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The C_4-Pathway of Photosynthesis

EVIDENCE FOR AN INTERMEDIATE POOL OF CARBON DIOXIDE AND THE IDENTITY OF THE DONOR C_4-DICARBOXYLIC ACID

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1. Leaves were exposed to $^{14}CO_2$ under steady-state conditions for photosynthesis. The kinetics of entry or loss of label in pools of CO_2 and other compounds was examined during the period of the pulse and a 'chase' with $^{12}CO_2$. 2. With maize the kinetics of labelling of the major CO_2 pool and of depletion of label during a 'chase' was consistent with this pool being derived from the C-4 of malate and being the precursor of the C-1 of 3-phosphoglycerate. 3. Similar results were obtained for *Amaranthus* leaves except that the C-4 of aspartate rather than malate was apparently the primary source of CO_2. 4. The size and turnover time of the CO_2 and C_4 acid pools was calculated. These results provided the basis for estimating the concentration of CO_2 in the bundle-sheath cells or chloroplasts assuming the pool was largely restricted to one or other of these compartments. 5. These findings are considered in relation to current schemes for the C_4-pathway and the operation of a CO_2 concentrating mechanism to serve ribulose diphosphate carboxylase.

The view is now widely held (see Hatch, Osmond & Slatyer, 1971) that during the operation of the C_4-pathway, C_4-dicarboxylic acids are formed in the mesophyll chloroplasts and then transferred to the bundle-sheath cells where the C-4 is released as CO_2 and refixed by ribulose diphosphate carboxylase. This proposition, one of two previously considered (Hatch & Slack, 1966, 1970*a*), has been supported by studies on the activity and location of enzymes. It is now known that C_4-pathway species contain sufficient ribulose diphosphate carboxylase to account for its operation as an integral reaction of the photosynthetic process (Bjorkman & Gauhl, 1969; Andrews & Hatch, 1971). One group of C_4-pathway species contain high activities of 'malic' enzyme (Slack & Hatch, 1967; Downton, 1970; Hatch & Slack, 1970*a*; Andrews, Johnson, Slack & Hatch, 1971; Chen, Brown & Black, 1971) and this is located in the bundle-sheath chloroplasts together with ribulose diphosphate carboxylase (Slack, Hatch & Goodchild, 1969). In these species NADP–malate dehydrogenase and probably phosphoenolpyruvate carboxylase are located in the mesophyll chloroplasts (Slack *et al.* 1969). Other studies have provided similar information, at least about the intercellular distribution of these enzymes (Bjorkman & Gauhl, 1969; Berry, Downton & Tregunna, 1970; Edwards, Lee, Chen & Black, 1970; Edwards & Black, 1971). Thus, this particular group of species contains enzymes with sufficient activity that are also in a location suitable for supporting a mechanism of the type proposed above, involving transport and decarboxylation of malate (Andrews *et al.* 1971). However, several other C_4-pathway species contain little 'malic' enzyme (Hatch & Slack, 1970*a*; Downton, 1970; Andrews *et al.* 1971; Chen *et al.* 1971) and also less NADP–malate dehydrogenase (Andrews *et al.* 1971). These deficiencies are compensated for by much higher alanine and aspartate aminotransferase activities (Andrews *et al.* 1971) and it was concluded that aspartate may replace malate as the donor of CO_2 in these species. Recent studies of CO_2 fixation by isolated mesophyll and bundle-sheath cells have provided support for many of these concepts (Edwards *et al.* 1970; Edwards & Black, 1971). An additional concept is that the reactions unique to the C_4-pathway constitute a mechanism for concentrating CO_2 for fixation by ribulose diphosphate carboxylase (Andrews *et al.* 1971; Hatch, 1971).

In the present studies direct evidence for the existence of an intermediate pool of CO_2 in C_4-pathway species and evidence establishing the identity of its precursor was looked for. By using radiotracers it was possible to detect substantial

pools of CO_2 in both maize and *Amaranthus* leaves. The kinetic characteristics of this pool were consistent with the primary source being the C-4 of malate in maize and the C-4 of aspartate in *Amaranthus*.

MATERIALS

Fully expanded leaves were obtained from maize (*Zea mays*, var. DS606A) or *Amaranthus palmeri* plants grown in soil in a greenhouse with the temperature controlled between 22 and 30°C. $Ba^{14}CO_3$ (61 mCi/mmol), [U-^{14}C]-malate and [U-^{14}C]aspartate were obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and [4-^{14}C]aspartate and malate dehydrogenase from Calbiochem, Los Angeles, Calif., U.S.A. [4-^{14}C]Malate was prepared from $NaH^{14}CO_3$ plus phosphoenolpyruvate in a system containing phosphoenolpyruvate carboxylase (Hatch & Slack, 1968), NADH and malate dehydrogenase. 'Malic' enzyme was prepared from maize leaves by fractionation with $(NH_4)_2SO_4$ and gel chromatography on Sephadex G-200 as described by Johnson & Hatch (1970). The fraction from Sephadex G-200 was adsorbed on to a Hypatite C column (Clarkson Chemical Co., Williamsport, Pa., U.S.A.) and then eluted with potassium phosphate buffer, pH 7.5.

METHODS

Treatment of leaves with $^{14}CO_2$ and isolation and identification of radioactive compounds. Maize leaf segments were allowed to establish a steady rate of photosynthesis in a 3-litre Perspex chamber as described by Hatch & Slack (1966). The light-intensity was approx. 8000 ft-candles, measured as described by Hatch & Slack (1966). Experiments were commenced by providing 0.5 mCi (8 μmol) of $^{14}CO_2$ in 2.5 ml of air. For pulse–'chase' studies leaves were transferred to a second chamber that was flushed with humidified air after a period of 35 s in $^{14}CO_2$. Similar procedures were employed for *Amaranthus* leaves except that the petiole was allowed to protrude through the rubber gasket. Lateral portions of the lamina were removed so that the leaf width was approx. 2.5 cm. A wad of tissue paper wrapped around the top of the petiole was kept saturated with water.

At the times specified individual leaves were removed from the chamber, kept in the dark in a stream of air for 2–3 s and then killed by plunging them into tubes containing 30 ml of 85% (w/v) ethanol containing 2,4-dinitrophenylhydrazine–HCl kept at −80°C (Hatch & Slack, 1966). The transient period in the dark was introduced to allow removal of the $^{14}CO_2$ associated with the boundary air layer and intercellular spaces. There are insignificant changes in the distribution of fixed label during such a period in the dark (Hatch & Slack, 1966, 1970*a*). With this killing procedure oxaloacetate forms a stable hydrazone derivative, whereas free oxaloacetate would degrade to release C-4 as $^{14}CO_2$.

To retain the CO_2 located in the leaves the tubes were quickly sealed with a stopper fitted with closed inlet and outlet connexions. The inlet extended to the bottom of the tubes. The sealed tubes were kept at −20°C for 24 h to allow complete penetration of the killing mixture. The outlet tube was then connected to an evacuated 500-ml flask containing 40 ml of 0.1 M-Hyamine hydroxide in a dioxane–toluene–ethanol mixture identical in proportion with the mixture used for subsequent scintillation counting. The connexion between the two vessels was opened and gas was rapidly evolved from the leaves. The inlet valve of the tube containing the leaf was then opened to allow a steady stream of air to flow through the tube and into the Hyamine solution. Equilibration of pressure was reached in about 20 min. The flask containing the Hyamine was then sealed and shaken for a further 20 min to ensure complete absorption of $^{14}CO_2$.

Other soluble radioactive compounds were extracted from the leaves as described by Hatch & Slack (1966). Samples of the chloroform extract remaining after extraction of hydrazones into 0.5 M-Na_2CO_3 were used to determine phaeophytin and hence chlorophyll (Vernon, 1960). The insoluble residue was washed with water and then suitable portions were collected on glass-fibre discs, dried, and counted to determine the radioactivity in starch. In the case of maize the remaining material was dried at 80°C and weighed.

Samples of all fractions were counted in a scintillation mixture containing 8% (w/v) naphthalene and 0.5% (w/v) 2,5 - diphenyloxazole in dioxane – toluene – ethanol (377:377:246, by vol.).

The proportions of radioactivity in individual compounds was determined by chromatography as previously described (Hatch & Slack, 1966; Johnson & Hatch, 1969).

Purification and degradation of compounds. Radioactive malate, aspartate and glycerate were purified by chromatography as described by Johnson & Hatch (1969). Glycerate was degraded as described by Hatch & Slack (1966). Radioactivity in the C-4 of aspartate was determined by treatment with aspartate decarboxylase (contained in glutamate decarboxylase provided by Sigma Chemical Co., St Louis, Mo., U.S.A.). Reaction mixtures containing 50 mM-potassium acetate buffer, pH 5.0, 1 mM-L-aspartate, 0.1 mg of pyridoxal phosphate/ml and 5 mg of the enzyme preparation were incubated for 5 h at 35°C. Radioactivity appearing in CO_2 was determined by counting the radioactivity of samples taken before and after this treatment. The radioactivity in the C-4 of malate was determined by treating samples of malate for 30 min at 30°C in reaction mixtures containing 0.1 unit of 'malic' enzyme, 2 units of alanine aminotransferase, 50 mM-tris–HCl buffer, pH 7.5, 3 mM-$MnCl_2$, 5 mM-NADP and 10 mM-glutamate. After acidification with formic acid the radioactivity released as CO_2 was determined as described above. Degradation procedures were verified by treating samples of [U-^{14}C]- and [4-^{14}C]-malate and -aspartate.

RESULTS

Labelling of CO_2 and other intermediates in maize leaves. The pattern of labelling of intermediates from $^{14}CO_2$ in maize leaves was similar to that described for sugar cane (Kortschak, Hartt & Burr, 1965; Hatch & Slack, 1966). The C_4 acids, malate plus aspartate, were labelled at a maximum rate from zero time whereas 3-phosphoglycerate, hexose

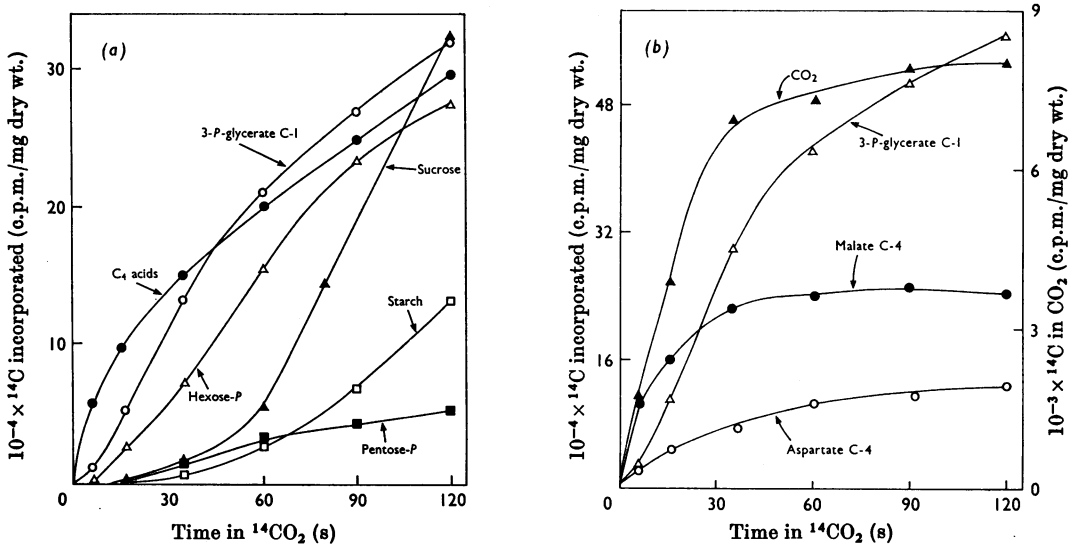

Fig. 1(*a*) and (*b*). Incorporation of radioactivity into compounds and specific carbon atoms of maize leaves exposed to $^{14}CO_2$. Experimental details are provided in the Methods section.

phosphates and pentose phosphates were rapidly labelled after increasing lag periods (Fig. 1*a*). The total radioactivity in the C_4 acids continued to increase up to 120s. In contrast, the C-4 of malate was almost completely saturated with ^{14}C by 30s whereas that of aspartate was saturated more slowly (Fig. 1*b*). The internal CO_2 pool was small compared with the malate pool but the kinetics of its labelling closely followed those of the C-4 of malate. The maximum rate of labelling of the C-1 of 3-phosphoglycerate occurred after about 20s by which time the CO_2 pool was approaching saturation.

During a 'chase' with $^{12}CO_2$ malate lost radioactivity much more rapidly than aspartate (Fig. 2*a*). The most rapid loss of label from aspartate occurred after the major loss of label from malate. Likewise, the maximum rate of loss of label from 3-phosphoglycerate occurred when the loss from malate and aspartate was almost complete. Labelling of other compounds was in accordance with previous proposals for the operation of the C_4 pathway (Hatch & Slack, 1970*a*; Andrews *et al.* 1971). The results in Fig. 2*b* confirm an earlier suggestion (Hatch & Slack, 1966) that the radioactivity remaining in the C_4 acids is due to label entering C-1, C-2 and C-3. In Fig. 2 radioactivity is expressed as % of that present at the start of the 'chase' with $^{12}CO_2$ to simplify the comparison of the kinetics of loss of label. The C-4 of malate and aspartate were almost free of label by 90s but the initial loss of label from the C-4 of malate was much more rapid. For instance after 10s more than 50% of the label in the C-4 of malate was lost compared with about 10% for the C-4 of aspartate. The initial loss of label from CO_2 proceeded at a rate intermediate between the rates for the C-4 of the C_4 acids, consistent with it being derived from these carbons. Subsequently, the loss of label from CO_2 was somewhat slower than from the C-4 of the C_4 acids. Considering the size and turnover time of this pool (see Table 1) it might have been expected, ideally, to have followed more closely the kinetic behaviour of the C-4 of the C_4 acids. The maximum rate of loss of label from the C-1 of 3-phosphoglycerate occurred after the major part of the label had been lost from the C-4 of C_4 acids and CO_2.

Labelling of CO_2 *and other intermediates in* Amaranthus *leaves.* In most respects the pattern of labelling of intermediates during the exposure of *Amaranthus* leaves (Fig. 3*a*) to $^{14}CO_2$ was similar to that observed for maize. One difference was the marked labelling of alanine in *Amaranthus*, a feature that has been observed in *Atriplex* (Johnson & Hatch, 1968). The rate of labelling of alanine closely followed that of C-1, C-2 and C-3 of the C_4 acids. Also, in contrast with maize, more label appeared in aspartate than in malate and the maximum labelling of the C-4 of aspartate occurred at least as rapidly as the C-4 of malate (Fig. 3*b*). The approach to radioactive saturation of the CO_2 pool closely followed that of the C-4 of the C_4 acids. As with maize the C-1 of 3-phosphoglycerate reached a maximum rate of labelling only as the CO_2 pool approached its maximum specific radioactivity.

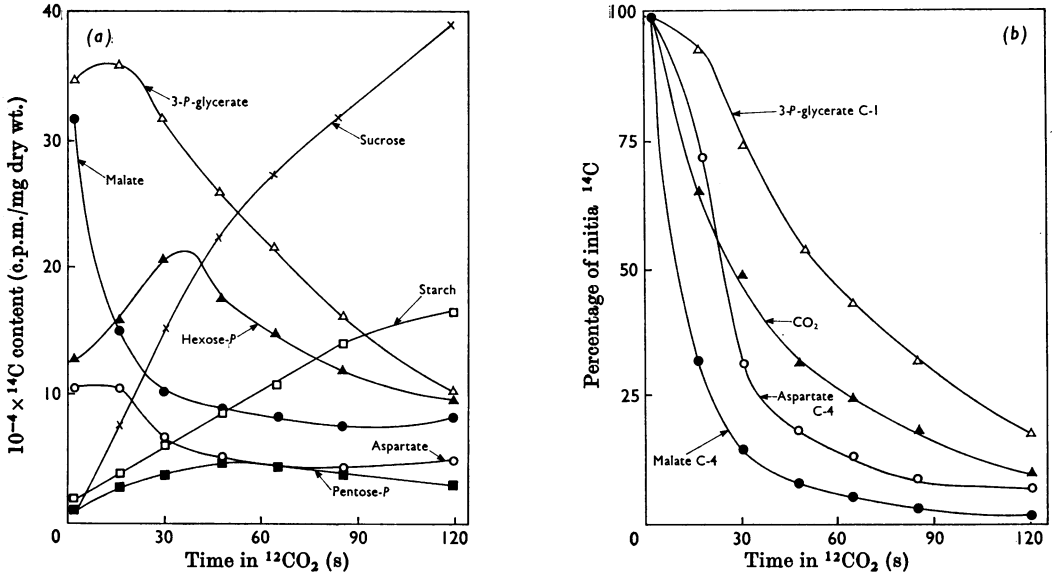

Fig. 2(*a*) and (*b*). Changes in radioactivity in compounds and specific carbon atoms of maize leaves during a 'chase' in $^{12}CO_2$. At zero-time leaves were transferred to $^{12}CO_2$ after a period of 35 s in $^{14}CO_2$. Other details are provided in the Methods section.

Table 1. *Size and concentration of* CO_2, C_4 *acid and 3-phosphoglycerate pools*

Pool sizes were calculated as described in the text from the results in Fig. 1(*a*) and Fig. 3(*a*). Turnover times were calculated from the pool size and the observed rates of CO_2 fixation in these experiments (5.5 and 6 μmol of CO_2/min per mg of chlorophyll for maize and *Amaranthus*, respectively). The turnover time for 3-phosphoglycerate was calculated on the assumption of a turnover rate twice that of the rate of CO_2 fixation. This value was an underestimate since the C-1 of 3-phosphoglycerate had not reached maximum specific radioactivity. The basis for the calculation of concentrations is discussed in the text.

Species	Compound	Pool size (μmol/g fresh wt.)	Turnover time (s)	Estimated concentration (mM)		
				Photosynthetic cells	Bundle-sheath cells	Bundle-sheath chloroplasts
Maize						
	CO_2 (dark)	0.007	—	0.017	—	—
	CO_2	0.072	0.8	0.17	0.8	1.6
	Malate	0.97	13.5	2.4	—	—
	Aspartate	0.55	—	1.4	—	—
	3-Phosphoglycerate	>2.4	>18	>6	—	—
Amaranthus						
	CO_2 (dark)	0.02	—	0.05	—	—
	CO_2	0.23	2.8	0.55	1.9	4.5
	Aspartate	1.02	14	2.4	—	—
	Malate	0.76	—	1.8	—	—
	3-Phosphoglycerate	>3.1	>22	>7	—	—

In contrast with maize, with *Amaranthus* leaves label was lost more rapidly from aspartate than malate during a 'chase' in $^{12}CO_2$ (Fig. 4*a*). The pattern of appearance of this label in 3-phosphoglycerate, hexose phosphates, pentose phosphates and the photosynthetic end products was consistent with the operation of the Calvin cycle as in the scheme proposed by Andrews *et al.* (1971). As with the time-course in $^{14}CO_2$ the change of label in alanine was similar to that observed for the sum of C-1, C-2 and C-3 of the C_4 acids.

With *Amaranthus* leaves the initial rate of loss

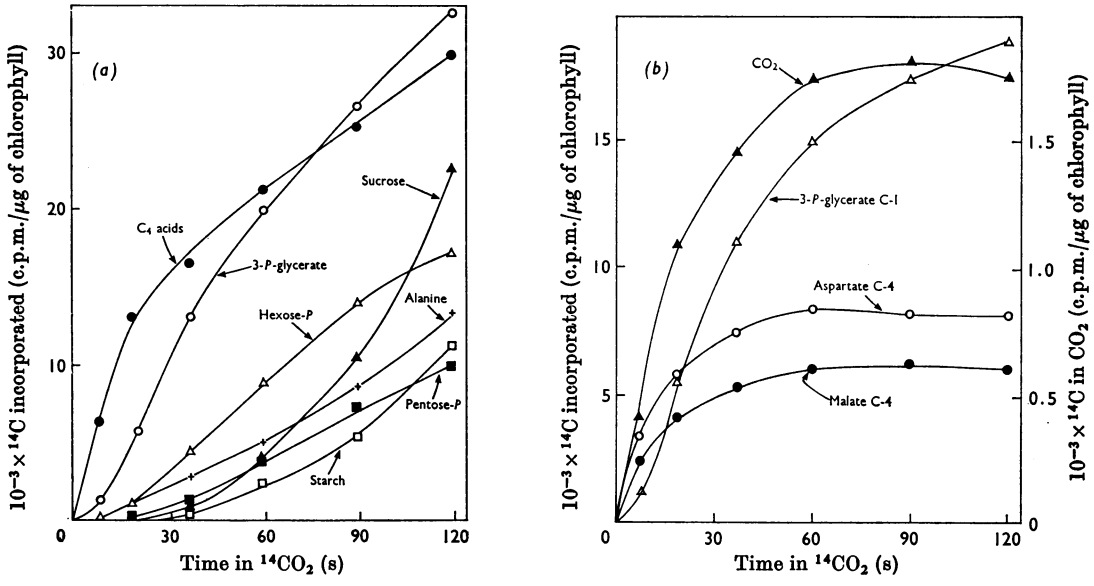

Fig. 3(*a*) and (*b*). Incorporation of radioactivity into compounds and specific carbons of *Amaranthus* leaves exposed to $^{14}CO_2$. Experimental details are given in the Methods section.

of label from the C-4 of aspartate during the 'chase' in $^{12}CO_2$ was much faster than that from the C-4 of malate (Fig. 4*b*). The rate of loss of label from CO_2 was intermediate between that of the C-4 of aspartate and of malate. As with maize the maximum rate of loss of label from the C-1 of 3-phosphoglycerate occurred only after the majority of the label had moved from the C-4 of the C_4 acids and the CO_2 pool.

Pool sizes, concentrations and turnover times. From the specific radioactivity of the $^{14}CO_2$ supplied and the amount of radioactivity in specific carbons when they were saturated with ^{14}C (Figs. 1*b* and 3*b*), it was possible to calculate the pool size of CO_2, C_4 acids and 3-phosphoglycerate (Table 1). In separate experiments the size of the CO_2 pool in leaves placed in the dark was also determined. Leaves were allowed to establish a steady rate of photosynthesis by illuminating them at 8000 ft-candles for 45 min and then placing them in the dark. After 35 s $^{14}CO_2$ was provided and individual leaves were removed for analysis at intervals thereafter. The maximum labelling of CO_2 in both maize and *Amaranthus* leaves was found in the first leaf samples, taken 30 s after providing $^{14}CO_2$, and it remained constant up to 120 s. During this period any closure of stomata caused by placing the leaves in the dark would be insignificant (R. W. Downes, unpublished work). With both species the rate of fixation into C_4 acids was initially about 12% of the rate in the light but declined to 1–1.5% by 120 s. Fixed label was only detected in oxaloacetate, malate and aspartate. Thus until about 2 min after illumination C_4 acid synthesis is enhanced compared with the steady rate in the dark. Some of the CO_2 found in leaves in the dark may have been derived from acids formed during this period of enhanced synthesis. Nevertheless, the total CO_2 pool in the dark was only about 10% of that in the light.

The pools of C_4 acids and 3-phosphoglycerate were relatively large compared with the CO_2 pool (Table 1). The values for 3-phosphoglycerate were based on the radioactivity in C-1 at 120 s (Figs. 1*b* and 3*b*). Approximations made by extending the curves for the C-1 of 3-phosphoglycerate indicated that it was probably about 80% saturated at this time. From the turnover time of the C_4 acid pools it was possible to calculate the expected half-time for loss of radioactivity from the C-4 in a 'chase' experiment, assuming this loss obeyed first order kinetics. The calculated values were 9 s for the C-4 of malate in maize and 10 s for the C-4 of aspartate in *Amaranthus*. Observed values were about 10 s (Fig. 2*b*) and 15 s (Fig. 4*b*), respectively.

Enzyme studies have been interpreted to indicate that CO_2 is released in the bundle-sheath chloroplasts of maize and either the bundle-sheath cells or chloroplasts of *Amaranthus* (Andrews *et al.* 1971). It was therefore useful to calculate the concentration

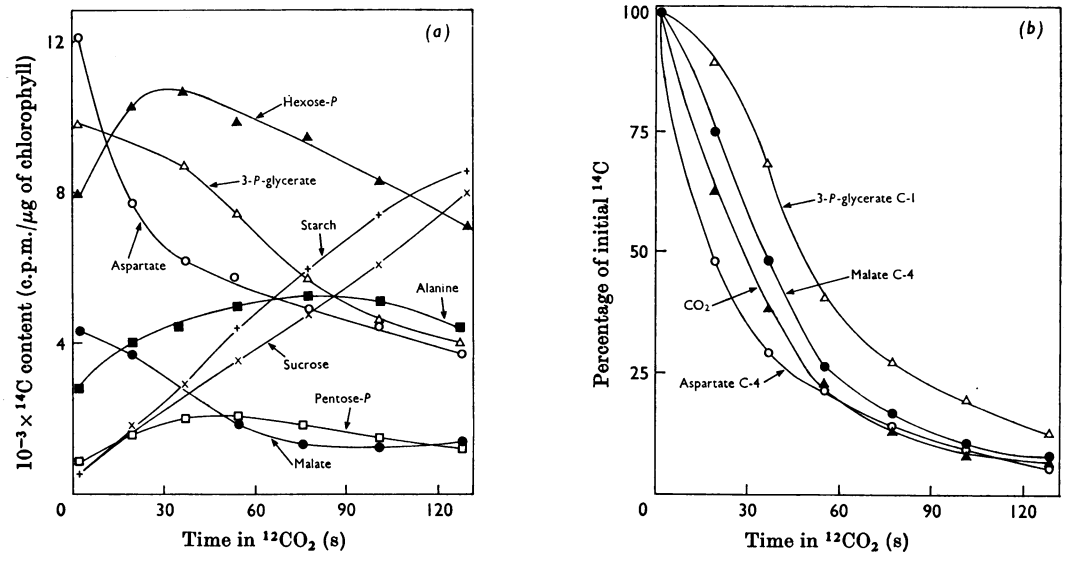

Fig. 4(*a*) and (*b*). Changes in radioactivity in compounds and specific carbons of *Amaranthus* leaves during a 'chase' in $^{12}CO_2$. At zero-time leaves were transferred to $^{12}CO_2$ after a period of 35s in $^{14}CO_2$. Further experimental details are provided in the Methods section.

of CO_2 by using the assumption that it is confined largely to one or other of these compartments. Estimates of the proportion of the total leaf volume occupied by the bundle-sheath cells and chloroplasts were made from light micrographs of leaf cross sections. For this calculation it was assumed that both cells and chloroplasts approximated to spheres. The values obtained were 10% for the bundle-sheath cells and 5% for the bundle-sheath chloroplasts of maize and 12 and 5% respectively for *Amaranthus*. No correction was made for vacuolar volumes. The concentrations quoted in Table 1 assume that all the CO_2 detected was located in the respective compartments indicated.

Assuming that the CO_2 in leaves placed in the dark provides a maximum estimate of the size of non-specific pools then at least 90% of the CO_2 in illuminated leaves could represent a specific pool involved in photosynthesis. Alternatively, by using simple physical data (Umbreit, Burris & Stauffer, 1959), estimates can be made of the amounts of CO_2 gas and of $(CO_2+HCO_3^-)$/g of leaf in equilibrium with atmospheric CO_2. The amount of CO_2 gas in the liquid phase would be about 6nmol/g fresh wt. Assuming a cytoplasmic pH of 7.0, a cytoplasmic volume of 20% of the leaf volume, and equilibration between CO_2 and HCO_3^-, the concentration of CO_2 plus HCO_3^- would be about 15nmol/g fresh wt. It should be noted that during steady-state photosynthesis the concentration of CO_2 in the freely accessible liquid space of the cell would be less than that at diffusion equilibrium.

DISCUSSION

It has been proposed that the unique reactions of the C_4-pathway, those leading to the release of CO_2 from C_4 acids, have the main function of concentrating CO_2 for fixation by ribulose diphosphate carboxylase (Andrews *et al.* 1971; Hatch, 1971). Even with the liquid phase concentration of CO_2 in diffusion equilibrium with atmospheric CO_2, the turnover for the reaction catalysed by ribulose diphosphate carboxylase would be quite inadequate to account for its operation as an integral reaction of photosynthesis (Hatch & Slack, 1970*b*). The equilibrium concentration of CO_2 in solution would be about 7 μM but the K_m of ribulose diphosphate carboxylase for CO_2 is at least 50 times this concentration (Cooper, Filmer, Wishnick & Lane, 1969; Andrews & Hatch, 1971). In contrast, even with CO_2 concentrations well below 7 μM the activity and K_m of phosphoenolpyruvate carboxylase for CO_2 in C_4-pathway species would permit a turnover sufficient to account for its operation in the primary CO_2-fixing process (Hatch, 1971).

The present studies provide evidence that, in the light, the size of the leaf CO_2 pool in C_4-pathway species is much higher than would be expected from simple diffusion of CO_2 into the liquid phases of

the tissue. The most direct evidence for this was that the size of the pool developed during photosynthesis was about ten times greater than that found in leaves placed in the dark. Calculations of the maximum concentrations of CO_2 gas or CO_2 plus bicarbonate likely to occur in leaves equilibrated in air with CO_2 supported this conclusion. Other calculations (Table 1) showed that if the CO_2 pool in illuminated leaves was largely confined to the bundle-sheath cells or bundle-sheath chloroplasts its concentration would be about 1 mM or more. Such concentrations would sustain near-maximum operation of ribulose diphosphate carboxylase. Assuming the operation of a process for concentrating CO_2 in the bundle-sheath cells or chloroplasts it appears likely that a permeability barrier may exist to restrict dissipation of this CO_2. As a consequence, movement of atmospheric CO_2 into the bundle-sheath chloroplasts would also be restricted. This would accord with the observations that most, if not all, of the label entering the C-1 of 3-phosphoglycerate from $^{14}CO_2$ is derived from the C-4 of C_4 acids (Johnson & Hatch, 1969) and that the rate of transfer of label from the C-4 to 3-phosphoglycerate is not decreased by increasing the $^{12}CO_2$ concentration (Hatch & Slack, 1966).

The inferences drawn above concerning the nature of the CO_2 pool in illuminated leaves were supported by the kinetics of labelling of CO_2. The results of these studies are consistent with the idea that the pools in both maize and *Amaranthus* are at least largely derived from the C-4 of a C_4 acid and are the precursor of the C-1 of 3-phosphoglycerate. These kinetics were not those expected if the CO_2 pool was derived directly from atmospheric CO_2 to serve either phosphoenolpyruvate carboxylase or ribulose diphosphate carboxylase. With this origin, and with the size and consequent turnover time of this pool, its half-time for saturation during a pulse in $^{14}CO_2$, and for depletion of radioactivity during a chase in $^{12}CO_2$, should have been about 2s or less. In contrast, the observed half-times were between 15 and 20s.

As indicated in the introduction the C_4-pathway species containing high 'malic' enzyme activity probably transport malate to the bundle-sheath chloroplasts to provide CO_2. An associated character of these particular species is the deficiency of both grana and photosystem 2 activity in the bundle sheath chloroplasts (Downton, Berry & Tregunna, 1970; Woo *et al.* 1970; Anderson, Boardman & Spencer, 1971). Significantly the operation of 'malic' enzyme to release CO_2 would also yield NADPH. A second group of C_4-pathway species were found to contain little 'malic' enzyme but high aminotransferase activities and were assumed to transport aspartate instead of malate (Andrews *et al.* 1971). The conclusion that the C_4 acid donating CO_2 may vary in different species received support from the present studies. Chen *et al.* (1971) deduced from earlier findings (Hatch & Slack, 1966) that the gross label in malate declined more rapidly than the label in aspartate during a 'chase' experiment with sugar cane leaves and observed the reverse with bermuda grass. They related these differences to differences in 'malic' enzyme activity and suggested that the labelling behaviour may be indicative of the C_4 acid functioning as the primary source of the C-1 of 3-phosphoglycerate. In the present studies maize, a species with high 'malic' enzyme activity, behaved like sugar cane. In contrast, in *Amaranthus*, a species with low 'malic' enzyme activity, label was lost more rapidly from aspartate, as was the case with bermuda grass. During the present studies the labelling changes in the specific carbon (C-4) involved in this transfer were also examined, thereby eliminating the complicating effect of label entering C-1, C-2 and C-3. Generally speaking, the changes observed in the C-4 label qualitatively followed those observed in the gross label of the C_4 acids in spite of the substantial influence of label entering C-1, C-2 and C-3.

The results discussed above are consistent with the scheme for the C_4-pathway formulated earlier (Andrews *et al.* 1971; Hatch, 1971). Further it is notable that alanine was only slightly labelled in maize but markedly labelled in *Amaranthus*. The labelling of alanine closely resembled that of C-1, C-2 and C-3 of the C_4 acids. According to the scheme, alanine functions as an intermediate in the regeneration of phosphoenolpyruvate, being derived in turn from the C-1, C-2 and C-3 of aspartate. In relation to this scheme it should also be emphasized that at present there is no evidence for the exclusive operation of either malate or aspartate as a CO_2 donor in any species. Enzyme results suggest the predominance of one route or the other in many species (Downton, 1970; Andrews *et al.* 1971; Chen *et al.* 1971). However, malate and aspartate may contribute about equally in *Gomphrena* (Andrews *et al.* 1971). With the species examined during the present studies label was rapidly lost from the C-4 of both malate and aspartate during 'chase' studies. However, the kinetics of loss from the C_4 acid assumed to be in the side pool was quite consistent with label being transferred to the donor C_4 acid via oxaloacetate. It is not possible to say whether all the loss of label from the C_4 acids in the side pool occurred in this manner.

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