# Metabolism of 2'-Carboxyarabinitol in Leaves[1]

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## ABSTRACT

Results presented here indicate that 2'-carboxyarabinitol (CA) is the *in vivo* precursor and product of 2'-carboxyarabinitol 1-phosphate (CA1P) metabolism in leaves. When [2-$^{14}$C]CA was fed in the light to leaves of five species known to be highly active in CA1P metabolism (*Phaseolus vulgaris, Lycopersicon esculentum, Helianthus annuus, Petunia hybrida*, and *Beta vulgaris*), [$^{14}$C]CA1P was formed in the dark. Reillumination of a *Phaseolus* leaf caused this [$^{14}$C]CA1P to be rapidly metabolized to [$^{14}$C]CA ($t_{1/2}$ = 1 min). The epimer 2'-carboxyribitol could not substitute for CA in the dark synthesis of CA1P, and CA in the anionic form was a better substrate than CA in the lactone form. In leaves of *Phaseolus vulgaris*, the active CA pool size used in the dark synthesis of CA1P is between about 70 and 110 nanomoles per milligram of chlorophyll. The photosynthetic electron transport inhibitor diuron did not affect the dark synthesis of [$^{14}$C]CA1P, but did greatly reduce the rate of its subsequent light degradation ($t_{1/2}$ = approximately 10 min). Dark synthesis of [$^{14}$C]CA1P was inhibited by dithiothreitol and NaF. From the present data, we suggest that CA1P and CA participate in a metabolic substrate cycle *in vivo*.

CA1P[2] (also known as hamamelonic acid 2-P, ref. 1) is a branched chain, phosphorylated sugar acid that is apparently unique to plants (see ref. 19 for review). CA1P is a tight-binding inhibitor of Rubisco activity (2) and can be degraded *in vitro* by a specific chloroplast phosphatase to yield CA plus Pi (4, 6). Using MS and isotope dilution procedures, we have shown that CA does occur in leaves of most, if not all, plants (11), thus supporting the hypothesis that CA1P may be metabolized *in vivo* in the light to CA (4, 6).

The precursor used for CA1P synthesis is unknown. Beck *et al.* (1) have suggested that CA1P may be derived from hamamelose 2',5-bisP, which in primrose is made from fructose 1,6-bisP (3). However, because we were unable to demonstrate the formation of [$^{14}$C]CA1P in leaves of various species (including primrose) that were fed [$^{14}$CO_2$ (10), we have concluded that the precursor to CA1P synthesis is far "downstream" from Calvin cycle intermediates.

Leaves of *Phaseolus vulgaris* have the highest levels of both CA1P and CA of any species yet examined, up to approxi-

mately 67 and 1000 nmol mg$^{-1}$Chl$^{-1}$, respectively (9, 11). In most species examined, levels of CA are relatively greater than those of CA1P (9,11). This may indicate that there is a source of CA other than CA1P and/or that there is a relatively slow turnover of the CA pool. That CA metabolism may be more complex than CA1P metabolism is possible because about 63% of the CA in an illuminated bean leaf occurs outside the chloroplast (11), whereas CA1P is thought to occur strictly within the chloroplast (18).

In this study, we have fed [2-$^{14}$C]CA through petioles of leaves from various species to examine its metabolism under both light and dark conditions. We demonstrate that CA is phosphorylated in the dark to form CA1P and that this CA1P can be converted back to CA if the leaf is reilluminated. We propose a biochemical model for this metabolism in which CA and CA1P function in a metabolic substrate cycle, and carbon entry and exit to the cycle occurs through CA.

## MATERIALS AND METHODS

### Plant Material

Plants were grown in a naturally illuminated greenhouse (9) and received a modified half-strength Hoagland solution daily.

### Synthesis and Purification of [2-$^{14}$C]CA and [2-$^{14}$C]CR

[2-$^{14}$C]CABP and [2-$^{14}$C]CRBP (specific radioactivities usually about 1.5 Ci mol$^{-1}$)were synthesized and each purified as described elsewhere (5, 9, 13). [2-$^{14}$C]CA and [2-$^{14}$C]CR were prepared by treating the free acid form of [$^{14}$C]CABP or [$^{14}$C]CRBP, respectively, with alkaline phosphatase (Type VII-NL, Sigma Chemical Co.) as described previously (11).

### Leaf Metabolism of CA

Plants were kept in a dark growth chamber overnight, and leaves were taken the following day after the plants had experienced at least 2 h in the light (28°C, 800 $\mu$mol quanta m$^{-2}$ s$^{-1}$). Leaf petioles were cut under H$_2$O and then placed in an adjacent, submerged microcentrifuge tube (1.5 mL). Leaves were illuminated at 500 ± 50 $\mu$mol quanta m$^{-2}$ s$^{-1}$ filtered through a 14 cm H$_2$O bath (15°C). Water in the tube was lowered to just above the cut surface, and [$^{14}$C]CA then added (about 0.6 $\mu$mol, 1.5 Ci mol$^{-1}$). The leaf was allowed repeatedly to deplete the H$_2$O level to just above the cut surface, with small volumes of H$_2$O added over a period of 2 h. About 45 to 60 min was sufficient for complete uptake of the supplied $^{14}$C label. Leaves were then transferred to a darkened chamber (usually for 4 h) prior to killing them in

[2] Abbreviations: CA1P, 2'-carboxyarabinitol 1-P; CA, 2'-carboxyarabinitol; CABP, 2'-carboxyarabinitol bisP; CR, 2'-carboxyribitol; CRBP, 2'-carboxyribitol bisP; CR1P, 2'-carboxyribitol 1-P; 2D-TLE/TLC, two-dimensional thin-layer electrophoresis/thin-layer chromatography.

boiling 80% (v/v) ethanol. Variations in leaf feeding or illumination conditions are indicated in the figure legends and tables.

Leaves were extracted as described previously (10). Extracts were partitioned with $CHCl_3$, dried by rotary evaporation, resuspended in 1.7 mL of $H_2O$, brought to 15% (v/v) ethanol, and cooled to 4°C. The sample was centrifuged at 17,000g for 20 min, and the supernatant fluid was stored at 4°C. About 10,000 dpm of each sample (with or without about 5,000 dpm of appropriate standards) were spotted onto a cellulose-coated plate. Metabolites were separated by 2D-TLE/TLC (10, 17), located by autoradiography, and removed from the plates, and the $^{14}C$ radioactivity was measured by liquid scintillation spectroscopy.

In some experiments, leaf extracts were fractionated using a 12.5-mL column of QAE-Sephadex (formate form, ref. 14). The resulting neutral, organic acid, and sugar phosphate fractions were concentrated and examined by 2D-TLE/TLC (10). This procedure allowed a reduction in the detection limit from about 6 nmol of a singly labeled metabolite (1% of the supplied label) to about 0.5 nmol (0.08% of the supplied label). In other experiments, leaf extracts were further processed to purify $[^{14}C]CA1P$ and measure its specific radioactivity (9). The largely purified CA1P preparation was treated with alkaline phosphatase, and the products were separated using QAE-Sephadex chromatography (11). The CA specific radioactivity was then measured by HPLC (11) using a flow-through scintillation counter (Radiomatic Instruments).

## RESULTS

We first examined metabolism of $[^{14}C]CA$ by feeding it in the anionic form to bean leaves via the transpiration stream and analyzing the subsequent metabolite distribution of $^{14}C$ label by 2D-TLE/TLC. After a 2-h uptake period in the light, the $^{14}C$ label in the leaf remained exclusively in CA. This result was observed at both a 1% and an 0.08% limit of detection of supplied label (see "Materials and Methods"). However, if the leaf was placed in the dark for 4 h after having been fed $[^{14}C]CA$ in the light, part of the $^{14}C$ label was found in CA1P. This dark synthesis of $[^{14}C]CA1P$ was measured as a function of the amount of $[^{14}C]CA$ fed to the bean leaf (Fig. 1). The amount of $[^{14}C]CA1P$ formed was somewhat greater when relatively small amounts of CA were fed (0-200 nmol). Such a response would be expected if an endogenous pool of CA were becoming increasingly saturated with $^{14}C$ label. The specific radioactivity of the dark synthesized CA1P was also measured, and it exhibited a similar pattern of partial saturation when fed increasing amounts of $[^{14}C]CA$ (Fig. 1). Significantly, the CA1P specific radioactivity measured after feeding 600 nmol of labeled CA (0.72 Ci/mol) was diluted about 50% from the CA supplied to the leaf (1.5 Ci/mol), indicating that the active CA pool size may be quite large.

To measure the active pool size of CA within the bean leaf, we fed a small amount of CA of varying, but relatively high, specific radioactivity (Fig. 2.). By using this approach, the amount of CA fed was estimated to be <10% of the total amount of CA1P that would be formed, thereby presumably not perturbing too greatly the endogenous pool and its sub-
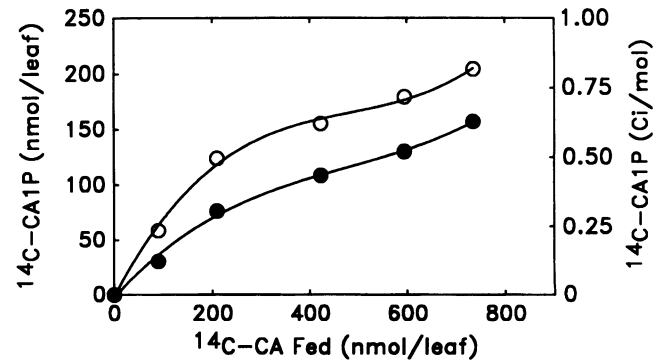


**Figure 1.** Dark synthesis of $[^{14}C]CA1P$ as a function of the amount of $[2-^{14}C]CA$ fed to leaves of *P. vulgaris*. Detached leaves were fed the indicated amounts of CA anion for 2 h at 500 $\mu$mol quanta $m^{-2}$ $s^{-1}$, and then were darkened for 4 h. $^{14}C$ metabolites were analyzed by 2D-TLE/TLC, and CA1P specific radioactivity was measured by HPLC. ●, Amount of $[^{14}C]CA1P$. ○, CA1P specific radioactivity.

sequent metabolism. Under these conditions, about 60% of the $[^{14}C]CA$ was converted to CA1P, and the resulting CA1P specific radioactivity varied linearly with that of the supplied $[^{14}C]CA$. From these data, one can calculate by isotope dilution that the $[^{12}C]CA1P$ pool size in these experiments was 195 nmol/leaf, and the total active CA pool size during this time is then at least the same size (see below). For convenience, most of the experiments described hereafter were done by feeding 600 nmol of $[^{14}C]CA$ (1.5 Ci/mol), which provided a more substantial level of labeled CA1P.

The synthesis of $[^{14}C]CA1P$ was examined over a 24-h dark period in bean leaves that had been fed $[^{14}C]CA$ in either its anionic or lactone forms (Fig. 3A). In both cases, $[^{14}C]CA1P$ was detected after 2 h in the dark. However, during the first 4 h in the dark, the percentage of $[^{14}C]CA1P$ formed was greater in leaves fed CA anion than CA lactone (37 *versus* 10%, respectively). The percentage of $[^{14}C]CA1P$ formed from the CA anion declined thereafter such that by 24 h, similar amounts of $[^{14}C]CA1P$ were present after feeding either the CA anion or lactone forms. However, four other products containing 5 to 10% of the total label were also formed in the dark from the $[^{14}C]CA$ lactone (data not shown). All of these products are as yet unidentified, but none are apparently phosphorylated. Three of these unknown products were sometimes observed after feeding the CA anion, but generally only after partially purifying the organic acid fraction from the dark extract (0.08% detection limit). These same unknown products were observed at similar levels after feeding small amounts (20 nmol) of CA anion with much higher specific radioactivity (50 Ci/mol). When the free acid of $[^{14}C]CR$ (an epimer of CA) was fed to bean leaves, neither CR1P nor any other product was detected (1% detection limit) (Fig. 3A).

We also calculated the unlabeled CA1P pool size in the same bean leaves fed CA free acid in the previous 24-h time course (Fig. 3B). This was done by measuring the CA1P specific radioactivity and calculating the amount of $[^{12}C]CA1P$ present (9). The leaf $[^{12}C]CA1P$ pool was found to increase most rapidly during the first 4 h of the dark treatment, and then at a more gradual rate throughout the re-
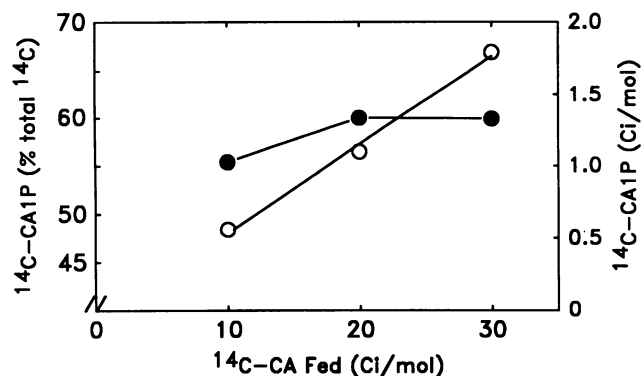
**Figure 2.** Dark synthesis of [$^{14}$C]CA1P as a function of the specific radioactivity of [2-$^{14}$C]CA fed to leaves of *P. vulgaris*. Detached leaves were fed 20 nmol of CA anion as described in Figure 1. ●, Percent [$^{14}$C]CA1P. O, CA1P specific radioactivity.

**Table I.** *Amount of [$^{14}$C]CA1P Formed after Feeding [$^{14}$C]CA to Leaves of Selected Species*

Leaves were fed 600 nmol of [$^{14}$C]CA anion (except as indicated) via the transpiration stream for 2 h in the light (500 µmol quanta m$^{-2}$ s$^{-1}$), and then were transferred to the dark for 4 h. Leaf metabolites were extracted in hot ethanol, and the distribution of $^{14}$C label was determined by 2D-TLE/TLC. Where indicated, values are given ± 1 SD.

| Species | No. of Samples | Amount of [$^{14}$C]CA1P | |
|---|---|---|---|
| | | nmol leaf$^{-1}$ | nmol (g fresh weight)$^{-1}$ |
| *P. vulgaris* | 7 | 130 ± 45 | 76 |
| | 9[a] | 62 ± 23 | 36 |
| *Lycopersicon esculentum* | 2 | 85 | 94 |
| *Helianthus annuus* | 1 | 31 | 20 |
| *Petunia hybrida* | 1 | 26 | 25 |
| *Beta vulgaris* | 5 | 8.7 ± 1.8 | 1.6 |
| *Spinacea oleracea* | 1 | 0 | 0 |

[a] Leaves were fed [$^{14}$C]CA in the lactone form.

maining dark period. However, the total leaf CA1P pool ([$^{12}$C]CA1P + [$^{14}$C]CA1P) was quite constant from 4 to 24 h of dark treatment (about 275 nmol/leaf). During this time, the [$^{14}$C]CA1P pool decreased by an amount comparable to the increase in the [$^{12}$C]CA1P pool (120 nmol).

In repeated experiments in which leaves of different bean plants were fed different preparations of [$^{14}$C]CA anion, an average of 130 nmol of [$^{14}$C]CA1P was formed per leaf after a 4-h dark treatment (Table I). Leaves of tomato, sunflower, petunia, and sugar beet also formed [$^{14}$C]CA1P in the dark
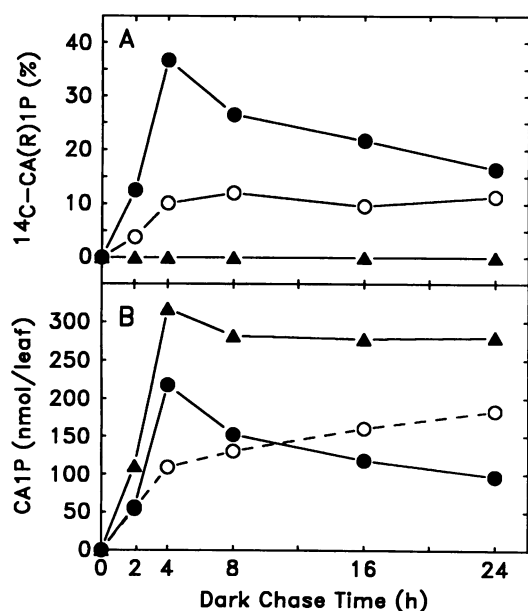
from [$^{14}$C]CA anion (Table I), but made lesser amounts than did bean leaves. However, in spinach we could not detect any dark formation of [$^{14}$C]CA1P from [$^{14}$C]CA anion.

Because CA1P is rapidly degraded at high light intensities *in vivo* ($t_{1/2}$ = approximately 2.5 min; ref. 8), we examined the metabolic fate of the labeled CA1P that was formed from [$^{14}$C]CA in a darkened bean leaf after return of the leaf to the light. The [$^{14}$C]CA1P formed after 4 h in the dark was rapidly metabolized in the light to [$^{14}$C]CA, with a $t_{1/2}$ of about 1 min (Fig. 4). After 10 min in the light, there was no detectable [$^{14}$C]CA1P remaining, and there was no $^{14}$C label observed in any metabolite other than CA or the above mentioned three minor products (0.08% detection limit).

In additional experiments, several possible effectors were also fed to bean leaves in the light (Table II). DCMU had no influence on the dark synthesis of CA1P but did greatly decrease the rate of its degradation in the light ($t_{1/2}$ = approximately 10 min). Mannose had no influence on either the dark synthesis or subsequent light degradation of CA1P.
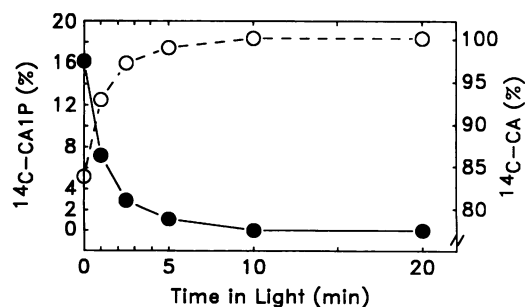


**Figure 3.** Dark metabolism in leaves of *P. vulgaris* fed [$^{14}$C]CA or [$^{14}$C]CR. Detached leaves were fed in the light for 2 h and then transferred to darkness for the indicated times. A, The percent conversion of [$^{14}$C]CA anion (●), [$^{14}$C]CA lactone (O), or [$^{14}$C]CR anion (▲) to the respective products, [$^{14}$C]CA1P or [$^{14}$C]CR1P. B, The absolute amounts of [$^{14}$C]CA1P (●), [$^{12}$C] CA1P (O), and total CA1P (▲) formed by those bean leaves of panel A that were fed [$^{14}$C]CA anion.



**Figure 4.** Metabolism of dark synthesized [$^{14}$C]CA1P by leaves of *P. vulgaris* returned to the light. Leaves were fed [$^{14}$C]CA anion in the light for 2 h, transferred to darkness for 4 h, and then returned to light (500 µmol quanta m$^{-2}$ s$^{-1}$) for the indicated times. No loss of $^{14}$C label occurred during the light/dark treatments. ●, [$^{14}$C] CA1P; O, [$^{14}$C]CA.

**Table II.** *Amount of [$^{14}$C]CA1P Formed by Leaves of Phaseolus vulgaris under Varying Conditions*

Leaves were fed [$^{14}$C]CA anion in the light as in Table I. After 30 min, the solution in the transpiration reservoir was replaced with the solution indicated, and the leaf was allowed to transpire another 90 min prior to changing the light conditions.

| Treatment | Amount of [$^{14}$C]CA1P | |
| --- | --- | --- |
| | 4 h dark | 4 h dark, then 10 min 500 $\mu$mol m$^{-2}$ s$^{-1}$ |
| | *nmol leaf$^{-1}$* | |
| No additions | 107 | 0 |
| 100 DCMU | 123 | 68 |
| 25 mM mannose | 110 | 0 |
| 50 mM DTT | 1 | ND[a] |
| 100 mM NaF | 0 | ND |

[a] Not determined.

However, relatively high exogenous levels of DTT and NaF each completely inhibited the dark formation of [$^{14}$C]CA1P.

## DISCUSSION

We have demonstrated that CA is both a precursor to CA1P synthesis and a product of CA1P degradation *in vivo* (Table I and Fig. 4). The dark formation of [$^{14}$C]CA1P was initially more rapid when CA was fed in the anionic form *versus* the lactone (Fig. 3A). The rate of CA1P formation from CA lactone may be limited by a relatively slow *in vivo* conversion of CA to its anionic form prior to phosphorylation. Whether CA (as the anion) is the immediate precursor for CA1P synthesis is not yet certain, but the simplest and most likely route for CA1P synthesis would be direct phosphorylation of CA at the C-1 position.

After feeding small amounts of [$^{14}$C]CA of varying specific radioactivity (Fig. 2), we have calculated that the pool size of CA within the leaf that is utilized for CA1P synthesis is at least 195 nmol/leaf. Because in this experiment 40% of the label was not incorporated into CA1P, the actual pool size could be proportionately larger (*i.e.* 325 nmol/leaf). On a Chl basis, the active CA pool size, then, is between about 70 and 110 nmol/mg. This amount is about 50 to 75% of the amount of CA that we have shown to occur in the light within the chloroplast (11), and indicates that the chloroplast is probably the intracellular site of CA1P synthesis.

The present data support a general model for metabolism of CA and CA1P in which the two are interconverted in a light-dependent manner. We hypothesize that the CA ↔ CA1P conversions are catalyzed by distinct proteins, as opposed to a single bifunctional enzyme. This hypothesis is supported by the facts that spinach leaves contain moderate levels of CA1P phosphatase activity (J. Kobza and J.R. Seemann, unpublished data, see also ref. 16), but made no [$^{14}$C]CA1P and when fed [$^{14}$C]CA (Table I), and that we have as yet been unable to get partially purified bean CA1P phosphatase to catalyze synthesis of CA1P from CA and possible phosphate donors such as ATP (T. Charlet and B. Moore, unpublished data). We also suggest that carbon entry

to or exit from the CA/CA1P cycle occurs exclusively through CA. This hypothesis that CA is a metabolic branch point is consistent with the following observations. In certain species such as sugar beet, the pool size of CA in the light is much less than the pool size of CA1P in the dark (11). That is, in the light there likely is net carbon flow from CA1P to CA to some unknown metabolite(s), whereas in the dark the carbon path presumably is from an unknown substance(s) to CA and then to CA1P. Additional support for this hypothesis is provided by the observation that [$^{14}$C]CA is metabolized in the dark to several minor products other than CA1P, but CA1P is converted in the light only to CA (Fig. 4).

The rate of total CA1P formation by darkened, excised leaves of *P. vulgaris* was linear for about 4 h (Fig. 3B) and equivalent to that previously reported for intact leaves (8). Between 4 and 24 h of dark treatment, the total leaf CA1P pool was a constant 275 nmol, yet the amount of [$^{14}$C]CA1P decreased by 120 nmol. The basis for the decline in CA1P specific radioactivity is uncertain if CA is both precursor and product of CA1P metabolism. The simplest explanation is that the [$^{14}$C]CA that is produced by hydrolysis of labeled CA1P is diluted by a large amount of [$^{12}$C]CA before being again utilized in the resynthesis of CA1P. Nonetheless, during this time, there was turnover of a substantial portion of the CA1P pool. From previous measurements (9), we would predict that the total leaf Rubisco catalytic site content would be about 160 nmol/leaf, and, therefore, of the total CA1P pool, a large portion may occur unbound within the stroma. The observed dark turnover of CA1P thus might be due to metabolism of this unbound pool.

The accumulation of CA1P in leaves is strongly dependent on the level of irradiance (*e.g.* ref. 19). The biochemical mechanisms that regulate its accumulation are not at all clear, but the leaf level of CA1P under a given light level has been suggested to reflect the prevailing rates of both its synthesis and degradation (19, 20). The observed dark turnover of CA1P (Fig. 3B) provides the best evidence to date for this suggestion. From the present data, CA1P phosphatase activity in the dark may be estimated as ≥9 nmol h$^{-1}$ g fresh weight$^{-1}$ (depending on the amount of [$^{14}$C]CA that is reconverted to CA1P), whereas the rate of CA1P synthesis in the dark is ≥55 nmol h$^{-1}$ g fresh weight$^{-1}$ (adjusted for CA1P phosphatase activity). Because both CA1P synthesis and degradation do occur in the dark, a substrate cycle exists that presumably consumes ATP (or the equivalent). One possible regulatory benefit for a CA/CA1P substrate cycle may be to provide a more responsive metabolic control for preventing CA1P accumulation in moderate to high light, and perhaps for facilitating CA1P accumulation in certain species under low light (*e.g.* see ref. 12).

Other aspects of physiological control on CA1P metabolism were probed by feeding various possible effector metabolites to the detached leaves (Table II). DCMU did not affect the rate of [$^{14}$C]CA1P accumulation in the dark, but did greatly reduce the rate of its degradation in the light, a result comparable to a previous observation made using leaf discs (18). Although CA1P phosphatase activity *in vitro* is inhibited about 50% by Pi (7), the lack of any observed mannose effect on CA1P metabolism (Table II) indicates that Pi may not normally have any regulatory function, contrary to a previous

suggestion (7, 15). The inhibition of CA1P accumulation in the dark by DTT and NaF were quite unexpected (Table II). The reasons for these effects await further investigation.

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