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Carbon Dioxide Fixation in Sugarcane Leaves [1, 2]

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Sugarcane is one of the most efficient plants for producing carbohydrates by photosynthesis (12); it is therefore of great interest to compare its photosynthetic cycle with that of other plants. Our preliminary experiments (not reported here), in which sections of sugarcane blades were exposed to $C^{14}O_2$ for periods up to 15 seconds showed that the radioactivity in 3-phosphoglyceric acid (PGA) was never over 34 % of the total activity incorporated. In no case was this as much as the sum of the activities in malic plus aspartic acids. As a check on our methods of analysis, we exposed a soybean leaf to $C^{14}O_2$ for 15 seconds; PGA accounted for 80 % of the total radioactivity.

The experiments reported here are part of a continuing study of sucrose synthesis in the sugarcane plant.

Materials and Methods

Leaves were selected from 1-year old, field-grown plants of variety H 37-1933. This is a complex interspecific hybrid involving *Saccharum officinarum* L., *S. spontaneum* L., and *S. robustum* Brandes and Jeswiet ex Grassl (13). Leaf No. 3 or No. 4 (the youngest fully expanded leaves) was cut off, and sections were cut under water from the middle part of the leaf.

Tungsten light, filtered through water to reduce heat, gave an illumination of 2000 ft-c at the surface of the leaf. This is well below light saturation for sugarcane (10) and about the same intensity used by many other investigators (1, 2, 3, 4).

Room temperature, 27 to 30°, was maintained by air conditioning. This is a normal range of outdoor temperatures in Hawaii, though well below those obtaining in many other sugarcane-growing areas. Photosynthesis in sugarcane is essentially constant from 30 to 40° (15).

Leaf sections were preilluminated for at least 10 minutes to eliminate induction effects.

Three methods of exposing leaf sections to $C^{14}O_2$ were used.

1. The porous filter method. For short exposures requiring accurate timing leaf sections 2 inches long were placed on a porous metal filter on the bottom of a beaker and illuminated from above. $C^{14}O_2$ was injected through the stem of the filter, and photosynthesis was terminated by pouring in boiling 95 % ethanol. Duration of photosynthesis was taken to be from the moment of $C^{14}O_2$ injection until the alcohol hit the leaf, as timed with a stopwatch. Errors in timing are undoubtedly negligible compared to errors in estimating the time required to "kill" the leaf.

2. The steady-state method. For longer exposures, leaf sections 3 inches long were placed on a rack in an 18-liter aquarium, the lower ends of the leaves being supported by a trough filled with water. (We have established that there is no transfer of radioactive material from the leaf to the water.) A slit in the cover was closed with cellophane tape. A small electric fan maintained air circulation.

CO_2 was injected to raise the concentration to 0.10 %. Ten minutes later, 500 μc of $C^{14}O_2$ were injected. At each scheduled time 1 leaf section was withdrawn and dropped into boiling alcohol; this operation required 1 to 2 seconds.

3. The dip method. The procedure was to inject $C^{14}O_2$ into a closed glass chamber with a narrow, cellophane-tape-covered slit in the top. The tape was removed, a leaf quickly lowered through the slit, and withdrawn after the selected time.

Extractions. Leaf sections were boiled in 95 % ethanol for 5 minutes. Whereas a leaf such as soybean is completely extracted and colorless after this treatment, the sturdier sugarcane leaf appears unchanged although some green color can be seen in the alcohol. Blending with the further addition of alcohol to 50 ml in a Waring blendor resulted in the extraction of most of the sugars and chlorophyll.

After filtration, the alcohol-insoluble material was twice boiled for 3 minutes with 20 ml of water and filtered. The residue was again washed with alcohol to facilitate drying. Aliquots of each solution were counted on planchets at infinite thinness. The resi-

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dues were counted at infinite thickness; an experimentally determined factor was used to convert these to infinite thinness.

To test the effects, if any, of high temperature of killing, a leaf exposed to $C^{14}O_2$ was dropped into a mixture of dry ice and acetone and blended in this mixture at a temperature below −10°. The acetone was evaporated in vacuo, the temperature remaining below 0°. The resulting fine powder was poured into boiling 95 % alcohol and boiling continued for 5 minutes.

Chromatography. The following solvents were used, all on Whatman No. 41 H paper. Composition is given by volume, except where noted. 1) Phenol-water (80:20 by wt); 2) Isoamyl alcohol-isopropanol-formic acid-water (5:7:2:3); 3) Isoamyl alcohol-isopropanol-water (4:4:1); 4) *n*-butanol-acetic acid-water (10:2:5); 5) *n*-butanol-benzyl alcohol-formic acid-water (5:5:0.1:1); 6) *n*-butanol-benzyl alcohol-diethylamine-water (5:5:0.1:1); 7) Di-isopropyl ether-formic acid (2:1). Aliquots of each extract were spotted on strips of Whatman No. 41 H paper, and run with 80 % phenol, descending, until the front had moved 14 inches. The strips were dried in air at room temperature, and then were scanned at 2.5-mm intervals with a Geiger counter using a 5-mm slit. Active areas were eluted and rechromatographed with solvent 2 for further separation and identification. After the second separation, areas showing activity which could be due to phosphorylated compounds were again eluted. These eluates were treated with alkaline phosphatase and the chromatography repeated.

Completeness of elution was checked by counting the eluted sections. In order to elute phosphates quantitatively 0.001 N HCl was used instead of water.

All identifications were checked by cochromatography in each of the 7 solvents used, except that solvents 3, 5, and 6, in which most phosphorylated compounds remain near the origin, were omitted for these compounds.

Chemical and Physical Identification. The identity of sucrose was confirmed by invertase hydrolysis and cochromatography of the hydrolysate with known glucose and fructose.

Malic acid isolated from several samples was heated to 100° for 1 hour with concentrated H_2SO_4; the resulting maleic acid was cochromatographed with authentic maleic acid. Other samples were mixed with known malic acid and gave ether distribution coefficients of 61 to 62 both by weight and by radioactivity, in agreement with the published value (14).

The ether extract was evaporated to dryness, and the recovered acid had a neutralization equivalent of 68 (malic acid, 67). The water phase was precipitated with barium acetate and alcohol: found, Ba 47 %; Ba $C_4H_2O_5 \cdot 2H_2O$, 45 %.

The identity of sugars was checked by spraying chromatograms with silver nitrate, *P*-anisidine, or aniline phthalate.

Aspartic acid eluted from the chromatograms was mixed with authentic l-aspartic acid. The distribution coefficient between water and *n*-butanol was 59 to 63 both by weight and by radioactivity. The ninhydrin reaction was positive.

The identification of phosphomalic acid as an early product of photosynthesis in sugarcane, reported in preliminary tests (11), is now corrected. Our original reference glyceric acid, obtained by purchase and used in our preliminary tests, proved to be not glyceric acid but mainly glycerol; authentic glyceric acid was later obtained from A. A. Benson, and was also synthesized by deamination of serine. The glycerol is widely separated from malic acid on chromatography with 80 % phenol. Glyceric acid has an R_F value close to that of malic, and its presence after phosphatase treatment of the whole extract was overlooked, being considered as "trailing" of the much larger amount of malic acid. The phosphorylated compound called phosphomalic has been correctly identified as 3-phosphoglyceric acid both by cochromatography and by distribution coefficient after phosphatase treatment.

This subject is explained in detail because of the recent paper of Bonnet (7).

Results and Discussion

Figure 1 shows the averaged results of 16 experiments run over a period of many years for widely varying primary purposes, and under widely varying conditions. CO_2 concentration ranged from 0.3 to 2 %. Each point represents 1 to 6 determinations. The predominance of the 4-carbon acids at short photosynthesis time is striking.

Methods of Killing. To eliminate the possibility that this predominance could be an artifact due to the killing in boiling alcohol, duplicate leaf sections were exposed to $C^{14}O_2$ for 10 seconds by the dip method. One was killed by boiling alcohol, the other frozen in dry ice acetone. Analysis showed no qualitative difference between the two. Percentage composition also agrees closely with the 10-second results in table IV.

Specific Activity. In order to recover amounts sufficient for purification, 10 leaf sections about 12 inches in length were exposed to 0.3 % $C^{14}O_2$, 20 μc/ml, for 3 seconds by the dip method. The water extracts were chromatographed on Whatman No. 3 MM paper with solvent 2. The areas containing PGA (R_F·18) and malic acid (R_F·61) were eluted. PGA was purified by the method of Bassham et al (3).

The eluate containing malic acid was evaporated to 1 ml, an excess of calcium acetate added, and calcium malate precipitated by addition of alcohol to 80 %, and this procedure repeated twice.

Recovered were 0.33 mg Ba PGA, cpm 223, and 0.24 mg Ca malate, cpm 500. This is equivalent to a specific activity of 2300 cpm/mg for glyceric acid, 3200 for malic acid. On the assumption that all activity was in C-1 of glyceric and C-4 of malic acid,

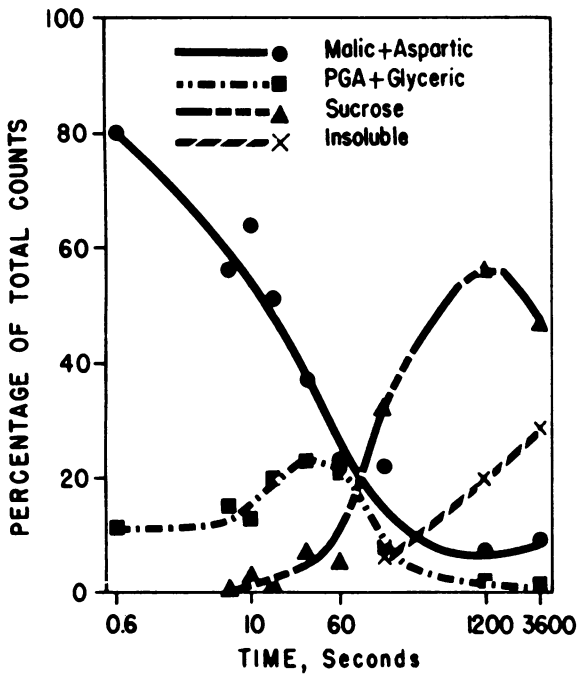

FIG. 1. Distribution of photosynthetic products in sugarcane leaves emphasizing predominance of 4-carbon acids at short time intervals. Results averaged from 16 separate experiments.

these carbon atoms had activities of 20,000 and 36,000 cpm/mg respectively.

Dark Metabolism. Because it is well known that malic acid is a major product of CO_2 fixation in the dark, the rates of synthesis of malate in dark and light (2000 ft-c) were compared by exposing matched leaf sections to the same $C^{14}O_2$ atmosphere by the dip method.

One leaf was kept in total darkness overnight, inserted in the chamber for 10 minutes, and killed in boiling alcohol; the entire operation was carried out in a darkroom. The other leaf was preilluminated for 10 minutes, and exposed to the $C^{14}O_2$ for 10 seconds in the light to obtain fixation of the same order of magnitude.

The rate of fixation of C^{14} in the dark was 0.5 % of that in the light. The rate of fixation into malate was 1 % of that in the light (table I).

Table I. *Carbon Dioxide Fixation in Sugarcane Leaves*

Leaf sections of equal area exposed to the same $C^{14}O_2$ in light (2000 ft-c) for 10 seconds, or complete darkness for 600 seconds.

	Total counts × 10^{-3}	
	Light	Dark
Malic	167	98
Aspartic	112	12
PGA	55	0.1
All other	162	36
Total	496	146

When dark fixation is followed by exposure to light, results are complicated because an induction period intervenes before normal photosynthesis can proceed. A striking feature of this is a temporary accumulation of glucose-1-P (table II). It is apparent that the radioactivity found in sucrose has come mainly from the malic acid synthesized in the dark.

Table II. *Ten Minutes of Dark Fixation Followed by Photosynthesis*

Leaf sections of equal area exposed to $C^{14}O_2$ in the dark for 10 minutes, then placed in air in light (2000 ft-c) for the indicated time.

	Total counts × 10^{-3} Minutes light			
	0	1	3	5
Malic	21	15	8	8
Glucose-1-P	0	4	9	1
Sucrose	0	6	15	16
All others	8	9	4	8
Total	29	34	36	32

Photosynthesis, 0.6 to 810 Seconds[3]. The porous filter method was used to allow leaf sections to photosynthesize for 0.6, 3.5, 6.3, and 9.8 seconds. In order to obtain sufficient activity for analysis, relatively high concentrations of radioactive CO_2 were used. To give comparable numerical results, the absolute values of radioactivity found must be corrected for size of leaf, specific activity, and concentration of CO_2. This last is based on the assumption that uptake is proportional to the logarithm of the CO_2 concentration (6). Corrections were made to correspond to the conditions of the 10-second exposure of the series described below.

These experiments are steady-state with respect to light but not with respect to CO_2 concentration, and in this resemble the experiments of Benson et al. (5) in that a high concentration of $C^{14}O_2$ follows preillumination in air.

The steady-state method was used for photosynthesis times of 10 to 810 seconds. The only correction factor applied here was for size of leaf, based on the weight of the extracted residue. These longer exposures are essentially steady-state, as the addition of $C^{14}O_2$ raised total CO_2 concentration only from 0.10 to 0.13 %.

The overall photosynthesis rate (table III) decreases at the longest times, perhaps due to decrease in CO_2 concentration. However, except for the value at 0.6 second where timing uncertainty and correction factors are maximal, all values are within 15 % of the mean, a variation less than might be expected from biological variation in samples of this kind.

[3] These data are briefly mentioned by Burr (8).

Table III. *Calculation of Photosynthesis Rates in Sugarcane Leaves*

Time, sec	CO_2 μc/cc	CO_2 %	Residue mg	Total counts × 10^{-3}	Total counts corr. × 10^{-3}	Corr. total counts/sec/ mg residue
0.6	702	8.9	24.7	91	2.0	101
3.5	463	5.4	23.0	192	7.8	67
6.8	391	4.8	26.9	357	15.1	67
9.3	391	4.8	19.8	400	22.9	76
10	21.3	0.13	32.7	25	25	76
30	21.3	0.13	34.4	82	78	80
90	21.3	0.13	35.2	222	203	70
270	21.3	0.13	27.6	511	601	67
810	21.3	0.13	31.7	1662	1696	64

In table IV the results for these 2 series are presented together. The best justification for this is the good agreement between results at 9.3 and at 10 seconds.

Experiments of this kind can be interpreted most easily on the assumption that the introduction of $C^{14}O_2$ causes no change in the reactions of photosynthesis already proceeding. This assumption may well be taken as essentially correct for the steady-state series (photosynthesis of 10 to 810 seconds); in the porous filter method, however, the leaf samples were exposed to a sudden drastic increase in CO_2 concentration. We have 3 reasons for believing that the assumption is nevertheless justified.

1. The experiments of Wilson (reported in reference 3) show that changes in reservoir size of PGA and ribulose 1.5-diP on changing CO_2 concentration from 1 to 0.003 %, are of the order of 100 %, and require 1 to 2 minutes for completion. Changes in 10 seconds would thus be relatively small.

2. The same experiments show that 1 effect of increased CO_2 concentration should be an increased concentration of PGA at the expense of ribulose 1,5-diP. This would imply a large temporary increase in the rate of entry of CO_2 into PGA, which we do not find.

3. Our results at 9.3 seconds using 4.8 % CO_2 agree closely with those at 10 seconds using 0.13 % CO_2.

It is evident that for the first 10 seconds most of the activity is in malic and aspartic acids. Even at 30 seconds these account for 40 % of the total activity. Only these 2 acids show a finite slope at zero time (fig 2), a criterion for primary products of carboxylation (2). The percentage of radioactivity in PGA is at first low, increasing with time to a maximum but never equalling the amount in the 4-carbon acids. This cannot be due to phosphatase activity (7), for activity would then appear in free glyceric acid; actually, radioactivity found in glyceric acid was in no case greater than 15 % of that in PGA.

Malonate Inhibition. In view of the importance attached to the results of malonate inhibition of photosynthesis (4, 9), a leaf section was allowed to stand in 10^{-2} M malonic acid for 1 hour in the light in air, then exposed to 0.1 % $C^{14}O_2$ by the dip method. In contrast to the results of Bassham et al. (4), the inhibitions of total photosynthesis, of malic acid, and

Table IV. *Distribution of Photosynthetic Products in Sugarcane Leaves*

(Total counts per minute × 10^{-3} in identified compounds and other fractions).

	Time (sec)								
	0.6	3.5	6.8	9.3	10	30	90	270	810
PGA	0.11	0.65	3.11	3.11	4.32	16.61	23.3	32.5	27.1
Glyceric	0	0.02	0.37	0.41	0.54	0.54	1.0	3.0	0.5
Aspartic	0.43	1.26	2.25	3.43	4.47	9.55	15.2	45.1	37.3
Malic	1.25	4.91	7.57	12.34	10.55	21.50	37.8	61.3	120.4
Sucrose	0	0.06	0.15	0.24	0.20	3.88	50.6	292	1089.0
Glucose	0	0	0	0	0	0.39	1.8	5.4	30.5
Fructose	0	0	0	0	0	0	2.8	8.4	17.0
Glucose-1-P	0	0.01	0.08	0.11	0.54	4.19	12.2	24.0	30.5
Fructose-di-P	0	0.06	0.20	0.30	0.22	2.48	4.3	8.4	11.9
Sedoheptulose-P	0	0	0	0	0	0.23	0.2	0.7	0.7
ATP	0	0	0	0	0.05	0.23	0.7	0.4	0.5
Insoluble	0.05	0.39	0.68	1.35	1.04	5.28	11.0	32.5	113.6
Lost in wash	0.09	0.32	0.74	0.99	0.96	4.50	8.9	16.2	67.8
Undetermined	0.07	0.18	0.0	0.6	1.8	8.2	33	70	149
Total	2.0	7.85	15.1	22.9	24.7	77.6	203	601	1696

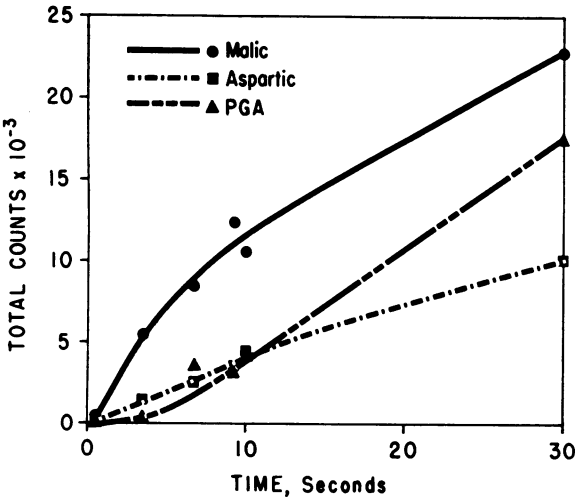

FIG. 2. Early products of photosynthesis in sugarcane leaves pointing out the difference in slope at zero time of malic, aspartic, and phosphoglyceric acids. Leaf sections were exposed to $C^{14}O_2$ for the indicated time, then killed in boiling alcohol.

of PGA are identical (table V). The effect of malonate inhibition of photosynthesis in sugarcane is thus qualitatively different from the effect in *Scenedesmus,* where malic acid was strongly inhibited with only a minor effect on PGA and the overall photosynthesis rate.

Table V. *Malonate Inhibition of Photosynthesis in Sugarcane Leaves*

Leaf sections of equal area stood in water or 10^{-2} M malonic acid for 1 hour, then placed in 0.1 % $C^{14}O_2$ for 1 minute in light, 2000 ft-c.

	Total counts × 10^{-3}		
	Control	Malonate	% Inhibition
Malic	195	23	88
Aspartic	40	8	80
PGA	235	25	89
Glucose-1-P	334	16	95
Fructose-1,6-diP	53	6	89
Sugars	95	21	78
All others	660	85	87
Total	1612	184	89

Summary

In sugarcane, carbon assimilation proceeds by a path qualitatively different from many other plants in that the first stable compounds formed in photosynthesis are malic and aspartic acids.

These acids are converted to sucrose via 3-phosphoglyceric acid and hexose phosphates.

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