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Incorporation of carbon from photosynthetic products into 2-carboxyarabinitol-1-phosphate and 2-carboxyarabinitol

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The synthesis of 2-carboxy-D-arabinitol-1-phosphate (CA1P), the naturally occurring inhibitor of ribulose-1,5-bisphosphate carboxylase/oxygenase, was studied in leaves of the French bean *Phaseolus vulgaris*, L. Leaves were supplied with air containing $^{14}CO_2$ in the light then the plants were transferred to normal air in the light or in the dark. Leaf samples were frozen in liquid nitrogen, ground to a powder and extracted with acid. Lipids, pigments and cations were removed from the extract and CA1P and 2-carboxy-D-arabinitol (CA) recovered by anion exchange chromatography. The CA1P was further purified by its specific binding to purified ribulose-1,5-bisphosphate carboxylase/oxygenase. CA and CA1P were identified by chromatographic properties and n.m.r. spectra. When plants were kept for 15 h in darkness after exposure to $^{14}CO_2$, up to 2.2% and 5.5% of the radioactivity in the extracts was present in CA1P and CA, respectively. The most radioactivity appeared in these compounds when photosynthesis from $^{14}CO_2$ took place at low photosynthetic photon flux density (PPFD). Under such conditions, radioactivity was detected in CA1P after only 10 min. During subsequent exposure to normal air ($^{12}CO_2$) at low PPFD the amount of radioactivity in CA1P remained almost constant for 6 h; in darkness the rate of incorporation of radioactivity into CA1P reached a maximum after 2 h and the radioactivity was still increasing 6 h later. At low PPFD, the amount of CA1P in the leaves reached a maximum after 2 h. In darkness, the amount of CA1P began to increase rapidly after a lag of almost 1 h, well ahead of the increase in radioactivity in CA1P.

INTRODUCTION

2-Carboxy-D-arabinitol-1-phosphate (CA1P) (Gutteridge et al., 1986; Berry et al., 1987) is a naturally occurring tight-binding inhibitor of D-ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39) (Rubisco), the enzyme catalysing the first step in the incorporation of inorganic carbon into sugar derivatives in plants. CA1P regulates the activity of Rubisco when photosynthetic photon flux density (PPFD) is the factor limiting photosynthesis. CA1P was discovered following the observation that in many plant species there is a diurnal variation in the activity of extracted Rubisco (Vu et al., 1983; Servaites et al., 1984). This was because there was a phosphorylated compound of low molecular weight bound to the enzyme extracted from leaves collected at night. The phosphorylated compound could be co-purified with Rubisco provided that CO_2 and Mg^{2+} were present in the solution and that steps such as ion exchange or salting out of protein were avoided. It was dissociated from Rubisco by removing the CO_2 and Mg^{2+}, by adding ammonium sulphate, or by denaturing the protein. Purification from potato leaves (Gutteridge et al., 1986) and French bean leaves (Berry et al., 1987) was followed by characterization as CA1P. Much of the subsequent published research has relied heavily on the extent to which extracted Rubisco is inhibited as an indication of the amounts of CA1P present in the leaves. Thus Kobza and Seemann (1989) have deduced that CA1P is synthesized and broken down in the light.

An isotope dilution method (Moore et al., 1991) showed that CA1P was present in all 10 C_3 plants, and in the two C_4 plants tested. Only relatively small amounts were present in species for which diurnal variation in the activity of extracted Rubisco could not be demonstrated. A phosphatase has been purified from tobacco leaves (Holbrook et al., 1989; Salvucci and Holbrook, 1989) and from French bean leaves (Kingston-Smith et al., 1992) that hydrolyses CA1P to 2-carboxy-D-arabinitol (CA) and is assumed to be a component of the mechanism by which CA1P is removed from leaves as the light intensity increases in the morning. It is likely that another component is an enzyme known as Rubisco activase since this releases CA1P from the catalytic site of Rubisco making it available to the phosphatase (Robinson and Portis, 1988).

CA is present in 121 species of vascular plants examined using isotope dilution and chromatography (Moore et al., 1993). It is the aldonic acid corresponding to the sugar hamamelose. Hamamelose and hamamelitol, the corresponding sugar alcohol, are present in species from 104 families of angiosperms as well as in mosses and gymnosperms (Sellmair et al., 1977). There is some evidence that hamamelose bisphosphate is an early product of carbon assimilation by spinach (Beck et al., 1971) and the labelling of individual carbon atoms is consistent with an origin in an aldolase type of condensation between two triose sugar phosphates, possibly a rearrangement of the carbon chain of fructose bisphosphate (Gilck and Beck, 1974). There has been little progress in establishing the route of biosynthesis of CA1P and no evidence that hamamelose bisphosphate is involved. Moore et al. (1992) showed that CA is mainly in the vacuole at night and in the vacuole and cytoplasm in the day. French bean leaves have converted [^{14}C]CA into CA1P (Moore and Seemann, 1992) but Moore and Seemann (1990) have been unable to demonstrate the incorporation of radioactivity into CA1P from

Abbreviations used: CA, 2-carboxy-D-arabinitol; CA1P, 2-carboxy-D-arabinitol-1-phosphate; PPFD, photosynthetic photon flux density; Rubisco, D-ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39); PAD, pulsed amperometric detector; PEG, polyethylene glycol.

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the products of photosynthesis in the presence of $^{14}CO_2$. This led them to suggest that regulation of Rubisco might depend on a cyclic process of dephosphorylation of CA1P in the morning and rephosphorylation of CA at night. This cycle may well be involved, but the origin of the branched chain of CA and CA1P from known metabolites needs to be explained.

Gutteridge and Parry (S. Gutteridge and M. A. J. Parry, unpublished work) have found that CA1P is considerably labelled with ^{14}C in potato (*Solanum tuberosum*) leaves by photosynthesis from $^{14}CO_2$ followed by darkness for several hours. We describe below, experiments showing that, as with potato, it is relatively easy to demonstrate incorporation of carbon from CO_2 into CA1P and also into CA. Synthesis and turnover of CA1P in dim light is indicated by the results. We consider possible reasons for the failure of Moore and Seemann (1990) to detect synthesis of CA1P from assimilated CO_2.

METHODS

Plant material

All experiments used attached leaves of *Phaseolus vulgaris* L. (cv. Tendergreen) grown under artificial light with a 14 h/20 °C day (310 μmol quanta $\cdot m^{-2} \cdot s^{-1}$) and a 10 h/16 °C night. Plants were used between 14 and 18 days following sowing, inclusive of any pretreatments listed in Tables 1 and 2.

Other materials

Wheat Rubisco was prepared as described by Bird et al. (1982) and the concentration determined from the absorbance at 280 nm (Paulsen and Lane, 1966). CA1P phosphatase was isolated from *P. vulgaris* according to Kingston-Smith et al. (1992). Standard CA1P and ribulose 1,5-bisphosphate were synthesized as described previously (Kingston-Smith et al., 1992). $NaH^{14}CO_3$ was obtained from Amersham. Alkaline phosphatase (bovine intestine) was supplied by Sigma. Dowex resins (AG 50W-X8 and AG 1-X8) were supplied by BioRad. All other chemicals were of analytical grade and used without further purification.

Exposure to $^{14}CO_2$

Air containing 350 μmol/mol $^{14}CO_2$, was generated by passing 2 litres of CO_2-free air through 1 ml of 28 mM $NaH^{14}CO_3$ (4 μCi/μmol) immediately after addition of 1 ml of 1 M H_2SO_4. The gas was dried by passage through anhydrous magnesium perchlorate, and stored in a sealed, double-walled, polythene bag. Plants were placed under a mercury-vapour lamp (200 μmol quanta $\cdot m^{-2} \cdot s^{-1}$) at 22 °C within a fume hood for 30 min. This 'background' level of illumination was maintained throughout the experiment. A Parkinson leaf chamber (Analytical Development, U.K.) was used to enclose 5 cm^2 of primary leaf. Assimilation rates were measured with a model LCA 2 infrared gas analyser with an air supply unit (Analytical Development, U.K.) Assimilation of CO_2 by the enclosed leaf was induced by illumination (800 μmol quanta $\cdot m^{-2} \cdot s^{-1}$) from a quartz-halogen light source through a fibre-optic cable (Hansatech Instruments, U.K.). The temperature of the leaf in the chamber was 25 °C. After 40 min, the rate of CO_2 assimilation from air was maximal, with a CO_2 depletion of 25–35 μmol/mol, at a flow rate of 200 ml/min. The PPFD within the leaf chamber was either maintained at 800 μmol quanta $\cdot m^{-2} \cdot s^{-1}$ or reduced to 60 μmol quanta $\cdot m^{-2} \cdot s^{-1}$, and the gas flow adjusted to give approx. 20 % depletion of CO_2. Air (2 l) containing $^{14}CO_2$ was pumped through the leaf chamber using a diaphragm pump (Charles Austin U.K.) at the required flow rate, measured by a calibrated flow meter (Rotameter Manufacturing, Croydon, U.K.); unassimilated $^{14}CO_2$ was absorbed by a soda-lime tower. When the storage bag was almost exhausted, the mercury-vapour and quartz-halogen lamps were turned off and the leaf chamber removed. The whole plant was then kept in complete darkness for 15 h at 22 °C, after which the treated leaf was rapidly removed, weighed, and then frozen and stored in liquid nitrogen.

For the experiment of Figure 4, several changes to the procedure were made. Induction was by illumination giving 200 μmol quanta $\cdot m^{-2} \cdot s^{-1}$ for 40 min. The PPFD at the surface of the experimental leaf was then decreased to 60 μmol quanta $\cdot m^{-2} \cdot s^{-1}$, and a whole leaf was enclosed by a gas-tight polythene bag, which was collapsed onto the leaf by evacuation and then immediately filled with 750 ml of air containing $^{14}CO_2$ (52 μCi/μmol). Illumination was continued for 10 min, the polythene bag was removed, and the whole plant either removed to total darkness or kept at the same low light intensity. Leaf discs of 5 cm^2 were rapidly removed, frozen and stored in liquid nitrogen, at the indicated times.

Isolation of CA and CA1P from leaf tissue

CA and CA1P were extracted from leaves with an acidic solution to avoid enzymic degradation. Recovery and quantification of the compounds depended heavily on h.p.l.c. Retention times and signal strengths were first determined for each system of h.p.l.c. using authentic CA and CA1P. This information formed the basis for the selection of fractions of column effluent containing the compounds and for estimating the amounts. The purification steps and identification methods are described below.

Extraction of CA1P and CA

Frozen leaves and leaf discs were ground to a fine powder in liquid nitrogen and 3.5 % (v/v) trifluoroacetic acid/0.15 % (w/v) 8-hydroxyquinoline was added to a final ratio of 2 ml/g fresh wt. of whole leaves, or 2.5 ml per 5 cm^2 leaf disc and grinding continued. The 8-hydroxyquinoline prevented adsorption of some phosphate esters to the tissue residue (Isherwood and Barrett, 1967). On thawing, the mixture was kept for 20 min at 0 °C with occasional stirring and then clarified by centrifugation (15800 *g* for 5 min at 4 °C). The clear supernatant was applied to a Bond Elut C_{18} column (Analytichem International) at 1 ml supernatant per 1 ml bed volume and washed through with 1 ml of H_2O. The resulting colourless solution was evaporated to dryness *in vacuo* over NaOH pellets and anhydrous $CaCl_2$. The residue was dissolved in H_2O and passed through a 0.75 cm × 2 cm column of Dowex-50 (H^+). The eluate was collected and dried down as before. Recovery of CA and CA1P at this stage was greater than 90 %.

Preparative h.p.l.c.

The residue from the above procedure was redissolved in 1 ml of H_2O. Aliquots of 0.1 ml were diluted 10-fold in 50 mM NaOH and loaded immediately onto a CarboPac PA1 column (4 mm × 250 mm) (Dionex U.K.) attached to a Bio-LC chromatography system (Dionex U.K.), including a pulsed amperometric detector (PAD), with post-column addition of 0.2 M NaOH (1 ml/min). The column was pre-equilibrated with 32 mM sodium acetate and CA was eluted isocratically at this acetate concentration (flow rate 1 ml/min; retention time 10 min). A 32–600 mM sodium acetate gradient eluted CA1P. CA- and CA1P-containing eluate was collected in 0.4 ml fractions. Recovery of CA1P following this treatment was typically 80 %. Fractions containing CA or CA1P were neutralized by addition of 2 equiv. of Bicine (acid) and the solution frozen at −20 °C.

Table 1 Effect of conditions before and during photosynthesis on incorporation of ^{14}C from $^{14}CO_2$ into CA1P

Seedlings, 7 (expts. 1–4) or 9 days (expt. 5) after sowing, were subjected to the sequence of treatments shown under the various experiments. Initially the plants were kept in the glasshouse day/night regime (310/0 PPFD*), or in continuous light of the intensity shown (310 or 70 PPFD*), or in darkness. In experiment 5, after 6 days in darkness the plant was illuminated for 600 min at 200 μmol quanta $\cdot m^{-2} \cdot s^{-1}$ followed by 300 min in the dark before a satisfactory rate of photosynthesis could be induced.

		Experiment number				
		1	2	3	4	5
Plant growth conditions	PPFD*	310/0	310	310	70	0
	Time (days)	8	8	8	8	6
Induction period (before exposure to $^{14}CO_2$)	PPFD*	800	800	800	800	800
	Time (min)	40	40	40	40	40
During exposure to $^{14}CO_2$	PPFD*	800	800	60	800	800
	Time (min)	20	20	50	30	40
After exposure to $^{14}CO_2$	PPFD*	0	0	0	0	0
	Time (h)	15	15	15	15	15
Analyses						
Total d.p.m. extracted ($\times 10^{-6}$)		3.90	6.26	3.50	6.94	5.48
D.p.m. in CA1P: (i) total ($\times 10^{-3}$)		4.7	3.1	20	2.8	9.3
(ii) % of total		0.12	0.05	0.57	0.04	0.17
CA1P content (nmol/g fresh wt.)		108	120	84	54	72

* μmol quanta $\cdot m^{-2} \cdot s^{-1}$.

Table 2 Effect of conditions before and during photosynthesis on incorporation of ^{14}C from $^{14}CO_2$ into CA1P and CA

Seedlings were taken 8 days after sowing and given the sequence of treatments shown in different experiments. The plant of experiment 6, kept for 7 days in darkness, required the additional treatment (see Table 1) before a satisfactory rate of photosynthesis could be induced. In expt. 3, 4 l of air containing $^{14}CO_2$ was used to allow a longer exposure to $^{14}CO_2$.

		Experiment number					
		1	2	3	4	5	6
Plant growth conditions	PPFD*	310/0	310/0	310/0	310/0	0	0
	Time (days)	7	7	7	7	3	7
Induction period (before exposure to $^{14}CO_2$)	PPFD*	800	800	800	800	800	800
	Time (min)	40	40	40	40	40	40
During exposure to $^{14}CO_2$	PPFD*	800	60	60	60	800	800
	Time (min)	15	130	75	75	40	35
After exposure to $^{14}CO_2$	PPFD*	0	0	0	0	0	0
	Time (h)	15	15	15	15	15	25
Analyses							
Total d.p.m. extracted ($\times 10^{-6}$)		3.40	5.09	1.79	2.00	n.d.	n.d.
D.p.m. in CA: (i) total ($\times 10^{-3}$)		126	n.d.	116	142	114	57
(ii) % of total		3.7	n.d.	6.5	7.1	n.d.	n.d.
D.p.m. in CA1P: (i) total ($\times 10^{-3}$)		2.4	76	39	34	8.6	4.6
(ii) % of total		0.07	1.5	2.2	1.7	n.d.	n.d.
CA content (nmol/g fresh wt.)		790	n.d.	810	970	421	451
CA1P content (nmol/g fresh wt.)		90	116	101	105	95	70

* μmol quanta $\cdot m^{-2} \cdot s^{-1}$. n.d., not determined.

CA purification

Bicine in the CA-containing fractions was removed by Dowex-50 (H^+) and the effluent applied to the CarboPac column pre-equilibrated with 0.1 M NaOH containing 0.1 M sodium acetate, at a flow rate of 1.0 ml/min and eluted isocratically with the same eluent. Column effluent was monitored by using both the PAD cell and a radioactivity monitor (Berthold LB507A, equipped with a YG150 solid scintillant flow-cell). Post-column addition of disodium dihydrogen pyrophosphate immediately before the radioactivity monitor, prevented sample adsorption to the solid scintillant. The radioactivity in CA was calculated from the integrated signal.

CA1P purification

CA1P was further purified by binding it to ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39; Rubisco). Wheat Rubisco was activated in 0.1 M Tris/HCl (pH 8.2)/ 20 mM $MgCl_2$/10 mM $NaHCO_3$, by incubation for 40 min at 40 °C (Parry et al., 1987). This was added to a neutral solution containing the CA1P from h.p.l.c. with 20 mM $MgCl_2$ and 10 mM $NaHCO_3$. A 7.5-fold (Tables 1 and 2) or a 5-fold (Figure 4) excess of Rubisco catalytic sites was used. After 20 min at 22 °C, 60% (v/v) polyethylene glycol (PEG 6000) in 20 mM $MgCl_2$ was added to give a final concentration of 20% (v/v) PEG. The mixture was incubated for 20 min at 0 °C. Precipitated

Rubisco was sedimented by centrifugation (27000 *g*, 15 min, 4 °C). The supernatant was discarded and the pellet resuspended in half the original volume of 20% PEG in 20 mM $MgCl_2$/10 mM $NaHCO_3$/0.1 M Tris/HCl (pH 8.2). Following resedimentation, the pellet was drained thoroughly, and redissolved in H_2O. The protein was then denatured and CA1P released without detectable decomposition, by incubating at 100 °C for 4 min. Precipitated protein was sedimented and the clear supernatant frozen at −20 °C. Recovery from this step was approximately 76%.

Other methods

CA1P concentration was confirmed by the extent to which samples inhibited the carboxylase activity of Rubisco (Gutteridge et al., 1986). Proton (^1H) NMR spectra were obtained in D_2O using 3-trimethylsilyloxypropane sulphonic acid (sodium salt) as internal standard ($\delta = 0.0$) using a JEOL GNX 400 spectrometer (with fourier transform facility). To ensure that CA1P and CA were in the open-chain form, a 5-fold excess of NaOD was included in each sample. Some of the [^{14}C]CA1P sample subjected to n.m.r. analysis was subsequently treated with phosphatase (Figure 3).

RESULTS AND DISCUSSION

Gutteridge et al. (1986) extracted and purified CA1P from leaves in a buffered solution in which it was bound to Rubisco. In the present study, total CA1P was extracted in a strongly acidic solution. This was important because: (i) CA1P may not be synthesized in the chloroplast and hence may not all be bound to Rubisco *in vivo*, and (ii) after prolonged darkness, French bean leaves contain CA1P in excess of the number of catalytic sites on Rubisco so it cannot all be bound (Moore et al., 1991). Extraction with acid also recovers CA, allowing this also to be quantified. A simultaneous purification of CA and CA1P from acid extracts by h.p.l.c. is illustrated in Figure 1. The PAD signal provided an estimate of the CA1P concentration that was in close agreement with parallel estimations based on Rubisco inhibition. However, not all of the ^{14}C was associated with CA1P, so further purification was needed, as described above.

We first used ^{14}C to investigate conditions giving maximum incorporation of products of CO_2 assimilation into CA1P. Exposure to $^{14}CO_2$ was followed by a prolonged dark period,

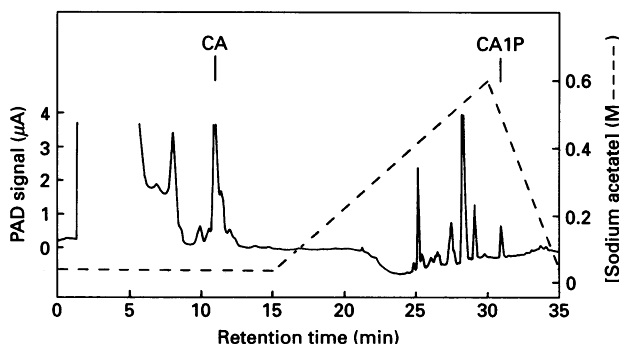

Figure 1 CA and CA1P from a leaf separated by anion exchange h.p.l.c.

An acid extract of a leaf that had been in darkness for 15 h was purified by passage through C_{18} Bond Elut and Dowex-50 (H^+) columns and analysed on a CarboPac PA1 (Dionex U.K.) column with sodium acetate, pH 7.5, as eluent. The PAD signal had been corrected for baseline drift.

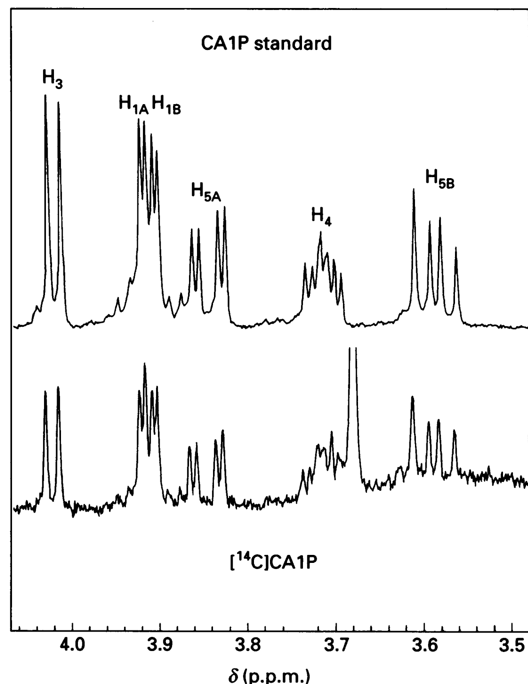

Figure 2 ^1H-n.m.r. analysis of [^{14}C]CA1P extracted from leaves of *P. vulgaris*

Upper trace: the spectrum of synthetic CA1P. CA1P (1.8 μmol) was dissolved in 0.55 ml of D_2O and subjected to 1000 scans. Lower trace: the spectrum of [^{14}C]CA1P from a leaf exposed to $^{14}CO_2$. Extracted CA1P (150 nmol) was dissolved in 0.55 ml of D_2O and subjected to 2000 scans. Excess of NaOD was added to ensure that CA1P was in the open chain form. The additional (degenerate) peak at 3.69 ppm was due to PEG, introduced during affinity purification using Rubisco. The assignment of chemical shifts to protons on specific carbon atoms (numbered 1–5) is based on the work of Gutteridge et al. (1986).

because most of the existing evidence showed high concentrations of CA1P in leaves after prolonged darkness. Of the different treatments shown in Table 1, experiments 3 and 5 produced more ^{14}C-labelled CA1P. The important differences between these and the other three experiments in Table 1, were low irradiance during the supply of $^{14}CO_2$ in experiment 3 and prolonged starvation by darkness before exposure to $^{14}CO_2$ in experiment 5. Since the CA1P in experiment 3 had a much higher incorporation of ^{14}C, as a proportion of the total d.p.m. extracted, than the other four experiments in this Table, we concluded that irradiance during exposure to $^{14}CO_2$ was the most important factor governing the flux of ^{14}C into CA1P.

Table 2 shows more evidence of the effects of photosynthesis in $^{14}CO_2$ at low PPFD. Further tests of the effect of a prolonged dark period have also been included. The extent of ^{14}C incorporation and the total amounts of both CA1P and CA, were measured in these experiments. Fourteen to 32 times more ^{14}C (between 1.5 and 2.2% of the total ^{14}C extracted) was found in CA1P when $^{14}CO_2$ was supplied at low rather than at high PPFD. Doubling the time of exposure to $^{14}CO_2$ (Table 2, compare experiment 2 with experiments 3 and 4) doubled the absolute amount of ^{14}C in CA1P, but did not significantly affect the proportion of the total ^{14}C in CA1P. Approx. 7% of the total radioactivity extracted co-purified with CA after exposure to $^{14}CO_2$ at low irradiance (experiments 3 and 4) compared with about 4% of the total after exposure at high PPFD (experiment 1). Thus, the effect of PPFD during exposure to $^{14}CO_2$ was somewhat smaller on incorporation of ^{14}C into CA than into

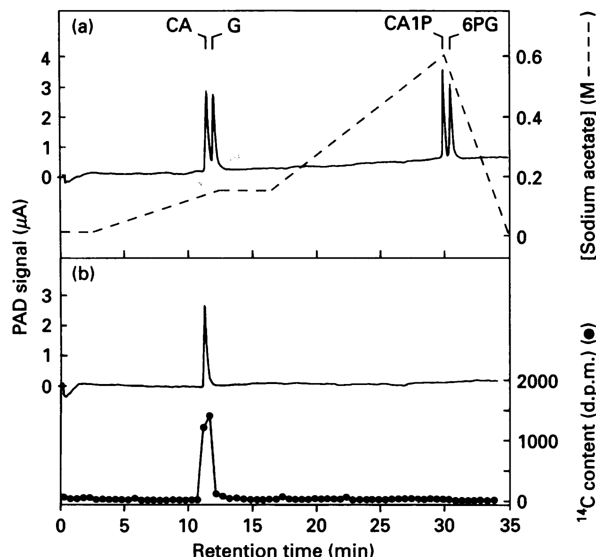

Figure 3 Resolving power of the CarboPac PA1 column and hydrolysis of CA1P to CA to confirm the presence of ^{14}C

(a) Resolution of CA1P and CA from isomeric compounds. Separation of 20 nmol each of CA, 6-phosphogluconate (6PG) and CA1P, and 12 nmol of gluconate (G). The broken line shows the concentration of sodium acetate. (b) A sample containing 20 nmol of CA1P and 3500 d.p.m. ^{14}C, purified from a leaf of *P. vulgaris* after photosynthesis from $^{14}CO_2$, was incubated in 0.2 ml of H_2O containing 12 units of alkaline phosphatase for 75 min at 22 °C. The products were separated as in (a). Fractions were collected at intervals of 0.5 min, and the ^{14}C content was measured using a liquid scintillation spectrometer. The signal from the PAD was corrected for baseline drift.

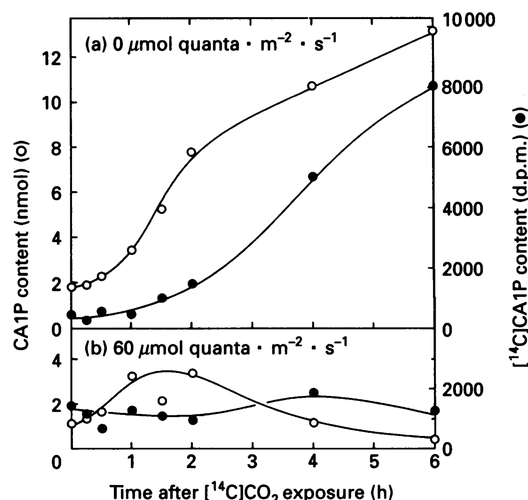

Figure 4 Amount and ^{14}C content of CA1P in French bean leaf in dim light or darkness following exposure to $^{14}CO_2$ at low irradiance

Photosynthesis was induced by illumination for 40 min at 200 μmol quanta·m^{-2}·s^{-1}, before exposure to $^{14}CO_2$ for 10 min at 60 μmol quanta·m^{-2}·s^{-1}. After exposure to $^{14}CO_2$, plants were kept in normal air either in darkness (a) or at 60 μmol quanta·m^{-2}·s^{-1} (b). At the times indicated, 5 cm^2 discs were cut from the leaves and frozen in liquid nitrogen. CA1P was acid-extracted and purified by absorption to Rubisco, as described in the Methods section. The concentration and the radioactivity of CA1P in the 5 cm^2 discs were determined from the integrated signals from the PAD and radiometric detectors, respectively.

CA1P. This suggested that there was a constant flow of carbon from pools of recent assimilate to CA regardless of light conditions during assimilation, but that these conditions did affect the source of carbon for CA1P synthesis. In every experiment, more radioactivity appeared in CA than in CA1P. The total amount of CA exceeded that of CA1P by 5- to 10-fold. In all experiments the amounts of CA1P after 15 h of darkness were similar (Table 2) so it was the relative contributions of new and pre-existing precursors to the synthesis of CA1P that was sensitive to the conditions during exposure to $^{14}CO_2$. Plants kept under the normal glasshouse growth conditions prior to experiments contained about 900 nmol of CA and 100 nmol of CA1P/g fresh wt. after the 15 h of darkness. These amounts were similar to those measured by isotope dilution (Moore and Seemann, 1990; Moore et al., 1992) of 939 and 100 nmol/g fresh wt. for CA and CA1P, respectively. The amounts of CA1P in the leaves cannot be determined by the relative activities of CA1P phosphatase and CA kinase alone, as proposed by Moore and Seemann (1992), because new carbon was being incorporated into both compounds.

The proton NMR spectrum obtained with [^{14}C]CA1P isolated from *P. vulgaris* (Figure 2, lower trace) was identical to that obtained for standard CA1P (Figure 2, upper trace), except for the presence of a signal at 3.69 ppm caused by traces of PEG introduced during the affinity purification by absorption on Rubisco. Outside the spectral range shown in Figure 2 the only resonances were those due to residual ^1H_2O. The extracted CA1P had, therefore, been separated from other leaf components. Moreover, ^{14}C was proved to be in CA1P since treatment with a phosphatase, followed by h.p.l.c., showed that the radioactivity was then entirely associated with CA (Figure 3b). Although alkaline phosphatase was used to produce the result in Figure 3, a similar result was obtained when the specific CA1P phosphatase was used. The h.p.l.c. system employed resolved closely related compounds, e.g. 6-phosphogluconate and gluconate, from CA1P and CA, respectively (Figure 3a). The n.m.r. spectrum, the conversion of [^{14}C]CA1P to [^{14}C]CA, and the evidence that the same ^{14}C-labelled compound binds to Rubisco, confirmed that ^{14}C was incorporated into CA1P. N.m.r. spectroscopy of purified CA from leaves (results not shown) was consistent with CA being the major component.

Thus CA1P synthesis accounted for a significant proportion of recently assimilated carbon; this conclusion was contrary to that of Moore and Seemann (1990). They used a different cultivar of *P. vulgaris* (cv Linden) but CA1P-dependent inhibition of Rubisco is conserved among cultivars and species of *Phaseolus* (Sage, 1993). Another difference was the much higher daytime PPFD during the growth of the plants used by Moore and Seemann (1990). Whether this would increase the proportion of CA1P synthesized from unlabelled precursors such as CA is unknown. No absolute measures of ^{14}C in their leaf tissue were reported, but only the percentages of the total assimilated found in sucrose and sugar phosphates. We suspect that Moore and Seeman (1990) would have been limited by the amount of extract that could be loaded on to t.l.c./thin-layer electrophoresis plates without adverse effects on resolution.

Tables 1 and 2 provide evidence that photosynthesis at low PPFD provides a precursor of CA1P and CA but do not show whether CA1P was synthesized in the period of low PPFD or in the subsequent long dark period. A pulse-chase experiment was therefore performed, in which a short period of photosynthesis with $^{14}CO_2$ and low PPFD was followed by a chase with $^{12}CO_2$ either in darkness or in continuing low light. The results show (Figure 4) that CA1P was radioactive after only 10 min of exposure to $^{14}CO_2$ at low PPFD but became much more

radioactive in the 6 h of darkness. Immediately after exposure to $^{14}CO_2$ at low PPFD, detectable amounts of CA1P were present (1.1–1.8 nmol/cm^2). The subsequent increase in CA1P content with time was sigmoidal. These changes in CA1P were consistent with changes observed in apparent $k_{cat.}$ of Rubisco in extracts from leaves of *P. vulgaris* following transfer from light to darkness (Sage et al., 1993). After a 30 min lag, synthesis of CA1P increased considerably (Figures 4a and 4b), the highest rate being between 1 and 2 h after exposure to $^{14}CO_2$. However, the highest rates of incorporation of ^{14}C into CA1P were recorded later (Figure 4a). This indicated the presence of a pre-existing pool of non-radioactive precursors which were used for CA1P synthesis initially. One likely precursor is CA, which is thought to be reversibly phosphorylated, in a light-sensitive manner.

Moore and Seemann (1992) administered [^{14}C]CA to detached leaves of *P. vulgaris* and measured the [^{14}C]CA1P formed in an ensuing dark period. The appearance of [^{14}C]CA1P proceeded at the same rate as that of [^{12}C]CA1P. Thus phosphorylation of CA was unlikely to be the cause of the lag in [^{14}C]CA1P synthesis observed in Figure 4(b). Presumably, synthesis of a precursor other than CA would be rate limiting.

The rates of change of [^{14}C]CA1P and total CA1P in both time courses (Figures 4a and 4b) indicate that the specific radioactivity of CA1P falls over the first 2 h of the chase period in $^{12}CO_2$ followed by a sustained increase over the next 4 h. Without further experiments of this type the conclusions must be tentative but the results suggest that two sources of assimilated carbon give rise to CA1P, one which is exhausted of ^{14}C soon after photosynthesis from $^{14}CO_2$, the other being more remote is mobilized slowly in the early period of darkness. More work is needed to identify these sources of carbon. The former may be an intermediate of the Calvin cycle and the latter a more stable product of assimilation which is more abundant as a product of photosynthesis at low irradiance. The amount of ^{14}C in CA1P after 6 h in low PPFD was much the same as it was at the end of the pulse of $^{14}CO_2$. The fall in total CA1P after 2 to 6 h incubation at low irradiance suggests an adaptation to low light.

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