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Further Studies on a New Pathway of Photosynthetic Carbon Dioxide Fixation in Sugar-Cane and its Occurrence in other Plant Species

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1. The pathway of photosynthesis in sugar-cane, which gives most of the radioactivity fixed during short periods in $^{14}CO_2$ in C-4 of oxaloacetate, malate and aspartate, was examined under varied conditions. 2. The pattern of labelling was essentially the same with leaves of different ages and with leaves equilibrated at carbon dioxide concentrations in the range 0–3·8% (v/v) and light-intensities in the range 1400–9000ft.-candles before adding $^{14}CO_2$. 3. Radioactive products were examined after exposing leaves of 33 different plant species to $^{14}CO_2$ for 4sec. under standard conditions. 4. A labelling pattern typical of sugar-cane was found in several species of Gramineae but not in others. Of 16 species from other Families only a species of Cyperaceae contained a large proportion of the fixed radioactivity in oxaloacetate, malate and aspartate.

Enzymes other than ribulose diphosphate carboxylase may be operative for photosynthetic carbon dioxide fixation by some bacteria (Losada, Trebst, Ogata & Arnon, 1960; Fuller, Smillie, Sisler & Kornberg, 1961; Buchanan, Bachofen & Arnon, 1964; Buchanan & Evans, 1965) and recent evidence indicates that photosynthesis in sugar-cane proceeds by an alternative process (Kortschak, Hartt & Burr, 1965; Hatch & Slack, 1966). Our studies with sugar-cane leaves, conducted under steady-state conditions, showed that after approx. 1sec. in $^{14}CO_2$ as much as 93% of the fixed radioactivity was located in oxaloacetate, malate and aspartate. Radioactivity appeared first in C-4 of the C_4 dicarboxylic acids and then in C-1 of 3-phosphoglycerate. We concluded that C-1 of 3-phosphoglycerate is derived from C-4 of either oxaloacetate or possibly malate by a transcarboxylation reaction. These studies were conducted with leaves of the same age, at the concentration of carbon dioxide in air, and with a relatively high artificial light-intensity of 8200 ft.-candles. Two important questions arise from this work: (*a*) are the early products of photosynthesis in sugar-cane the same with leaves of different age and under varied conditions of carbon dioxide concentration and light-intensity?; (*b*) what other plant species have a pathway of photosynthesis similar to that in sugar-cane? The present paper provides information relating to these questions.

MATERIALS

Except where otherwise indicated leaves of sugar-cane (hybrid variety, Pindar) were used. The commercial sources of enzymes, reagents and radioactive compounds were as described by Hatch & Slack (1966).

METHODS

Photosynthesis in $^{14}CO_2$

Segments of leaf-blades were cut, equilibrated and exposed to $^{14}CO_2$ in either a large tube or the Perspex chamber as described by Hatch & Slack (1966). Subsequent treatments including the killing procedure are described in the legends of individual Tables. The light-source and the method for measuring light-intensity were as described by Hatch & Slack (1966). Light-intensities are expressed as ft.-candles but the equivalent value in terms of cal./cm.^{2}/min. may be obtained by multiplying ft.-candles by $4{\cdot}4 \times 10^{-5}$.

Extraction of tissue

Leaves were killed by adding either boiling 80% (v/v) ethanol or 85% (v/v) ethanol containing HCl (0·2N) and

2,4-dinitrophenylhydrazine (0·04%, w/v) at −80° when oxaloacetate was to be determined. Extraction and counting procedures were as described by Hatch & Slack (1966) except for the following modification when the latter killing method was used. Approx. 25 ml. of the mixture was added to stop photosynthesis and the pooled tissue extracts, including extractions with 50% (v/v) ethanol and water, were evaporated under reduced pressure at 30° to about 20 ml. After the extraction of 2,4-dinitrophenylhydrazones from this solution with chloroform (Aronoff, 1956) the aqueous layer was further concentrated to about 5 ml. This extract was used to determine the radioactive water-soluble compounds.

Identification and estimation of radioactive products

Compounds labelled during photosynthesis by sugar-cane leaves were identified and estimated as described by Hatch & Slack (1966).

For the studies on photosynthetic products of leaves of different species the following standard procedure was used. Paper-chromatography solvents employed were: *A*, butan-1-ol–propionic acid–water (10:5:7, by vol.); *B*, pentan-1-ol saturated with 5N-formic acid; *C*, ethyl acetate–pyridine–water (8:2:1, by vol.); *D*, butan-1-ol–ethanol–0·5N-NH_3 (7:1:2, by vol.). The sources of the solvents and the methods for estimating radioactivity on developed chromatograms were described by Hatch & Slack (1966).

Malate. Malate was identified by its mobility on chromatograms developed with solvents *A* and *B* and estimated from the latter chromatograms. Its identity was confirmed by enzymic conversion into fumarate (Hatch & Slack, 1966).

Aspartate. Aspartate was identified and estimated from chromatograms developed in solvent *A*. To confirm its identity the area of the chromatograms corresponding to aspartic acid was cut out and its radioactivity determined with a Geiger–Müller tube. The paper was then sprayed with 2·5% (w/v) ninhydrin in 0·25M-sodium citrate buffer, pH 2·6, and heated in a humid atmosphere at 70° for 30 min. Trials with [1,4-^{14}C]aspartate established that the radioactivity located in the carboxyl groups of aspartic acid is lost as CO_2 during this treatment. For the different leaves examined the treatment released between 89 and 99% of the radioactivity from the areas of the chromatograms corresponding to the aspartic acid.

Oxaloacetate. The chloroform extract was concentrated to approx. 5 ml. and the radioactivity in a 0·05 ml. sample was determined by liquid-scintillation counting in the toluene–ethanol–phosphor mixture used to count aqueous extracts (Hatch & Slack, 1966). Other samples were co-chromatographed with authentic 2,4-dinitrophenylhydrazone markers in solvent *D* and the proportion of the total radioactivity in the oxaloacetate derivative was determined. The 2,4-dinitrophenylhydrazone of pyruvate was the only other labelled compound detected in the chloroform extracts.

The radioactivity in C-4 of oxaloacetate was determined by heating the 2,4-dinitrophenylhydrazone derivative in 0·5N-HCl. This treatment releases C-4 as CO_2, leaving the pyruvate derivative as the other product (Block, Durram & Zweig, 1958). Trials with the authentic derivative established that the reaction proceeded quantitatively in 25 min. at 85°. Samples of the oxaloacetate derivative were prepared by elution with 0·1N-NH_3 from chromatograms developed in solvent *D*. Loss of radioactivity as CO_2 was determined by counting samples before and after treatment.

3-Phosphoglycerate and phosphorylated hexoses. Samples of aqueous extracts were treated with *Escherichia coli* alkaline phosphatase and then chromatographed in solvents *B* and *C*. The proportions of the total radioactivity located in glyceric acid (solvent *B*) and glucose plus fructose (solvent *C*) provided a measure of the radioactivity originally located in 3-phosphoglycerate and hexose phosphates.

RESULTS

Effect of various conditions on the early products of photosynthesis in sugar-cane leaves

Effect of leaf age. The amount of $^{14}CO_2$ fixed by leaf segments increased with increasing leaf maturity, but there was little difference in the distribution of the incorporated radioactivity (Table 1). In the different leaves malate and aspartate contained between 63 and 70% of the total radioactivity after 5 sec. in $^{14}CO_2$. In the same experiment segments of leaves were also exposed to $^{14}CO_2$ for 70 sec. As with the shorter treatment, the distribution of radioactivity in individual compounds was very similar for the different leaves.

Effect of carbon dioxide concentration. Leaves were equilibrated with different concentrations of

Table 1. *Early products of photosynthesis in sugar-cane leaves of different age*

Leaves are numbered from the youngest fully-expanded leaf as zero, the younger leaf above this leaf being −1. Leaf segments were exposed to $^{14}CO_2$ for 5 sec. in a Perspex chamber (Hatch & Slack, 1966) and killed in boiling 80% (v/v) ethanol. The light-intensity was 8900 ft.-candles. Other details are described in the Methods section.

Leaf no.	10^{-4} × Total ^{14}C incorporated (counts/min./100 mg. of residue)	Distribution of total ^{14}C in individual compounds (%)				
		Malate	Aspartate	3-Phospho-glycerate	Hexose mono-phosphates	Other compounds
−1	133	63	7	16	8	6
0	221	49	14	18	5	14
2	212	52	17	19	5	6
5	326	48	17	18	7	10

carbon dioxide and then a standard quantity of $^{14}CO_2$ was supplied. With increasing carbon dioxide concentrations there was an approximately proportional decrease in the amount of $^{14}CO_2$ fixed, but the amount of the total radioactivity in malate plus aspartate remained high with all treatments (Table 2).

At least for leaves equilibrated at carbon dioxide concentrations in the range 0·03–1·47% (v/v) the pattern of labelling was almost identical, although the magnitude of the increase in carbon dioxide concentration due to the addition of $^{14}CO_2$ varied widely. To obtain sufficient radioactivity for the analyses conducted in some previous time-course studies (Hatch & Slack, 1966) it was necessary to supply an amount of $^{14}CO_2$ that increased the total concentration of carbon dioxide from 0·032 to 0·055% (v/v). We have now shown in a separate experiment that the proportion of radioactivity in different compounds at 5, 10 and 30 sec. is the same when the carbon dioxide concentration was increased from 0·032 to only 0·034% (v/v), or to 0·055%, by adding $^{14}CO_2$.

Effect of light-intensity. As the light-intensity was reduced from 9100 to 1470 ft.-candles the proportion of the fixed radioactivity in malate plus aspartate rose from 87 to 100% (Table 3). Though the rate of carbon dioxide fixation was considerably

Table 2. *Effect of carbon dioxide concentration on the early products of photosynthesis in sugar-cane leaves*

After the standard equilibration treatment at 8900 ft.-candles, individual segments were transferred to a test tube and flushed for 12 min. with humidified gas mixtures containing different concentrations of CO_2. As soon as the flushing was discontinued 2·1 μmoles of $^{14}CO_2$ gas containing $3·9 \times 10^7$ counts/min. were injected and after a further 3 sec. the leaf segments were killed and analysed as described in the Methods section. Gas mixtures were obtained by proportioning standard CO_2+N_2 mixtures with either air or O_2 so that the concentration of O_2 was always approx. 20% (v/v).

Concn. of CO_2 (%, v/v)		$10^{-4} \times$ Total ^{14}C incorporated	Distribution of total ^{14}C in individual compounds (%)			
For 12 min. before $^{14}CO_2$ injected	After $^{14}CO_2$ injected	(counts/min./100 mg. of residue)	Malate	Aspartate	Phosphorylated compounds	Other compounds
0	0·06	99	35	32	27	6
0·03	0·09	390	51	30	16	3
0·075	0·135	144	58	26	16	0
0·404	0·464	48	60	28	9	3
1·47	1·53	28	62	28	10	0
3·87	3·93	5	35	36	28	1

Table 3. *Effect of light-intensity on the early products of photosynthesis in sugar-cane leaves*

All leaf segments were equilibrated at 9100 ft.-candles under the standard conditions. One segment was then transferred to a tube at the same distance from the light-source, exposed to $^{14}CO_2$ for 3 sec. and killed with boiling 80% (v/v) ethanol. The equilibration chamber containing the remaining leaves was then moved away from the light-source in a stepwise manner to obtain the lower light-intensities. After 5 min. at each new light-intensity another leaf segment was exposed to $^{14}CO_2$ at that intensity. Other details including the method for measuring light-intensity are described in the Methods section.

Light-intensity (ft.-candles)	$10^{-4} \times$ Total ^{14}C incorporated (counts/min./100 mg. of residue)	Distribution of total ^{14}C in individual compounds (%)				
		Malate	Aspartate	3-Phospho-glycerate	Hexose mono-phosphates	Other compounds
9100	354	55	32	9	3	1
6900	284	56	32	9	1·1	2
5570	307	56	31	7	1·2	5
3680	262	60	31	6	0·7	2
2000	169	67	33	0	0	0
1470	53	65	35	0	0	0
0	1·5	—	—	—	—	—

decreased at 1470ft.-candles it was still 35 times the rate for leaves in the dark. In the dark, essentially all the radioactivity fixed during a period of 5min. remains in malate and aspartate (Hatch & Slack, 1966). However, a separate study showed that after longer period at 1500ft.-candles the sequence of labelling of other compounds was the same as previously observed with higher light-intensities (Hatch & Slack, 1966). After 14sec. in $^{14}CO_2$ 9% of the radioactivity was located in 3-phosphoglycerate, and after 20sec. hexose phosphates were labelled.

Early products of photosynthesis in different species and other genera

The radioactive products formed after leaves of several monocotyledonous and dicotyledonous plants were exposed to $^{14}CO_2$ for 4sec. are shown in Table 4. The commercial sugar-cane variety, Pindar, used for our previous studies is a hybrid of *Saccharum officinarum* and *S. spontaneum*. Varieties of these two species and *S. sinense*, and also of plants belonging to several other genera of Gramineae, showed the same labelling pattern as Pindar. However, with several other members of the Family Gramineae, and with other monocotyledonous and dicotyledonous plants, the C_4 dicarboxylic acids were not labelled. A few of these species contained a trace of radioactivity in malate but in all cases most of the radioactivity was located in 3-phosphoglycerate and hexose phosphates. An exception was the species of *Cyperus* that showed a pattern of labelling typical of sugar-cane. A fern (*Nephrolepis cordifolia*) that was also examined contained 47% of the radioactivity in 3-phosphoglycerate and 48% in hexose phosphates.

DISCUSSION

We have proposed a scheme (Hatch & Slack, 1966) for photosynthesis in sugar-cane that differs, at least in the initial steps, from the pathway that is generally accepted to operate in plants (Calvin & Bassham, 1962). During the present studies we varied leaf age, carbon dioxide concentration and light-intensity and obtained essentially the same labelling pattern as observed previously with our standard conditions (Hatch & Slack, 1966). The identification of the sugar-cane type of pathway in plants apparently does not depend on the use of a precise set of experimental conditions.

We have proposed that the labelling of 3-phosphoglycerate proceeds via C_4 dicarboxylic acids during photosynthesis of sugar-cane leaves in $^{14}CO_2$ (Hatch & Slack, 1966). If this is the only pathway operative it should be possible, with a sufficiently brief exposure to $^{14}CO_2$, to obtain label in C_4 dicarboxylic acids but not 3-phosphoglycerate. However, in the previous experiments relatively high light-intensities were used and after exposure to $^{14}CO_2$ for only 1sec. about 5% of the radioactivity was located in 3-phosphoglycerate. We have now found that with light-intensities of 1500–2000ft.-candles no label can be detected in 3-phosphoglycerate until about 9sec., but that qualitatively the time-sequence of labelling is the same as that previously observed. At lower light-intensities the primary carboxylation reaction apparently proceeds at a relatively more rapid rate than the transfer of radioactivity from C_4 dicarboxylic acids to 3-phosphoglycerate.

We have emphasized the improbability of sugar-cane's being unique in terms of its pathway of photosynthetic carbon dioxide fixation (Hatch & Slack, 1966). The present studies show that species from several of the tribes of Gramineae display a similar pattern of labelling to that observed with sugar-cane. These tribes are taxonomically related (Stebbins, 1956). Some classifications divide the tribes of Gramineae into two main sub-groups (Prat, 1960), and all the species showing the pathway similar to sugar-cane belong to tribes in one of these groups. Members of the other group including wheat, oat, bamboo and a rice relative contained little or no label in C_4 dicarboxylic acids.

A feature of the survey of different plant species is the clear distinction between the sugar-cane type of pathway and the pathway that features 3-phosphoglycerate as a major early product. Hence the evidence for the sugar-cane type of pathway in the *Cyperus* species emphasizes that it may be more widely operative than this relatively limited survey suggests.

The present studies confirmed the assumption (Hatch & Slack, 1966) that the distribution of radioactivity in the individual carbon atoms of malate would reflect the labelling of oxaloacetate. With all plants in which radioactive oxaloacetate was detected, between 90 and 100% of the ^{14}C was located in the C-4 position. However, labelled oxaloacetate was only detected in leaves containing a high proportion of the total radioactivity in malate and aspartate. Between 1 and 10% of the radioactivity was located in oxaloacetate but as little as 0·05% would have been readily detected. These studies have not provided support for the suggestion (Hatch & Slack, 1966) that in other plants the sugar-cane type of pathway may operate to give labelling of oxaloacetate but not of malate or aspartate. The possibility that malate and aspartate are side products of the main pathway is still not excluded.

Recent studies of maximum photosynthesis rates and photo-respiration demonstrate differences

Table 4. *Survey of the early products of photosynthesis in several plant species*

Leaves or leaf segments were equilibrated at approx. 8000 ft.-candles, exposed to $^{14}CO_2$ for 4 sec., and then killed and analysed as described in the Methods section. The tribes to which the following genera belong are given in parentheses: *Saccharum*, *Erianthus* and *Sorghum* (Andropogoneae); *Zea* (Maydeae); *Paspalum*, *Oxonopus* and *Digitaria* (Paniceae); *Chloris* (Chlorideae); *Eragrostis* (Eragrosteae).

Family	Genus	Species	Common name or variety	Distribution of total ^{14}C in individual compounds (%)					% of ^{14}C of oxaloacetate in C-4
				Oxaloacetate	Oxaloacetate + aspartate + malate	3-Phospho-glycerate	Hexose phosphates	Other compounds	
Monocotyledoneae									
Gramineae	*Saccharum*	hybrid	Pindar	3·3	69	15	6	10	94
Gramineae	*Saccharum*	*officinarum*	Badilla	10·6	76	11	0	13	96
Gramineae	*Saccharum*	*spontaneum*	Mandalay	2·1	67	19	6	8	93
Gramineae	*Saccharum*	*sinense*	Chunee	1·2	64	15	—	—	88
Gramineae	*Erianthus*	*maximus*	Mindinao	0·9	85	7	4	4	96
Gramineae	*Sorghum*	hybrid	Sorghum	2·7	70	18	2	10	96
Gramineae	*Sorghum*	*halepense*	Johnson grass	6·2	70	18	4	8	97
Gramineae	*Zea*	*mays*	Maize (corn)	5·2	88	12	2	0	98
Gramineae	*Paspalum*	*dilatatum*	Paspalum	6·3	72	12	5	11	97
Gramineae	*Axonopus*	—	Carpet grass	1·7	66	12	11	11	93
Gramineae	*Digitaria*	—	—	6·7	72	10	7	11	98
Gramineae	*Chloris*	*gayana*	Rhodes grass	2·3	70	15	8	7	98
Gramineae	*Eragrostis*	*brownii*	—	1·2	100	0	0	0	97
Gramineae	*Triticum*	*sativum*	Wheat	0	0	46	36	18	—
Gramineae	*Avena*	*sativa*	Oats	0	2	45	35	17	—
Gramineae	*Leersia*	*hexandra*	Rice-type	0	0	44	38	18	—
Gramineae	*Bambusa*	*vulgaris*	Bamboo	0	0	55	28	17	—
Cyperaceae	*Cyperus*	—	Sedge	1·9	79	15	2	4	93
Musaceae	*Musa*	—	Banana	0	0	37	49	14	—
Cannaceae	*Canna*	—	Canna	0	0	25	52	23	—
Palmae	*Phoenix*	*dactylifera*	Date palm	0	2	48	37	13	—
Liliaceae	*Lilium*	—	Spider lily	0	0	28	52	20	—
Orchideae	*Cymbidium*	—	Cym-doris	0	2	26	39	33	—
Dicotyledoneae									
Compositae	*Tithonia*	*diversifolia*	Japanese sunflower	0	0	53	38	9	—
Compositae	*Lactuca*	*savita*	Lettuce	0	4	47	40	9	—
Chenopodiaceae	*Beta*	*vulgaris*	Silver beet	0	7	34	39	20	—
Leguminosae	*Phaseolus*	*vulgaris*	French bean	0	2	48	37	15	—
Leguminosae	*Glycine*	*soja*	Soya bean	0	2	54	23	23	—
Geraniaceae	*Pelargonium*	—	Geranium	0	0	33	46	21	—
Rosaceae	*Prunus*	*persica*	Peach	0	0	33	49	17	—
Myrtaceae	*Eucalyptus*	—	Gum tree	0	0	28	33	39	—
Solanaceae	*Nicotiana*	—	Tobacco	0	0	45	42	13	—

between tropical grasses and several other plants including temperate grasses. The grasses that we showed to have similar products of photosynthesis as sugar-cane are all of tropical origin. Hesketh and co-workers (Hesketh & Moss, 1963; El-Sharkawy & Hesketh, 1965; J. D. Hesketh, personal communication) found that the maximum rates of photosynthesis at light-saturation for all tropical grasses were approximately double the rates for temperate grasses and several dicotyledonous plants. Attempts to explain these differences in terms of resistance to carbon dioxide diffusion were inconclusive. However, the absence of detectable photo-respiration from the tropical grasses (El-Sharkawy & Hesketh, 1965; Forrester, Krotkov & Nelson, 1965) may be indicative of a more efficient process for fixing carbon dioxide. Clearly, further studies will be necessary to establish the significance of the sugar-cane type of photosynthetic pathway in the tropical grasses in relation to these observations.

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