Conversion of d-Hamamelose into 2-Carboxy-D-arabinitol and 2-Carboxy-D-arabinitol 1-Phosphate in Leaves of Phaseolus vulgaris L.*

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[1-14C]Hamamelose (2-hydroxymethyl-D-ribose) was synthesized by reaction of ribulose 5-phosphate with potassium [14C]cyanide, catalytic hydrogenation of the resulting cyanohydrin, and dephosphorylation of the product. Its identity was established by a chromatographic comparison with hamamelose isolated from the bark of witch hazel (Hamamelis virginiana L.). Following vacuum infiltration of the [1-14C]hamamelose into leaf discs from Phaseolus vulgaris L., 14C-labeled 2-carboxy-D-arabinitol 1-phosphate (CA1P) were formed, in the dark. Conversion of hamamelose to both CA and CA1P in the leaf discs was inhibited by dithiothretol and sodium fluoride, although at high concentrations of these inhibitors conversion into CA was still evident when conversion into CA1P was totally inhibited. Wheat (Triticum aestivum L.) leaves converted hamamelose into CA without formation of CA1P. Leaves from P. vulgaris contained 68 nmol g⁻¹ fresh weight of hamamelose in the light and 35 nmol g⁻¹ fresh weight in the dark. A pathway for the biosynthesis of CA1P from Calvin cycle intermediates is proposed which includes the sequence: hamamelose → CA → CA1P.

The activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco), in many but not all plant species, is modulated by the transition state analogue, 2-carboxy-D-arabinitol 1-phosphate (CA1P) in response to decreased light intensity (1–3). CA1P, synthesized in chloroplasts during darkness or low irradiance, is a potent tight binding inhibitor of carbamylated Rubisco. CA1P is a branched chain, phosphorylated sugar acid (1, 4) also known as hamamelonic acid 21-phosphate (5), whose parent sugar is commonly known as hamamelose. At dawn, CA1P is released from the catalytic site of Rubisco by Rubisco activase (6) and rendered noninhibitory by a specific CA1P phosphatase, which converts it to 2-carboxy-D-arabinitol (CA) and inorganic phosphate (7–9). CA occurs in many plants and is not confined to the chloroplast (10). CA, administered to leaves through the petiole, is converted to CA1P in a subsequent period of darkness (11). However, since CA is present in species that make little CA1P, such as wheat (Triticum aestivum L.) (10, 12), it may serve other purposes, apart from its role as a precursor of CA1P.

The ready conversion of CA into CA1P in the leaf tissue of some plants, taken together with the identification of CA1P phosphatase, led to the conclusion that CA1P and CA participate in a metabolic substrate cycle in vivo, remote from mainstream metabolism (11). However, Andralojc et al. (13) demonstrated that up to 8% of recently assimilated carbon could be incorporated into CA1P and CA. This indicates a considerable flux of new carbon from Calvin cycle intermediates into CA and CA1P. Of the Calvin cycle intermediates, the most obvious potential precursor is fructose 1,6-bisphosphate (FBP) which undergoes intramolecular rearrangement in vivo and in vitro to form hamamelose 21,5-bisphosphate (HBP) (14). Consistent with this suggestion is the increased incorporation of newly assimilated radiolabel into CA1P when assimilation takes place at low irradiance (13); conditions under which FBP concentrations would be relatively high (15). In leaves of an alpine primrose (Primula clusiana Tausch), HBP is metabolized to hamamelose 21-phosphate, hamamelose 5-phosphate and hamamelose (14, 16). Hamamelose (2-hydroxymethyl-D-ribose) is very widely distributed among plant species (17).

In this study, radiolabeled hamamelose was prepared in order to explore its metabolism in leaves. It was specifically metabolized to CA in the light and to CA and CA1P in the dark. These results therefore provide a link, through hamamelose, between CA1P (or CA) and reports of the synthesis of HBP from FBP.

MATERIALS AND METHODS

Purification of Hamamelose—Hamamelitannin (2 molecules of gallic acid esterified to hamamelose) (18) was isolated from the bark of witch hazel (Hamamelis virginiana) according to the method of Glick et al. (19). The tannin was hydrolyzed using tannase (ICN Biomedicals Ltd., UK), and the hydrolysate was treated with Dowex-1 carbonate. Such treatment removed the gallic acid and further decolorized the sample. Approximately 800 mg of the liberated sugar (dissolved in 40 ml of H2O) were resolved chromatographically using a (60 g/60 g) charcoal/Celite column (46 cm × 2.5-cm diameter), eluted with a gradient of 0–10% (v/v) ethanol in water at 0.7 ml/min⁻¹, in 24 h, at room temperature. Approximately 300 mg of pure hamamelose were recovered from the effluent collected between 11 and 14 h. The 1H and 13C NMR spectra obtained for this purified product (in D2O) were diagnostic of hamamelose (20).

Synthesis of [14C]Hamamelose—We adapted the methods of McFadden et al. (21) and Serianni et al. (22). 0.2 ml of 80 mCi K14CN (58

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 Biosynthesis of 2-Carboxyarabinitol 1-Phosphate

McCormick et al. (1) used a Bio-LC chromatography system (Dionex, UK) with a pulse amperometric detector (PAD; Dionex, UK) or a radioisotope detector (RID; Berthold LB507A fitted with solid scintillant flow cell YG 150; EG & G Berthold, UK). The absolute radioactivity (disintegrations/min) of resolved solutes was determined by subjecting the fractionated eluate to liquid scintillation counting, in Ultima Gold scintillation mixture (Canberra Packard, UK). Anion exchange HPLC utilized a Carbopac PAI column (4 mm x 250 mm; Dionex, UK) with a flow rate of 1 ml/min, equilibrated, and developed as follows. (i) Isoacrylic elution using 0.04 M NaOH. Sample (in H$_2$O) was applied through a 0.05-ml injection loop, and the column was regenerated with 0.20 M NaOH (10 min) then 0.04 M NaOH (10 min) (see Fig. 1). (ii) Gradient elution with 0.05–1.00 M sodium acetate, pH 7.0 (5–15 min), 1.00 M sodium acetate, pH 7.0 (15–20 min). The sample (in H$_2$O) was carefully neutralized with NaOH and applied through a 1.0-ml injection loop. The column was regenerated with 0.05 M sodium acetate, pH 7.0. (iii) Isocratic elution using 0.05 M sodium acetate, pH 6.0. Samples (in H$_2$O) were applied through a 1.0-ml injection loop. After sample elution, no further regeneration needed (see Fig. 4). (iv) Gradient elution with 0.15 M NaOH (0–2 min), 0.15–0.30 M NaOH (2–12 min), 0.30 M NaOH (12 min-end). Samples (in 0.01 M NaOH, pH 10) were applied through a 0.1-ml injection loop. The column was regenerated for 20 min with 0.15 M NaOH (see Fig. 5). (v) Gradient elution with 0.10–1.0 M sodium acetate, pH 7.0 (0–30 min), a sample was applied through a 1.0-ml injection loop. The column was regenerated for 10 min with 0.10 M sodium acetate. (These conditions were used in the separation of products during $^{14}$C-hamamelose synthesis.)

Ion-moderated partitioning (IMP) HPLC utilized a Bio-Rad Aminex HTP 200 anion column, equilibrated and developed with 5 mM H$_2$SO$_4$ at a flow rate of 0.4 ml/min. Samples (in H$_2$O, unless stated otherwise) were applied through a 0.05-ml injection loop (Figs. 2 and 6).

In spite of differences in sample size and detector, HPLC elution profiles obtained using the same column chemistry were displayed with common axes, by expressing the detector signal as a percentage of the highest signal in the same chromatogram.

**Radioisotope Dilution Assay for Hamamelose**—A known weight (between 0.90 and 1.50 g, fw) of leaf material from *P. vulgaris* was extracted as described above in 2 ml of 3.5% (v/v) trifluoroacetic acid, containing 10.5 mmol of radiolabeled hamamelose (2.8 mCi mmol$^{-1}$). Hamamelose was purified from these extracts and the endogenous hamamelose deduced from the dilution of radiolabel, as evidenced by the presence of a PFD/RED/14C peak with a similar retention time to that described for the quantitation of CA1P (12).

**Lactonization of Resolved Extracts**—The peak of radioactivity with an identical retention time to CA, derived from leaf material infiltrated with radiolabeled hamamelose (Fig. 3, track B), was collected and treated with an excess of Dowex 50-H$^+$. Hydrochloric acid was then added, to a final concentration of 10 mM (pH = 3), and the sample was evaporated in vacuo over NaOH pellets and anhydrous CaCl$_2$. Just before subsequent HPLC analyses, the samples were rehydrated in water (Fig. 4) or 5 mM H$_2$SO$_4$ (Fig. 6).

**Phosphatase Treatment of Resolved Components**—The peak of radioactivity with an identical retention time to CA1P, derived from leaf material infiltrated with radiolabeled hamamelose (Fig. 3, track B), was treated with Dowex 50-H$^+$. Hydrochloric acid was then added, to a final concentration of 10 mM (pH = 3), and the sample was evaporated in vacuo over NaOH pellets and anhydrous CaCl$_2$. The sample was dissolved in 0.5 ml of 10 mM TEA. Two units of alkaline phosphatase (bovine intestine, Sigma, UK) were added, and the mixture was kept at 25°C for 2 h, after which it was mixed with 0.02 ml of 0.2 M NaOH (Fig. 5). The resulting solution was subjected to HPLC (Fig. 5).

**Rubisco Inhibition Assay**—Acid-stable neutral plus anionic metabolites, derived from hamamelose-infiltrated leaf material, were resolved by anion exchange HPLC (Fig. 3) and collected in a series of 0.5-ml fractions as they emerged from the column. Aliquots (35 ml) were incubated for 5 min in 0.5 ml of 100 mM Bicine, pH 8.2 (NaOH), 20 mM MgCl$_2$, 10 mM NaHCO$_3$, and 10 mg of Rubisco (previously carbamylation by exposure to the same concentrations of Bicine, MgCl$_2$, and NaHCO$_3$, for 40 min at 37°C). Rubisco activity was then determined, following addition of 0.5 ml of 100 mM Bicine, pH 8.2 (NaOH), 20 ml MgCl$_2$, 10 mM NaHCO$_3$ (0.5 mCi mmol$^{-1}$), and 0.66 mM ribulose 1,5-bisphosphate. The assay was stopped with 0.1 ml of 10 mM formic acid after 5 min. The samples were oven-dried, and the acid-stable 14C was determined by liquid scintillation counting.

**RESULTS**

**Purified and Chemically Synthesized $^{14}$C-Labeled Hamamelose**—Authentic hamamelose, from hamamelitannin, was resolved from common sugars by two distinct HPLC procedures. Thus, using anion exchange HPLC with 40 mM NaOH as eluent, hamamelose (retention time 16.0 min; Fig. 4, track C, peak 5) was fully resolved from galactose, glucose, fructose, and sucrose. It was also resolved from glucose, sucrose, and ribose.
by means of IMP HPLC, using 5 mM H₃SO₄ as eluent (retention time 15.5 min; Fig. 2, track A). These two HPLC procedures exploit different properties of hamamelose, and so their combined use provides a powerful tool in the separation of this sugar from complex mixtures (see below).

Reduction of the products of the reaction of K¹⁴CN and D-ribulose 5-phosphate (see "Materials and Methods") yielded two components that were resolved by anion exchange HPLC, with retention times similar to sugar monophosphates and each accounting for 20–25% of the detected radioactivity. Radioactivity was also incorporated into an unresolved mixture of the 5-phosphates of 2-carboxyarabinitol and 2-carboxyribitol (30–40% of radiolabel) and an unidentified neutral component (10–15% of label). The sugar constituent of only one of the sugar phosphate peaks had identical chromatographic properties to hamamelose, as evidenced by HPLC (Figs. 1, track B, and 2, track D) and also by TLC (not shown) and so was identified as 2-C-(hydroxymethyl)-D-ribose 5-phosphate (hamamelose 5-phosphate). The sugar liberated by phosphatase treatment of this compound was therefore [¹⁴C]hamamelose (using nomenclature adopted by Beck et al. (5)) and was used in the following experiments.

Metabolism of [¹⁴C]Hamamelose in Leaf Discs—Leaf discs were vacuum-infiltrated with solutions of the [¹⁴C]hamamelose, illuminated (PFD 200 µmol m⁻² s⁻¹) for 2 h, then placed in total darkness for 6 h. Analysis of the acid extract by anion-exchange HPLC at neutral pH revealed three radiolabeled peaks (Fig. 3, track B). The first radiolabeled compound to emerge from the column was neutral, having the same retention time as hamamelose (approximately 2.8 min; peak 1). This was followed by a weakly anionic component (peak 2), then a strongly anionic component (peak 3), and these had retention times identical to CA (6.2 min) and CA1P (15.7 min), respectively. Each of these peaks was subjected to further analysis to confirm their identities.

The Neutral Component Is Hamamelose—Material from the neutral peak (Fig. 3, track B) was shown exclusively to be hamamelose, by means of IMP (Fig. 2, track C). This demonstrates that the hamamelose administered had not been metabolized to any other neutral component in the course of the experiment.

The Weakly Anionic Component Is CA—When the component with the same retention time as CA (Fig. 3, track B, peak 2) was dehydrated in acid and then rehydrated immediately before anion exchange HPLC (at pH 6.0) it had a shorter retention time, consistent with a neutral compound. This is illustrated in Fig. 4, which shows the elution profiles of [¹⁴C]CA standards before (track A) and after (track B) such treatment, as well as of the treated product of [¹⁴C]hamamelose metabolism (track C). This behavior is consistent with the formation of a lactone (which is uncharged) and would be expected if the compound were CA.

Anion exchange HPLC at pH > 12 (i.e. at or above the pK₅ of sugar hydroxyl groups) (23), using sodium hydroxide as eluent, gives baseline resolution of CA from other weakly anionic components (Fig. 5, track A), including gluconic acid (GA), which can also form a lactone. Under these conditions, the weakly anionic compound derived from [¹⁴C]hamamelose (Fig. 5, track C) runs as a single peak with the same retention time as the [¹⁴C]CA standard (Fig. 5, track B).

Finally, both authentic CA and the weakly anionic product of hamamelose metabolism were dehydrated/rehydrated in dilute mineral acid (to ensure complete lactonization) prior to IMP.
Again, the compound in question yielded a single peak (Fig. 6, track C) with the same elution profile as the CA (lactone) control (Fig. 6, track B). Furthermore, 96% of the applied radiolabel was recovered in each of the peaks of tracks B and C. We concluded that this compound was exclusively CA.

The Strongly Anionic Component Is CA1P—The component with the same retention time as CA1P (Fig. 3, track B, peak 2) was collected as it emerged from the column, in a series of fractions, collected at 0.5-min intervals. Analysis of these fractions showed that radioactivity and ability to inhibit Rubisco coincided in three separate experiments (Fig. 7, A, B, and C), suggesting that radiolabeled CA1P had been formed. The amounts of CA1P present (60–80 nmol g⁻¹, fw) were estimated by reference to a standard CA1P-inhibition curve, constructed contemporaneously, and are similar to published values (12, 13).

Phosphatase treatment of this compound, followed by anion exchange HPLC, yielded a single peak of radioactivity (Fig. 5, track E) with a retention time identical to that of an authentic sample of CA1P treated in the same way (track D), and of authentic CA (track B). Additionally, 97% of the radiolabel associated with the strongly anionic component was accounted for beneath the peak of track E (Fig. 5), following phosphatase treatment. This confirms the identity of the strongly anionic peak as exclusively CA1P.

Hamamelose Content of P. vulgaris Leaves—The conversion of infiltrated hamamelose exclusively into CA and CA1P by leaves of P. vulgaris strongly suggests that hamamelose is a precursor of these compounds in vivo, for which it must be present in the leaves of P. vulgaris. A combination of anion-exchange and IMP HPLC of the acid-stable, neutral compounds extracted from leaf material demonstrated the presence of hamamelose and allowed an estimate of its amount. An acid extract of leaves was treated with Dowex 50-H⁻ and then neutralized using excess Dowex 1 carbonate, prior to HPLC. The supernatant was first analyzed by anion exchange HPLC at high pH, yielding several components with short retention times, followed by three large peaks corresponding to glucose, sucrose, and fructose. Finally, a single, small peak emerged with a retention time identical to that of hamamelose (Fig. 1, track A). This peak was collected and treated immediately with excess Dowex 50-H⁺ to remove Na⁺ ions and acidify. This was further resolved by IMP HPLC, revealing 2 major peaks (Fig. 2, track B). The first to emerge had a retention time similar to sucrose (track A), while the second, larger, peak had a retention time identical to that of hamamelose and was symmetrical, indicating base line resolution from other components. We concluded that this (latter) peak was hamamelose.

A radiotope dilution assay was conducted to measure the hamamelose content of leaves. This involved addition of ¹⁴C-labeled hamamelose of known specific radioactivity to the leaf material at the (initial) acid extraction, followed by isolation of...
the hamamelose in the extract by the two sequential HPLC fractionations. The specific radioactivity of the purified hamamelose was measured and used to determine the endogenous hamamelose. Table I shows that the hamamelose content of *P. vulgaris* was between 35 and 72 nmol g⁻¹, fw. Consistent with its proposed role as a precursor of CA1P (which accumulates in the dark) the hamamelose content of dark adapted leaves was 50% lower than that of light adapted leaves (treatment 3). Transfer from normal to low irradiance (treatment 2) did not significantly alter the hamamelose content (Table I).

**Reaction Sequence for Hamamelose Conversion into CA and CA1P**—We have shown that hamamelose is specifically converted into both CA and CA1P in leaf discs of *P. vulgaris*. Similarly, [¹⁴C]CA administered to leaf discs by vacuum infiltration is also converted into [¹⁴C]CA1P, in a subsequent period of darkness (Fig. 8). Since no other radiolabeled compounds were detected following infiltration of [¹⁴C]CA (not shown), we can conclude that CA is converted directly into CA1P in infiltrated leaf discs.

One approach to determining whether the conversion of hamamelose into CA and CA1P is sequential, and to establish a likely order of synthesis, is to find out if either CA or CA1P can be derived from hamamelose without the accompanying synthesis of either CA1P or CA, respectively. Moore and See-mann (10) reported that wheat contains substantial levels of CA1P (which accumulates in the dark) (3) of CA (4) still appeared in CA. In other words, in *P. vulgaris* as in wheat, conversion of hamamelose into CA does not require the synthesis of CA1P.

**Rate and Light Dependence of Hamamelose Metabolism**—Leaf discs were infiltrated with [¹⁴C]hamamelose, then incubated at room temperature in the light (PFD = 200 μmol m⁻² s⁻¹) for the indicated periods, after which the radiolabeled CA (●) or CA1P (■) was determined. After 2 h in the light, a proportion of the discs was transferred to darkness for the indicated periods, after which the amounts of [¹⁴C]-labeled CA (○) and CA1P (□) were determined.

![Figure 8](http://www.jbc.org/) **Appearance of [¹⁴C]CA1P in [¹⁴C]CA-infiltrated leaf material from *P. vulgaris*, in the dark.** [¹⁴C]CA was vacuum infiltrated into a series of (0.25 g, fw) leaf disc samples, which were then illuminated for 2 h, transferred to darkness for the indicated times, and then extracted, and the [¹⁴C]CA1P content determined.

![Figure 9](http://www.jbc.org/) **Light and time dependence of the conversion of hamamelose into CA or CA1P.** Leaf discs of *P. vulgaris* were infiltrated with [¹⁴C]hamamelose, then incubated at room temperature in the light (PFD = 200 μmol m⁻² s⁻¹) for the indicated periods, after which the radiolabeled CA (●) or CA1P (■) was determined. After 2 h in the light, a proportion of the discs was transferred to darkness for the indicated periods, after which the amounts of [¹⁴C]-labeled CA (○) and CA1P (□) were determined.

### Table I

| Pretreatment | Hamamelose content (nmol g⁻¹ fw) |
|--------------|---------------------------------|
| 1. 2 h: 10 μmol m⁻² s⁻¹ | 68.2 ± 8.2 |
| 2. As (1), then 0.5 h:60 μmol m⁻² s⁻¹ | 71.2 ± 5.1 |
| 3. As (2), then 4 h in dark | 35.4 ± 2.3 |

### Table II

| Treatment | [¹⁴C]Hamamelose | [¹⁴C]CA | [¹⁴C]CA1P |
|-----------|----------------|--------|----------|
| Control   | 31.2           | 64.3   | 4.5      |
| DTT 10 mm | 80.5           | 18.2   | 1.3      |
| NaF 20 mm | 93.5           | 6.3    | 0.1      |
| 100 mm    | 98.9           | 1.1    | 0        |
As in preceding experiments, $^{14}$C was detected exclusively in CA or CA and CA1P, depending on the conditions.

**DISCUSSION**

**Hamamelose Is Specifically Converted into CA and CA1P in Leaf Material of P. vulgaris**—Hamamelose was not metabolized to any other neutral component, following leaf infiltration (Fig. 2, track C). Therefore, the radiolabeled CA and CA1P must have been derived directly from hamamelose, rather than (indirectly) from some other neutral hamamelose derivative. The present work shows that CA and CA1P are the only significantly radiolabeled compounds to be derived from $[^{14}$C] hamamelose. Thus, hamamelose is a specific precursor of CA and CA1P, and in *P. vulgaris* is almost exclusively metabolized into these two compounds.

A small amount of radiolabel occasionally became incorporated into a component with a retention time consistent with a bisphosphate (Fig. 3, track B). However, the presence of $^{14}$C in this component was not always apparent, and the incorporation of radiolabel into CA or CA1P from hamamelose did not depend on its appearance.

**Hamamelose Is Converted into CA, Which Is Then Converted into CA1P**—Since the only compounds to consistently become radiolabeled, following the administration of hamamelose, were CA and CA1P (Fig. 3 track B), either CA and CA1P are both derived directly from hamamelose or only one is derived directly from hamamelose, the other being derived from the product of the initial conversion.

Hamamelose is converted exclusively into CA in the leaves of *T. aestivum* (Fig. 3, track C). The same applies using *P. vulgaris* in the presence of high concentrations of DTT or sodium fluoride (Table II). It therefore appears that hamamelose is converted directly into CA. The exclusive conversion of hamamelose into CA in the light (Fig. 9) supports this notion, but is not sufficient proof for a direct conversion of hamamelose into CA, since any CA1P made as an intermediate could be hydrolyzed to CA by the light-activated CA1P phosphatase (7, 9) and so escape detection. CA is converted exclusively into CA1P in isolated leaves, following vacuum infiltration of $[^{14}$C]CA (Fig. 8). This is in agreement with observations of Moore and Seemann (11), who investigated the metabolism of CA, following its uptake through the petiole. Since the interconversions, hamamelose $\rightarrow$ CA, and CA $\rightarrow$ CA1P have been demonstrated, but no conversion of hamamelose into CA1P has been observed in which CA was not also formed, the reaction sequence, hamamelose $\rightarrow$ CA $\rightarrow$ CA1P is likely. Absolute confirmation must await purification of the associated enzymes.

**Hamamelose Occurs Naturally in Leaves of P. vulgaris**—The occurrence of hamamelose in *P. vulgaris* would be expected, in the light of a survey by Sellmair et al. (17), which concluded that hamamelose was distributed widely, if not universally, throughout the plant kingdom. This survey depended on detecting radiolabeled hamamelose in plants following photosynthesis in $^{14}$CO$_2$.

We describe a method using HPLC for detecting and measuring hamamelose in leaf tissue directly. The smaller hamamelose content of darkened leaves (Table I) is consistent with its role in the (dark-dependent) synthesis of CA1P. Since hamamelose conversion into CA can take place at similar rates in the light and dark (Fig. 9), the lower hamamelose content of dark-adapted leaves (Table I) probably reflects a fall in the rate at which hamamelose is synthesized, rather than an accelerated conversion into CA/CA1P. Since hamamelose is converted almost exclusively into CA and CA1P (Fig. 3, track B), the difference between the hamamelose content of light and dark adapted leaves (of 36 mmol g$^{-1}$, fw) represents a minimum estimate for the amount of carbon converted into CA/CA1P at night. Our previous observation, that carbon assimilated at low irradiance is preferentially incorporated into CA1P (13), is not paralleled by an increase in the total pool size of hamamelose in leaves transferred from normal to low irradiance (Table I). However, it is possible that precursors of hamamelose accumulate, following such a transition (see below).

**Only the Net Phosphorylation of CA to Form CA1P Has an Absolute Requirement for Darkness, Not the (Prior) Conversion of Hamamelose into CA** (Fig. 9)—$[^{14}$C]Hamamelose is converted into $[^{14}$C]CA in the light and in the dark at rates much greater than that of its dark-dependent conversion into $[^{14}$C]CA1P (Fig. 9). This explains why the absolute amount of $[^{14}$C]CA formed was greater than that of $[^{14}$C]CA1P (Fig. 3, inset). Only after a prolonged period in the dark does the rate of $[^{14}$C]CA synthesis fall to that of $[^{14}$C]CA1P (Fig. 9). This is presumably due to substrate ($[^{14}$C]hamamelose) depletion having a greater effect on $[^{14}$C]CA synthesis than on $[^{14}$C]CA1P synthesis (consistent with the notion that CA is derived from hamamelose, but that CA1P is derived from CA).

**Hamamelose Is Also Converted into CA in Wheat**—The conversion of hamamelose into CA in wheat (Fig. 3, track C) is in agreement with the precursor/product relationship proposed for hamamelose and CA, and would be expected, judging by the considerable amounts of CA in wheat (10), the widespread distribution of hamamelose (17), and the structural similarity of the two molecules. The fact that no $^{14}$C was detected in CA1P in wheat is possibly due to the relatively low levels of CA1P present in wheat (10-fold lower than in *P. vulgaris*) (12) and/or a larger contribution of preexisting CA to its synthesis.

**A Putative Pathway for CA1P Biosynthesis**—We propose the following sequence of reactions for the biosynthesis of CA and CA1P:

\[
\text{CO}_2 + \text{RuBP} \rightarrow \text{FDP} \rightarrow \text{FPP} \rightarrow \text{HBP} \rightarrow H \rightarrow \text{CA} \leftrightarrow \text{CA1P} \quad \text{(Eq. 1)}
\]

This proposal is based on (a) the conversion of FDP into HBP by illuminated chloroplast material (Gilck and Beck (14)), (b) the present demonstration that hamamelose (H) can be metabolized to CA and CA1P, and (c) the phosphorylation of CA to CA1P in isolated leaves (More and Seemann (11)).

This reaction sequence is also supported by our previous observation of a lag in the appearance of radiolabeled CA1P (compared to total CA1P), following brief exposure to $^{14}$CO$_2$, in a pulse-chase experiment (13). Such a delay would correspond to the time taken for recently assimilated carbon to pass through the sequence of intermediates, following its initial incorporation into intermediates of the Calvin cycle. The flux of carbon from CO$_2$ through this pathway can be considerable: more than 8% of carbon assimilated at low irradiance became incorporated into CA + CA1P, in a subsequent period of darkness (13).

FDP is converted into HBP by chloroplast components (14), and CA1P has been shown to occur exclusively within the chloroplast (24). Therefore, the intracellular site of the FBP $\rightarrow$ HBP conversion and of the phosphorylation of CA, is thought to be the chloroplast. Neither hamamelose nor any hamamelose monophosphate have been detected in chloroplasts (16), and so it has been suggested that the site(s) of HBP dephosphorylation is/are outside the chloroplast (16). The site of the conversion, hamamelose $\rightarrow$ CA, remains to be established, although the proposed occurrence of hamamelose outside the chloroplast and the detection of CA in cytosol and vacuole (16) suggest that this reaction is either cytosolic or vacuolar. None of the enzymes of CA1P biosynthesis have been purified or characterized, except perhaps the CA1P phosphatase, which may be found to have an additional role in the dephosphorylation of HBP. However, this enzyme is located exclusively in the chloroplast (24).

The reaction sequence shown implies that factors influencing
the amounts of FBP in the chloroplast may affect the flux of photosynthate into CA and/or CA1P. Sassenrath-Cole and Pearcy (15) have shown that FBPase responds rapidly to changes in light intensity, and that this is reflected in the amounts of fructose and sedoheptulose bisphosphates. In particular, increased amounts of these two metabolites were shown to accompany a fall in light intensity (15). These observations are consistent with the greater incorporation of recently assimilated carbon into CA and CA1P at low PFD than at high PFD, reported previously (13). Hence, changes in the ambient concentration of FBP may well affect the amount of recently assimilated carbon incorporated into CA and CA1P.

If HBP is synthesized in the chloroplast, then its structural similarity to the transition state intermediate of the carboxylation reaction of Rubisco would suggest that it, too, would inhibit Rubisco. Indeed, it may prove to be the *daytime* inhibitor reported previously (25). This may be another reason why Rubisco activase is necessary, ensuring that Rubisco does not become irreversibly inhibited during the day.

It is puzzling that, in the light, there is a net flow of carbon away from CA1P (dephosphorylation to CA) while *de novo* precursors of CA1P are simultaneously being synthesized. CA1P, dephosphorylated to CA in the light, should provide sufficient carbon skeletons (along with the relatively large pool of vacuolar CA) (10) to meet the demand for rephosphorylation to CA1P, in an ensuing period of darkness. Perhaps these branch chain sugars serve some other purpose as well, for which a regular supply from recently assimilated carbon is needed.
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