14}C then moves from the C_4 dicarboxylic acid pool to 3-phosphoglycerate and subsequently to sucrose and a glucan via the hexose phosphates. Since the major products of $^{14}CO_2$ fixation in the dark are malate and aspartate the same carboxylation reaction may be operating for both processes. In the dark the rate of fixation of carbon dioxide is only 0·4% the rate in light and no radioactive phosphorylated compounds were detected. Hence light not only produces a 250-fold increase in the rate of fixation of carbon dioxide but is also necessary for the transfer of radioactivity from the C_4 dicarboxylic acid pool into 3-phosphoglycerate.

Support for this interpretation was obtained from experiments in which a period in $^{14}CO_2$ in the light was followed by periods in unlabelled carbon dioxide in either light or dark. In both cases the total radioactivity in leaf segments remained constant during the period in unlabelled carbon dioxide. In the light the radioactivity in malate and aspartate moved into other pools in the sequence already suggested. In contrast radioactivity in the C_4 dicarboxylic acid pool increased significantly in the dark and there was no movement of ^{14}C into hexose monophosphates or sucrose. The increase of radioactivity in the dicarboxylic acid pool and the appearance of label in alanine occurred at the expense of 3-phosphoglycerate. It appears unlikely that the transfer of carbon from the dicarboxylic acids to 3-phosphoglycerate in the light proceeds by a process involving decarboxylation and re-fixation of carbon dioxide.

The feature of photosynthesis is a net gain of carbon. This would be achieved only if the transfer of carbon from the C_4 dicarboxylic acids to 3-phosphoglycerate proceeded without the release and loss of carbon dioxide. Known pathways for this transformation involve the decarboxylation of oxaloacetate or malate with the appearance of their C-1, C-2 and C-3, as the C-1, C-2 and C-3 respectively, of 3-phosphoglycerate. Besides the problem of loss of carbon dioxide, the operation of this pathway is inconsistent with the fact that the labelling of the C-2 and C-3, relative to the C-1, is much more rapid for 3-phosphoglycerate than for malate. Rather, our results indicate that the C-4 of the C_4 dicarboxylic acids is the source of the C-1 of 3-phosphoglycerate. The earliest labelling of malate occurs in the C-4 and the total radioactivity in this carbon atom approaches an optimum value more rapidly than the radioactivity in the C-1 of 3-phosphoglycerate. The simplest interpretation is that the C-4 carboxyl group of a C_4 dicarboxylic acid is transferred by a transcarboxylation reaction to an acceptor substance, the latter providing the C-2 and C-3 of 3-phosphoglycerate. The carbon dioxide-acceptor for the initial carboxylation reaction could be regenerated from the remaining C_3 compound.

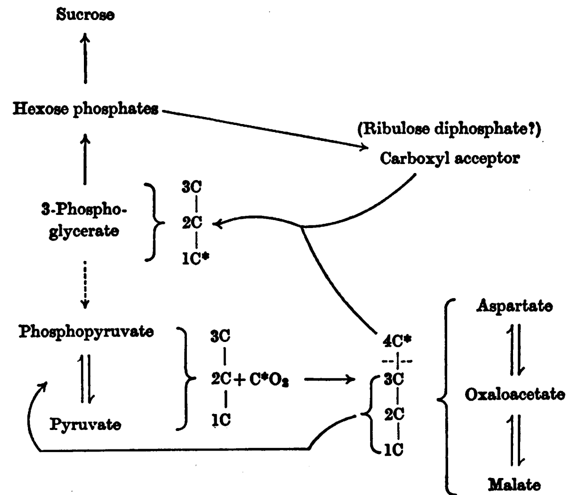
A definitive conclusion about the nature of the primary carboxylation reaction cannot be derived from the available data. Enzymes which carboxylate

pyruvate to give malate, and phosphopyruvate to give oxaloacetate, are known to occur in leaves. They are malate dehydrogenase (decarboxylating) (EC 1.1.1.40) ('malic enzyme') and phosphopyruvate carboxylase (EC 4.1.1.31). The operation of another enzyme, ATP-oxaloacetate carboxy-lyase (Mazelis & Vennesland, 1957) would not be favoured by the high ATP/ADP ratio which should prevail in the light. However, our data indicate that the C_4 dicarboxylation acids are in rapid equilibrium so that the primary carbon dioxide-fixation product need not be the acid involved in the subsequent transcarboxylation reaction.

Oxaloacetate is known to participate in several trans-carboxylation reactions (Wood & Stjernholm, 1961; Hülsmann, 1962) and therefore appears the more likely carboxyl donor. The carboxyl acceptor may be either a C_2 compound such as glycolaldehyde phosphate or the C_5 compound of the Calvin cycle, ribulose 1,5-diphosphate. The latter possibility is appealing since the subsequent steps may then be identical with those of the Calvin cycle (Calvin & Bassham, 1962). The rates of labelling of 3-phosphoglycerate and hexose monophosphates, and the distribution of radioactivity in their individual carbon atoms, are consistent with this possibility.

The above interpretations are summarized in Scheme 1, which shows two cyclic processes linked by a transcarboxylation reaction. The role of one cycle is to fix carbon dioxide and to provide carboxyl groups to react with a carboxyl acceptor in the other cycle. The latter cycle operates to regenerate the carboxyl acceptor for the transcarboxylation reaction. The relative rates of labelling of the C-2 and C-3, compared with the C-1, for malate and 3-phosphoglycerate is consistent with some of the latter compound being converted into the C_3 carbon dioxide-acceptor. While this movement of carbon is not a basically essential part of the scheme it may serve to replace carbon lost from the first cycle by side-reactions.

The possibility that the pathway outlined in Scheme 1 is peculiar to sugar cane appears remote. Hence the question why similar labelling patterns have not been observed during studies on other species arises. If malate and aspartate are not essential intermediates they may be labelled only if sufficient malate dehydrogenase and transaminase are present in the chloroplasts. Detection of radioactive oxaloacetate would depend upon an adequate pool size and preventing its breakdown during extraction. The only procedure we found satisfactory for measuring the radioactivity in oxaloacetate involved killing off leaves at $-80°$ in the presence of 2,4-dinitrophenylhydrazine. Hence, if standard killing procedures are used, the operation of the pathway described in Scheme 1 could give 3-phosphoglycerate as the first detectable radioactive product.



Scheme 1. Proposed pathway for photosynthetic fixation of carbon dioxide in sugar-cane leaves. The broken arrow indicates a minor pathway.

REFERENCES

Aronoff, S. (1956). *Techniques of Radiochemistry*. Ames: Iowa State College Press.

Bandurski, R. S. & Axelrod, B. (1951). *J. biol. Chem.* **193**, 405.

Bassham, J. A. (1964). *Annu. Rev. Plant Physiol.* **15**, 101.

Benson, A. A., Bassham, J. A., Calvin, M., Goodale, T. C., Haas, V. A. & Stepka, W. (1950). *J. Amer. chem. Soc.* **72**, 1710.

Bieleski, R. L. & Young, R. E. (1963). *Analyt. Biochem.* **6**, 54.

Block, R. J., Durram, E. L. & Zweig, G. (1958). *Paper Chromatography and Paper Electrophoresis*, p. 239. New York: Academic Press Inc.

Calvin, M. & Bassham, J. A. (1962). *The Photosynthesis of Carbon Compounds*, p. 10. New York: W. A. Benjamin Inc.

Friedemann, T. E. & Kendall, A. I. (1928). *J. biol. Chem.* **82**, 23.

Greenberg, D. M. & Rothstein, M. (1957). In *Methods in Enzymology*, vol. 4, p. 713. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Hülsmann, W. C. (1962). *Biochim. biophys. Acta*, **62**, 620.

Jermyn, M. A. (1955). In *Modern Methods of Plant Analysis*, vol. 2, p. 205. Ed. by Peach, K. & Tracey, M. V. Berlin: Springer-Verlag.

Kortschak, H. P., Hartt, C. E. & Burr, G. O. (1965). *Plant Physiol.* **40**, 209.

Mazelis, M. & Vennesland, B. (1957). *Plant Physiol.* **32**, 591.

Peterkofsky, A. & Racker, E. (1961). *Plant Physiol.* **36**, 409.
Pirie, N. W. (1955). In *Modern Methods of Plant Analysis*, vol. 4, p. 30. Ed. by Peach, K. & Tracey, M. V. Berlin: Springer-Verlag.
Racker, E. (1957). *Arch. Biochem. Biophys.* **69**, 300.
Racusen, D. W. & Aronoff, S. (1953). *Arch. Biochem. Biophys.* **42**, 25.
Stiller, M. (1962). *Annu. Rev. Plant Physiol.* **13**, 151.
White, L. M. & Secor, G. E. (1953). *Arch. Biochem. Biophys.* **43**, 60.
Wood, H. G. & Stjernholm, R. (1961). *Proc. nat. Acad. Sci., Wash.*, **47**, 289.
Zelitch, I. (1965). *J. biol. Chem.* **240**, 1869.
Ziegler, C. A., Chleck, D. J. & Brinkerhoff, J. (1957). In *Liquid Scintillation Counting*, p. 158. Ed. by Bell, G. C. & Hayes, F. N. Oxford: Pergamon Press Ltd.