Intramolecular Labelling of Sucrose Made by Leaves from [^{14}C]Carbon Dioxide or [$3\text{-}^{14}\text{C}$]Serine

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Pea leaves were illuminated in air containing 150 or 1000 p.p.m. of $^{14}\text{CO}_2$ for various times. Alternatively, segments of wheat leaves were supplied with [$3\text{-}^{14}\text{C}$]serine for 40 min in the light in air with 145, 326 or 994 p.p.m. of $^{12}\text{CO}_2$. Sucrose was extracted from the leaf material, hydrolysed with invertase, and ^{14}C in the pairs of carbon atoms C-3 + C-4, C-2 + C-5 and C-1 + C-6 in the glucose moiety was measured. The results obtained after metabolism of $^{14}\text{CO}_2$ were consistent with the operation of the photosynthetic carbon-reduction cycle; the effects of CO_2 concentration on distribution of ^{14}C in the carbon chain of glucose after metabolism of [$3\text{-}^{14}\text{C}$]serine is more easily explained by metabolism through the glycolate pathway than by the carbon-reduction cycle.

During photosynthetic assimilation of $^{14}\text{CO}_2$ by C_3 -pathway plants (Chollet & Ogren, 1975), the 3-phosphoglycerate produced is initially radioactive only in C-1, but the photosynthetic carbon-reduction cycle by which the acceptor ribulose 1,5-bisphosphate is regenerated (Bassham, 1964) causes ^{14}C to increase with time in C-2 and C-3 (Calvin *et al.*, 1951). As a consequence the hexose phosphates and hence hexose units of sucrose produced from the 3-phosphoglycerate are initially labelled in C-3 and C-4, but subsequently ^{14}C increase in C-1, C-2, C-5 and C-6. These conclusions were reached from experiments where $^{14}\text{CO}_2$ was supplied at a concentration greater than atmospheric, when the incorporation of carbon into glycolic acid, glycine and serine is decreased (Wilson & Calvin, 1955; Mortimer, 1959; Snyder & Tolbert, 1974; Lee & Whittingham, 1974). These substances form part of the cyclic metabolic pathway (glycolate pathway) responsible for photorespiration (Chollet & Ogren, 1975). Since much of the carbon in intermediates of this pathway may be subsequently incorporated into sucrose (Wang & Waygood, 1962), we investigated the effect of external CO_2 concentration on the intramolecular distribution of ^{14}C in the sucrose made either from $^{14}\text{CO}_2$ or from specifically labelled serine, hoping that this would show the extent to which sucrose synthesis from intermediates of glycolate metabolism involves carbon metabolism in the cytoplasm (Waidyanatha *et al.*, 1975). However, the extent of randomization of ^{14}C labelling in the hexose carbon chain in sucrose depends on the position of ^{14}C in glucose or in intermediates of the glycolate pathway supplied to the leaf (Kandler & Gibbs, 1959; Jimenez *et al.*, 1962; Mifflin *et al.*, 1966; Marker & Whittingham, 1967). We provide additional evidence for this and attempt an explanation.

Experimental

$\text{Na}_2^{14}\text{CO}_3$ and [$3\text{-}^{14}\text{C}$]serine were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Seedlings of *Pisum sativum* (var. Feltham First) were grown for 3 weeks at 20°C and illuminated for 12 h per day by fluorescent tubes giving $200\text{ }\mu\text{mol}$ quanta/ m^2 per s of photosynthetically active radiation (10400 lx). Wheat (*Triticum aestivum*, var. Kleiber) was grown under similar conditions but with a 16 h photoperiod for 2 weeks.

Illuminated, detached, pea leaves were supplied with $^{14}\text{CO}_2$ in air in a closed system similar to that of Porter & Martin (1952) in which steady-state photosynthesis was established and maintained. Segments of wheat leaves were supplied with [$3\text{-}^{14}\text{C}$]serine during steady-state photosynthesis by the methods described by Waidyanatha *et al.* (1975).

After exposure to $^{14}\text{CO}_2$ or [$3\text{-}^{14}\text{C}$]serine, leaves or leaf segments were dropped into boiling ethanol. Soluble compounds were extracted by grinding the tissue (0.15–5 g) with sand (1 g) in 25 cm^3 of ethanol/water (1:1, v/v). The ethanol used to kill the tissue was evaporated to dryness and the residue extracted with chloroform. The chloroform was washed with water and the water added back to the residue not soluble in chloroform. The water-soluble fractions from the extracts were dissolved in water and de-salted on columns (0.7 cm $\times$ 2.5 cm) of ion-exchange resins [Amberlite CG 120 (H^+ form) and CG 400 (CO_3^{2-} form)]. Sucrose in the non-ionic fractions was purified by paper chromatography on Whatman no. 1 paper with butan-1-ol / acetic acid / water (12:3:5, by vol.) as developing solvent. The sucrose was hydrolysed with invertase and the glucose and fructose produced were separated by paper chromatography in the same solvent mixture as above. The concentration of fructose was measured (Bacon &

Bell, 1948); its radioactivity was determined by liquid-scintillation counting (Patterson & Greene, 1965), and an estimate made of specific radioactivity.

The glucose was degraded as described by Rognstad & Woronsberg (1968) except for the following modifications. Initially, the concentration of the solution of lactate formed from glucose was increased to 0.4M by adding AnalaR 2M-lactate. However, the 2M solution contained anhydride, which was oxidized more slowly than lactate by acid permanganate and resulted in a spuriously high estimate of the specific radioactivity for C-3+C-4. The difficulty was overcome by heating the 0.4M-lactate at 80°C overnight in a stoppered tube. After the sample had cooled and water lost by evaporation had been replaced, the lactate content was checked by titrating a sample with NaOH to the phenolphthalein end point. For the acid permanganate oxidation we used phosphoric acid as recommended by Rognstad & Woronsberg (1968), but heated the evacuated reaction flask at 80°C for 30 min as described by Katz *et al.* (1955). The $^{14}\text{CO}_2$ that evolved at each of the three steps in the degradation was absorbed in 1 ml of 1M-NaOH contained in centre wells of the reaction flasks. Radioactivity in the CO_2 was estimated by liquid-scintillation counting of samples (0.1 ml). These were mixed with 1 ml of water and 10 ml of a mixture of 1 vol. of Triton X-100 with 2 vol. of toluene containing 2,5-diphenyl-oxazole (0.4%, w/v) and 1,4-bis-(4-methyl-5-phenyl-oxazol-2-yl)benzene (0.1%, w/v) (Patterson & Greene, 1965). The sample must contain less than 20 μmol of CO_2 to avoid precipitation of carbonate. The total amount of CO_2 absorbed was measured by micro-titration (Porter *et al.*, 1959) by using 0.25 ml samples taken from the centre wells. Reaction flasks (Porter *et al.*, 1959) had their centre wells protected from splashes of acid or alkaline solution during the initial acid permanganate oxidation of lactate, the alkaline permanganate oxidation of methylamine and the subsequent regeneration of CO_2 from the reaction mixture. For the Schmidt degradation, the dried heated sodium acetate was dissolved in conc. (98%) H_2SO_4 in special vials, 3 cm long, that fitted into the centre wells of the combustion flasks. These vials had a depression blown into the wall near the top in which the NaN_3 was lodged. The flasks were kept tilted so that the NaN_3 remained in the depression until the flasks had been stoppered. The NaN_3 was tipped a little at a time into the acid solution followed by careful mixing by gyrating the flasks until all had been added. The flasks were not evacuated either during addition of the NaN_3 or during the subsequent heating at 80°C for 1 h.

Results and Discussion

In Table 1 the ^{14}C in pairs of carbon atoms, C-3+C-4, C-2+C-5 and C-1+C-6, in the glucose

moiety of sucrose made by photosynthesis from $^{14}\text{CO}_2$ can be compared either after equal periods of time or after assimilation of equal amounts of $^{14}\text{CO}_2$. There was considerable randomization along the carbon chain from C-3 and C-4, but the comparison made when equal amounts of $^{14}\text{CO}_2$ had been assimilated shows that more $^{14}\text{CO}_2$ remained in C-3 and C-4 when the CO_2 concentration was 1000 rather than 150 p.p.m. This effect is probably caused by changes in the sizes of active pools of phosphorylated intermediates in the photosynthetic carbon-reduction cycle similar to those reported in *Chlorella* by Pedersen *et al.* (1966) rather than by decreased carbon flow through the glycolate pathway. Table 2 shows that wheat leaves incorporated radioactivity from $[3\text{-}^{14}\text{C}]\text{serine}$ mainly into C-1 and C-6 of the glucose moiety of sucrose. Randomization was much less than in Table 1 and was markedly decreased with increased CO_2 concentrations. Either more sucrose was made from serine by pathways not causing randomization, possibly entirely in the cytoplasm, as suggested by Waidyanatha *et al.* (1975), or the mechanisms responsible for randomization have less effect when C-1 and C-6 than when C-3 and C-4 are initially labelled in hexose phosphate intermediates. The latter possibility is the more probable, since the results are readily explained by reference to Scheme 1, showing which carbon atoms become radioactive in intermediates when $[3\text{-}^{14}\text{C}]\text{serine}$ is metabolized to 3-phosphoglycerate and then by the photosynthetic carbon-reduction cycle with recycling of the carbon via the glycolate pathway. Evidence that serine carbon is recycled in this manner has been obtained by Kumarasinghe *et al.* (1977). Glycolate production is a property of chloroplasts (Kearney & Tolbert, 1962), so the scheme implies that some serine carbon is metabolized in the chloroplasts and sucrose could be made from the triose phosphate formed. Increased CO_2 in the atmosphere would decrease randomization along the carbon chain by decreasing recycling through glycolate. Therefore it is impossible to decide from the results whether some sucrose was also made from serine carbon that did not enter the chloroplasts. Mifflin *et al.* (1966) and Marker & Whittingham (1967) showed that $[1\text{-}^{14}\text{C}]\text{glycolate}$ and $[1\text{-}^{14}\text{C}]\text{glycine}$ were metabolized by pea leaves to hexoses in sucrose that were almost equally radioactive in all carbon atoms, but the same precursors labelled with ^{14}C in C-2 resulted in hexoses in which C-3 and C-4 each contained only 5% of the ^{14}C . Jimenez *et al.* (1962) reached similar conclusions for wheat leaves. In our experiments $[3\text{-}^{14}\text{C}]\text{serine}$ was metabolized to hexose strongly radioactive in C-1 and C-6 especially if the atmosphere contained a high concentration of CO_2 . Conversely, from $^{14}\text{CO}_2$, which gives rise initially to carboxy-labelled 3-phosphoglycerate, there was a rapid spread of radioactivity to all the hexose carbon

Table 1. *Intramolecular distribution of ^{14}C in the glucose moiety of sucrose made by pea leaves from $^{14}\text{CO}_2$ in the light* Steady-state photosynthesis was established at 22.5°C at a photon flux density of 400 μmol quanta/ m^2 per s of photosynthetically active radiation (20800 lx) in a closed system in which air containing 150 or 1000 p.p.m. of CO_2 was circulated. Without significantly changing the conditions, $^{14}\text{CO}_2$ of high specific radioactivity was introduced into the system. Steady conditions were maintained for the time shown below by further additions of $^{14}\text{CO}_2$ of appropriate specific radioactivity. For Expt. A, eight pairs of leaflets (1.5 g fresh wt.) were enclosed in a chamber of 112.5 cm^3 total volume; for Expt. B, pea shoots (5 g fresh wt.) were used in which stems comprised much of the tissue, the chamber volume was 304.0 cm^3 and conditions were less satisfactory for gas exchange.

Expt.	Concn. of CO_2 (p.p.m.)	Time supplied (min)	Amount of CO_2 assimilated (μmol)	^{14}C in pairs of C atoms* (% of total in hexose)		
				C-3 + C-4	C-2 + C-5	C-1 + C-6
A	150	1	1	61.9	18.7	19.4
	150	2	2	49.7	21.0	29.3
	150	5	5	44.5	28.8	26.7
	150	10	10	43.5	27.7	28.3
B	150	22	12	39.2	28.9	31.9
	150	45	24	37.0	31.0	32.0
	150	85	48	37.0	30.2	33.4
A	1000	0.5	2.5	73.3	12.0	14.8
	1000	1	5	57.2	18.8	24.0
	1000	2	10	46.6	24.0	29.4
B	1000	4	12	46.3	26.0	27.2
	1000	8	24	42.6	29.5	27.9
	1000	16	48	37.0	31.7	31.3

Least significant difference ($P = 0.05$)†

Mean ^{14}C distribution for eight samples of lactate obtained from D-[U- ^{14}C]glucose ($\pm$ s.d.)

* Means of duplicate determinations.

† Values from one-way analysis of variance (Meddis, 1975).

Table 2. *Intramolecular distribution of ^{14}C in the glucose moiety of sucrose made by segments from wheat leaves given [3- ^{14}C]serine in the light*

Steady-state photosynthesis was established by three leaf segments, each 2 cm^2 in area, at 18°C in an open gas system supplied with air from cylinders containing 145, 326 or 994 p.p.m. of CO_2 at 420 cm^3/min . The leaf segments were illuminated at a photon-flux density of 1200 μmol quanta/ m^2 per s of photosynthetically active radiation (62300 lx). The cut bases of the segments were transferred from slots containing water to slots containing 0.4 μmol (3 μCi) of [3- ^{14}C]serine in 0.03 ml of water; steady-state conditions were maintained for a further 40 min.

Concn. of CO_2 in the atmosphere (p.p.m.) ...	Distribution of ^{14}C			Least significant difference† ($P = 0.05$)
	145	326	994	
^{14}C taken up by the leaf segments (μCi)	5.6	5.4	3.6	0.6
^{14}C in sucrose (%)	22	40	46	2.9
^{14}C in pairs of C atoms of glucose from sucrose (% of total in hexose)*	C-3 + C-4	11.0	5.9	3.8
	C-2 + C-5	24.1	18.0	11.2
	C-1 + C-6	64.9	76.1	85.0

* Means of single estimations on glucose from three independent experiments, each involving samples of three leaf segments.

† Values from one-way analyses of variance.

atoms and the concentration of CO_2 in the atmosphere had much less effect on intramolecular distribution of radioactivity. By using *Chlorella*, Kandler & Gibbs (1959) also found that ^{14}C from $^{14}\text{CO}_2$ quickly gave rise to radioactivity in all the carbon

atoms of hexose, whereas [1- ^{14}C]- or [6- ^{14}C]-glucose was metabolized to hexose in which C-1 and C-6 were the most radioactive. Thus photosynthetic metabolism results in a rapid displacement of carbon from C-1 of triose phosphates to C-2 and C-3, but the

reverse movement of carbon from C-2 and C-3 of triose to C-1 is slower. Kandler & Gibbs (1959) suggest that involvement in the carbon-reduction cycle of transaldolase activity could cause movement of carbon from C-2 and C-3 to C-1, but we have failed to produce a likely scheme to accommodate this suggestion. Scheme 1 does account for this pattern of randomization. After one cycle, C-2 as well as C-3 of serine is radioactive and thus [1,2,5,6- ^{14}C]hexose phosphates are formed. After a second cycle, the carboxy carbon atom of serine becomes radioactive and hexose phosphates become labelled in all carbon atoms. The relative labelling of carbon atoms would moreover be as we found them in hexose from sucrose.

The more rapid randomization from C-1 of triose phosphates by the photosynthetic carbon-reduction cycle than from C-3 by the glycolate pathway is probably because recycling in the reduction cycle involves smaller pools of intermediates than in the glycolate pathway and a more rapid turnover of carbon. Refixation of $^{14}\text{CO}_2$ released during metabolism of [1- ^{14}C]glycolate or [1- ^{14}C]glycine would not, as suggested by Jimenez *et al.* (1962) and Marker & Whittingham (1967), increase the rate of randomization, since refixation and metabolism through serine both give rise initially to 3-phospho[1- ^{14}C]glycerate.

Any attempt to decide the route by which carbon from intermediates of photorespiration reaches sucrose must take into account the interchange of carbon between the two interacting metabolic pathways shown in Scheme 1.

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