In C₄ plants, two enzymes of the C₄ cycle are known to be light activated, NADP-malate dehydrogenase and pyruvate-Pi dikinase (4-6, 8, 12). Although both enzymes are located in the mesophyll chloroplast of C₄ plants there is considerable evidence from in vitro studies that the mechanisms of light/dark-mediated activation/inactivation of the enzymes are quite different. Reducing conditions activate NADP-malate dehydrogenase (presumably via conversion of disulfide to sulfhydryl groups on the protein) while inactivation requires oxidizing conditions (6, 11, 13). Incubation of the inactive form of the enzyme with DTT plus thioredoxin leads to rapid activation. In vivo the apparent role of light is to generate a reductant through noncyclic electron flow which mediates activation of the enzyme, through either ferredoxin-thioredoxin reductase and/or a membrane-bound light effect mediator and ferraltein (1, 2). Recently, Chapman and Hatch (3) and Nakamoto and Sugiyama (15) showed that pyruvate-Pi dikinase could undergo activa-

1 This research was supported by a Herman Frasch Foundation Grant for Research in Agricultural Chemistry and National Science Foundation Grant PCM 82-04625.
number of treatments, the leaves were exposed to varying conditions prior to sampling and killing in liquid N₂. Thus, each enzyme assay was performed on a single leaf extract.

When leaves were taken from the chamber, the part of the leaf blade (about 1 g) which was under the gas treatment (i.e. N₂, 2% O₂-98% N₂, CO₂-free air) was cut and the midrib was removed. The leaf was immediately killed by immersion in liquid N₂ and then stored in liquid N₂ until extraction. The time from removal of the leaf from the chamber to killing in liquid N₂ was about 10 to 15 s (much shorter than the half-time for inactivation of the pyruvate-Pi dikinase). At the same time, the leaf blade of another plant in the same pot which was exposed to room air and darkness was sampled to compare with the treated leaf (Figs. 1 and 2). In one experiment, after exposure to N₂ gas in the dark, some leaves were further treated in room air prior to extraction (Fig. 3). The results of each experiment were obtained during a given day and leaves of similar age were sampled from several plants grown in the same pot.

In all in vivo experiments, sampled leaves were stored in liquid N₂. Comparisons of the leaves stored as described above in liquid N₂ with control experiments in which the enzymes were extracted without freezing the leaf showed that there was no significant difference in the extractable enzyme activities.

**Incubation of Leaves at Various Temperatures during Dark Treatment.** Before dark treatment, plants were illuminated as described previously for 1 h at 23 to 25°C. Following the illumination period, the plants were transferred to a room which was preset at the desired temperature. A leaf blade was immediately harvested for measurement of the initial activity (0 time) and other samples were taken at intervals during the dark; the midrib was removed, and the leaves were killed and stored under liquid N₂ as described above.
Enzyme Extraction and Assay. The extraction procedure was carried out at room temperature using a chilled mortar and grinding medium. The grinding medium contained 4 volumes/g tissue of 0.1 M Tris-HCl (pH 7.5, 4°C), 10 mM MgCl₂, 1 mM EDTA, 5 mM DTE² and 5% (w/w of leaf tissue) polyvinylpyrrolidone. The tissue was ground for 1.5 to 2 min and the homogenate was filtered through Miracloth. An aliquot of the filtrate was taken for Chl determination. For the assay of NADP-malate dehydrogenase, a 25-μl aliquot of the homogenate was added to the reaction mixture (25 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 0.2 mM NADPH₂) and centrifuged at 14,000 g for 1 min. The supernatant was assayed immediately at 25°C as previously described (9). The reaction was initiated by the addition of oxalacetate (0.5 mM) and the oxalacetate-dependent A decrease was measured at 340 nm.

For the assay of pyruvate, Pi dikinase, an aliquot of the same filtrate (400 μl) was rapidly desalted by passage through a column (0.8-cm diameter by 5-cm length) of Sephadex G-25 (superfine). The column was previously equilibrated with a buffer containing 50 mM Tris-HCl (pH 7.5, 25°C), 5 mM MgCl₂, 0.1 mM EDTA, 5 mM DTE, 0.2% (w/v) BSA, and 2.5 mM pyruvate. Desalting was accomplished by centrifuging the column at 1,400g for 3 min at room temperature after application of the sample (10). The eluate obtained with this method was not diluted and the yield was over 90% of the original sample. After Sephadex G-25 treatment, the

Abbreviation: DTE, diethioerythritol.

FIG. 3. Changes in the activity of NADP-malate dehydrogenase (MDH) and pyruvate, Pi dikinase under varying conditions following 1 h of illumination. Three leaves were treated under N₂ in the dark (one leaf per plant) as described in Materials and Methods. The leaves were sampled and extracted as follows: one leaf after 60-min N₂ treatment; one leaf after 60-min N₂ + 30-min air in the dark; and one leaf after 60-min N₂ + 30-min air in the dark + 15-min air in the light. The initial activities for NADP-malate dehydrogenase and pyruvate, Pi dikinase were 329 and 144 μmol mg⁻¹ Chl h⁻¹, respectively. The data are from a single experiment.

FIG. 4. ADP + ATP mediated in vitro inactivation of pyruvate, Pi dikinase in mesophyll chloroplast extracts of maize under N₂ or air. In the anaerobic treatments, the enzyme mixture was flushed with N₂ for 3 min in a small glass bottle (volume of 2.5 ml) before addition of ADP + ATP or water (in control). The bottle was covered with a rubber serum cap and two syringe needles were inserted, one serving as the inlet for the gas and the other for the outlet. The N₂ gas stream was humidified by passage through water in order to prevent sample evaporation. The enzyme samples were continuously flushed with N₂ except when samples were withdrawn with a microsyringe for enzyme assay. Samples were exposed to room air in the aerobic treatment. The temperature of incubation was 23°C and the initial enzyme activity was 0.42 unit/ml. The eluate was centrifuged at 14,000g for 1 min and aliquots (25 μl) of the supernatant were immediately assayed. The enzyme was assayed in the direction of pyruvate to phosphoenolpyruvate through coupling with phosphoenolpyruvate carboxylase and NAD-malate dehydrogenase as previously described (5). The reaction was initiated by addition of ATP.

Chl Determination. Chl was determined by the method of Wintermans and De Mots (21) following extraction in 96% ethanol.

Preparation of Chloroplasts and Chloroplast Extracts. The procedure was basically the same as previously described by Sugiyama and Hatch (19). Plants were pre-illuminated to activate pyruvate, Pi dikinase. The midrib was either removed from pre-illuminated leaves in the light or under room light at 4°C to minimize dark inactivation of the enzyme. The tissue was blended for 8 s (4 s twice) at 60% of line voltage in a Polytron. Chloroplasts were isolated as previously described and stored frozen in liquid N₂. For experimentation, the chloroplast extracts were treated on Sephadex G-25. For each assay, 180 μl of the eluate was added to the incubation mixture, giving a total volume of 200 μl. This medium used for preincubation (under N₂ or air and at varying temperatures; see Figs. 4 and 6) contained 18 mM Tris-HCl, pH 8.3, 4.5 mM DTE, 9 mM MgCl₂, 0.18 mM EDTA, 1.8 mg ml⁻¹ BSA, and in the presence or absence of 1 mM ADP and 50 μM ATP. At various time intervals, samples of 25 μl were removed and placed in cuvettes containing the complete assay system for pyruvate, Pi dikinase. These were immediately mixed and activity was measured by following the A change at 340 nm and 25°C.

RESULTS
The Influence of O₂ on the Inactivation of Pyruvate, Pi Dikinase and NAD-Malate Dehydrogenase. O₂ was required for inacti-
viation of both pyruvate, Pi dikinase and NADP-malate dehydrogenase in maize leaves in the dark following light activation (Figs. 1 and 2). The rate of inactivation under 2% O2 was only slightly lower than that at 21% CO2 (without CO2) or air (with CO2). N2 in the dark prevented inactivation of both enzymes. With NADP-malate dehydrogenase, there was a consistent although variable degree of increase in activity under N2 in the dark following light activation (Figs. 2 and 3 and data not shown) which did not occur with pyruvate, Pi dikinase.

The prevention of inactivation of both enzymes in the dark by the treatment with N2 is reversible (Fig. 3). Both enzymes were inactivated following transfer from the N2 treatment in the dark to air. When the leaves were re-illuminated in the air, the enzymes were reactivated after 15 min to the original light-activated level.

The effect of N2 versus air on the ATP + ADP-dependent inactivation of pyruvate, Pi dikinase in vitro was examined. The activated enzyme was obtained from chloroplasts isolated from pre-illuminated leaves. Inactivation occurred at similar rates under both air and N2 when incubated with ATP + ADP (Fig. 4). NADP-malate dehydrogenase immediately lost activity following desalting of the chloroplast extracts, which would remove DTT (results not shown). It was previously shown that the activated state of partly purified NADP-malate dehydrogenase of maize is maintained under N2 (13).

**Influence of Temperature on the Inactivation of Pyruvate, Pi Dikinase and NADP-Malate Dehydrogenase.** As the temperature was lowered during the postillumination period, there was an increasing lag period before inactivation of pyruvate, Pi dikinase proceeded in maize leaves (Fig. 5). The lag periods at 10, 17, and 25°C were more than 30, 25, and 10 min, respectively, while that at 32°C was less than 5 min. Following the lag period, the enzyme was inactivated and the rate of inactivation increased with increasing temperature. The half-life for dark inactivation was 7 min at 32°C, 20 min at 25°C, and 45 min at 17°C (Fig. 5). Therefore, in vivo inactivation of the enzyme proceeded in two phases, both showing a dependence on temperature. Inactivation of pyruvate, Pi dikinase in vitro was also strongly dependent on temperature, although there was no temperature-dependent lag period (Fig. 6). During the first 10 min of incubation, the ATP + ADP-dependent inactivation showed a slight lag, but this was independent of temperature. In the experiment of Figure 6, strict quantitative comparisons cannot be made between all temperatures inasmuch as results of Figure 6, A, B, and C, represent three different chloroplast preparations. In another experiment, the enzyme from one chloroplast preparation was incubated with ATP + ADP over a temperature range of 19 to 34°C for 30 min. An Arrhenius plot (log of the rate of inactivation versus 1/T) of this data gave a linear function over the temperature range of the experiment with a Q10 of 2.0.

In contrast to the results with pyruvate, Pi dikinase, NADP-malate dehydrogenase was rapidly inactivated in leaves during the postillumination period (half-time of about 3 min; Fig. 7) and there was little effect of temperature on the rate of inactivation between 10 and 32°C. Also, there was no lag in the inactivation of NADP-malate dehydrogenase upon transfer from light to darkness.

**DISCUSSION**

The Influence of O2 on the Inactivation of Pyruvate, Pi Dikinase and NADP-Malate Dehydrogenase. O2 was required for the dark inactivation of NADP-malate dehydrogenase in maize leaves. Previous studies indicate that the mechanism of activation/inactivation of the isolated enzyme is through a reductive/oxidative process involving enzyme thiol/disulfide interconversion (11, 13). In vivo in the light, reductive power from photosynthetic electron transport is apparently used to activate the enzyme. In the dark, this source of reductant is unavailable and the enzyme may be oxidized via a protein factor, possibly thioredoxin, with O2 as the final electron acceptor (13, 16). Therefore, in the dark under anaerobic conditions, the enzyme may be maintained in the reduced, activated state. The fact that the activated state of the enzyme in the dark under N2 tends to increase over that of the pre-illuminated state (Figs. 2 and 3) suggests that some reductant is available in the chloroplasm to further activate the enzyme. Whether this increase in the level of activation in the dark is associated with an increased level of reduced pyridine nucleotides in the chloroplasm under anaerobic conditions is uncertain. Lee-good and Walker (14) have found that fructose-1,6-bisphosphatase can be activated in isolated chloroplasts in the dark under anaerobic conditions provided that triose-P is added to generate reductive power in the chloroplasts. Therefore, it appears that reduced pyridine nucleotides can serve as the electron donor to activate certain enzymes independently of light.

Surprisingly, O2 is also required for inactivation of pyruvate, Pi dikinase in the dark in maize leaves. This cannot be readily interpreted mechanistically from current knowledge of the in vitro
mechanism of inactivation. Recently, Chapman and Hatch (3) and Nakamoto and Sugiyama (15) showed that the enzyme can be activated in the absence of a reducing agent, and therefore suggested that reduction/oxidation of the enzyme is not required. The requirement for reducing agents in early studies with the enzyme may have been to stabilize the protein and/or the activating factor (see 3, 7, 15, 20). Inactivation (ATP + ADP-dependent) can occur in the presence of a high concentration of DTT (10 mM: Refs. 3 and 19) in contrast to NADP-malate dehydrogenase which can be activated by DTT and inactivated in the absence of DTT with soluble protein factor (13). In the present study, the ATP + ADP-dependent inactivation of pyruvate-Pi dikinase occurred under anaerobic and aerobic conditions (Fig. 4), which further suggests oxidation of the enzyme is not required for inactivation. In vitro studies indicate that the relative levels of ATP, ADP, AMP, PPI, and Pi are involved in controlling the state of activation/inactivation of pyruvate-Pi dikinase (3, 7, 15, 19, 20). Therefore, maintenance of activation in the dark under N₂ might be mediated through an effect on the levels of these metabolites in the chloroplasts. Alternatively, there may be additional factors involved in mediating inactivation of the enzyme in vivo, possibly involving oxidation/reduction of the enzyme or regulation of the activity of the inactivating factor protein. Chapman and Hatch (3) found that, at relatively high pH, reducing conditions were required to stabilize the enzyme and give maximum activation, which might be relevant in vivo. Under certain conditions in the chloroplast, reducing conditions could have a stabilizing effect and favor activation of the enzyme.

**The Influence of Temperature on the Inactivation of**

**Pyruvate, Pi Dikinase and NADP-Malate Dehydrogenase.** The inactivation of pyruvate, Pi dikinase in leaves in the dark proceeded in two phases, both of which were influenced by temperature. First, there was a

![Fig. 7. Time course of in vivo inactivation of NADP-malate dehydrogenase in the dark at various temperatures following illumination of a maize plant for 1 h at 25°C. The average initial activity of NADP-malate dehydrogenase was 122 μmol mg⁻¹ Chl h⁻¹, respectively. The experiment was with the same plant extracts as that of Fig. 5 and the data are from a single experiment. Temperatures designated by the symbols are 10°C (○), 17°C (△), 25°C (□), and 32°C (○), respectively.](image-url)
lag period which increased with decreasing temperature (phase I) and was followed by a temperature-sensitive decrease in activity of the enzyme (phase II). In vitro the time-dependent inactivation of pyruvate, Pi dikinase proceeded without a lag period (lacked phase I) and exhibited temperature sensitivity similar to that of phase II in vivo. Phase I may represent the time taken for change in levels of certain effectors (e.g., relative levels of adenine nucleotides) which are substrates in the inactivation process or control the activity of the inactivating protein factor.

The strong temperature dependence of inactivation of pyruvate, Pi dikinase between 17 and 32°C may be due to the influence of temperature on catalysis of inactivation by the unidentified protein-inactivating factor (assuming inactivation is a catalytic process as it is for activation; Refs. 19 and 20). Below this temperature, both the in vivo and in vitro temperature limitations could be due to a combined effect on the protein-inactivating factor and dissociation of the dikinase enzyme from an active to inactive form. Below about 12°C, the activated pyruvate, Pi dikinase dissociates and is converted from an active to inactive form. This results in a break in the Arrhenius plot around this temperature (18). Hatch (7) also found a break in the Arrhenius plot for the ATP + ADP-dependent inactivation of pyruvate, Pi dikinase at a temperature of 18°C, indicating a greater $Q_10$ below this temperature.

The present study indicates differences in the temperature dependence of inactivation of pyruvate, Pi dikinase and NADP-malate dehydrogenase in maize leaves. It shows that $O_2$ has a role in the inactivating process with pyruvate, Pi dikinase as well as with NADP-malate dehydrogenase. More information about the mechanism of activation/inactivation is needed, particularly with pyruvate, Pi dikinase, in order to understand how the activation/inactivation in vivo is regulated by environmental factors.

Acknowledgments—The authors thank Dr. G. J. Williams, III for the use of his facilities for exposing plants to various environmental conditions.

LITERATURE CITED

5. EDWARDS GE, M UHOURA, T SUGIYAMA 1980 Light and temperature dependence of the rate and degree of activation of pyruvate, Pi dikinase in vivo in maize. Photosynthesis Research 1: 199–207
14. LEEGOOD RC, DA WALKER 1981 Activation of fructose 1,6-bisphosphatase in darkened intact chloroplasts by NADPH. Arch Biochem Biophys 212: 644–654