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PHOTOSYNT HATE PARTITIONING IN HIGHER PLANTS: I. THE EFFECT OF ELEVATED CARBON DIOXIDE LEVELS II. THE ROLE OF PYRUVATE KINASE

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Publication Date
1983-12-01
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I. THE EFFECT OF ELEVATED CARBON DIOXIDE LEVELS
II. THE ROLE OF PYRUVATE KINASE

C.W. Baysdorfer
(Ph.D. Thesis)

December 1983

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PHOTOSYNTHATE PARTITIONING IN HIGHER PLANTS:

I. The Effect of Elevated Carbon Dioxide Levels
II. The Role of Pyruvate Kinase

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This work was supported by the U.S. Department of Energy under Contract Number DE-AC03-76SF00098.
Photosynthate Partitioning in Higher Plants:
1. The Effect of Elevated Carbon Dioxide Levels.
11. The Role of Pyruvate Kinase.

Christoph William Baysdorfer

Abstract

Regulation of Photosynthetic Rates in a Simulated Alfalfa Crop. Long and short term CO$_2$ enrichment, $^{14}$CO$_2$ feeding, and partial defoliation were used to investigate source/sink interactions in a simulated alfalfa crop. Long term CO$_2$ enrichment did not increase the photosynthetic rate or the growth rate in mature alfalfa, in spite of the fact that photorespiration was substantially reduced. Short term CO$_2$ exposures did, however, increase mature crop photosynthetic rates as did partial defoliation of the crop. In contrast, seedling photosynthetic rates and growth rates were increased in response to long term CO$_2$ enrichment. These results suggest that, for the mature alfalfa crop, photosynthesis is limited by the demand for photosynthate. In seedlings, photosynthesis appears to be limited by photosynthate supply.

Spinach Pyruvate Kinase Isoforms; Partial Purification and Regulatory Properties. Pyruvate kinase from spinach (Spinacea oleracea L.) leaves consists of two isoforms, separable by blue agarose chromatography. Both isoforms share similar pH profiles and substrate and alternate nucleotide $K_m$ values. In addition, both isoforms differ in their response to three key metabolites; citrate, aspartate, and glutamate.
The first isoform is similar to previously reported plant pyruvate kinases in its sensitivity to citrate inhibition. The $K_i$ for this inhibition is 1.2 mM citrate. The second isoform is not affected by citrate but is regulated by aspartate and glutamate. Aspartate is an activator with a $K_a$ of 0.05 mM, glutamate an inhibitor with a $K_i$ of 0.68 mM. A pyruvate kinase with these properties has not been previously reported. Based on these considerations it is likely that the activity of the first isoform is regulated by respiratory metabolism. The second isoform, in contrast, may be regulated by the demand for carbon skeletons for use in ammonia assimilation.
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ACKNOWLEDGEMENTS

I thank the members of my thesis committee for their assistance during the course of this work. To Jim Bartholomew and Bob Buchanan, my special thanks for your patience in dealing with a crazed grad student in the throes of a deadline. To Melvin Calvin, your example has inspired me to maintain a broad range of scientific interests. Finally, I would like to extend my gratitude to Al Bassham. You have been a superb teacher and advisor. This manuscript is dedicated to you.
General Introduction

Regulation of the synthesis and partitioning of photosynthate is a complex and poorly understood process. The rate of carbohydrate production by the Calvin cycle must be coordinated with the rate of carbohydrate metabolism and translocation (partitioning) in order to maintain an equivalence of 'supply and demand'. Recent in vitro work has revealed several regulatory mechanisms that may be important control points for photosynthate production and partitioning.

The Calvin cycle is controlled by a complex and only partially understood interaction of pH, ionic composition, metabolite levels and ferredoxin mediated reactions (for reviews, see 1,9). The product of this pathway, triose phosphate, can be converted to starch or exported from the chloroplast. Starch synthesis is regulated by the intrachloroplast metabolite and inorganic phosphate levels (10). Export is controlled by both chloroplastic and cytoplasmic metabolite and inorganic phosphate levels (6). Triose phosphate metabolism branches again in the cytoplasm, one pathway leading to sucrose synthesis, the other via glycolysis to lipid or isoprenoid synthesis, amino acid synthesis, or to mitochondrial respiration.

Triose phosphate flow into sucrose may be regulated by a fructose 2,6-bisphosphate mediated control system (2,8). Sucrose transport in the phloem appears to be controlled by a feedback loop regulated by the extent of sucrose utilization in sink tissues (3).
Control of sucrose utilization in sinks is, at present, poorly understood.

Regulation of the other branch point for triose phosphate, flow through the glycolytic pathway, is also poorly understood. The major irreversible step from triose phosphate to pyruvate is catalysed by pyruvate kinase. This enzyme is, therefore, a likely control point for adjusting glycolytic flow to meet the needs of lipid or isoprenoid synthesis, mitochondrial respiration, and amino acid synthesis. In spite of the potential importance of the leaf enzyme, little is known about its regulatory properties.

Many potential rate limiting steps have been described in vitro. In order to increase crop photosynthetic rates we need to know which of these steps are rate limiting in vivo. Two prominent candidates are the light reactions and the Calvin cycle (4). Either or both of these pathways may be operating at maximal capacity in the leaf, if so, they would be rate-limiting for photosynthesis. The photorespiratory process is also a strong candidate due to its, apparently wasteful, consumption of energy (5,11). Another possible rate limiting step is the demand for sucrose by the sinks (7). The rate of sucrose utilization in the sinks can affect the rate of photosynthesis in the leaf by the regulatory mechanisms previously mentioned. If the sinks require less photosynthate than the maximum the leaves are capable of producing, photosynthate demand can become the rate limiting factor.

This work was designed to answer two questions about the regulation of photosynthate production and partitioning. The first asks, is photosynthesis limited by the rate of reactions that
supply photosynthate (light reactions, Calvin cycle, photorespiration) or by the demand for photosynthate in the sinks. The second question is concerned with the regulatory mechanisms controlling pyruvate kinase, specifically, how is this enzyme regulated by the demand for carbon skeletons for use in nitrogen assimilation.
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CHAPTER 1

REGULATION OF PHOTOSYNTHETIC RATES

IN A SIMULATED ALFALFA CROP
Introduction

The hypothesis that plant dry matter accumulation is limited by the rates of the reactions that supply photosynthate (that is, source limited) rather than by the rates of reactions that utilize photosynthate (sink limited) is quite controversial (for reviews, see 5,12,26). This hypothesis, if shown to be correct, has considerable implications for agriculture. There have been suggestions, for example, that agricultural yields could be increased by reducing photorespiration through either genetic or chemical means (10,25,26) or by increases in the atmospheric carbon dioxide concentration (24,25). These suggestions and the hypothesis imply that, under conditions of optimal water and nutrient supply, dry matter accumulation is limited by the maximum or potential rate of photosynthesis.

In evaluating the data used to support the hypothesis of source limitation it is important to keep in mind that, although the rate of dry matter accumulation is certainly a function of the rate of photosynthesis, it does not necessarily follow that the maximal photosynthetic rate limits the production of dry matter. That is, if photosynthesis is less than the maximum the plant is capable of, dry matter accumulation will be proportional to current photosynthesis but not limited by the maximal photosynthetic rate. Thus, crop photosynthesis must be presently occurring at maximal rates in order for future increases in the photosynthate supply to result in an increase in dry matter accumulation.
Carbon dioxide enrichment studies provide the best evidence that crop photosynthesis may, at times, approach maximal rates. Higher than ambient \( \text{CO}_2 \) levels have been conclusively shown to increase photosynthesis rates during short term exposures \((1,7,16,19)\) suggesting that, in these studies, \( \text{CO}_2 \) is the major factor limiting the rate of photosynthesis. Hence, at ambient \( \text{CO}_2 \) levels, photosynthesis may be at the maximum rate attainable for that \( \text{CO}_2 \) concentration.

Although \( \text{CO}_2 \) enrichment almost invariably increases photosynthetic rates in short term experiments (hours), there is doubt as to whether increased rates are always maintained over the long term \((\text{for review, see } 12)\). In addition, certain stages of plant growth appear to be more responsive to \( \text{CO}_2 \) enrichment than others \((12,16)\). Thus, if \( \text{CO}_2 \) enrichment is to be used to support the hypothesis of a source limitation of dry matter production, several precautions must be taken. First, a long term increase in the rate of photosynthesis must be demonstrated. Second, results obtained at one stage of plant growth should not be extrapolated to other stages.

The hypothesis can also be tested by changing the source/sink relationship of the plant. Partial defoliation, which would be expected to increase the 'demand' on the remaining leaves, should not result in an increase in photosynthetic rate if photosynthesis was already source limited.

The objective of this study was to test the hypothesis of source limitation of dry matter production using both long term \( \text{CO}_2 \) enrichment and defoliation. Alfalfa entirely dependent on
nitrogen fixation was used since nodulated alfalfa have a high demand for photosynthate and a correspondingly high photosynthetic rate (22,23).
Materials and Methods

Plant material. Alfalfa (*Medicago sativa* L. cv Moapa 69) plants were grown in a gas-tight controlled environment chamber (Conviron PGV 36) under a 12 hour photoperiod, a radiant flux density of 800\(\mu\)E m\(^{-2}\) s\(^{-1}\), and a 27\(^{\circ}\)/20\(^{\circ}\)C temperature regime. Plants were sown in vermiculite, inoculated with *Rhizobium meliloti* (Nitragin Company, Milwaukee) and watered daily with one-half strength modified Hoagland solution for five days. Thereafter plants were watered daily with one-half strength minus nitrogen solution (14). After initial thinning, a plant density of 80 plants m\(^{-2}\) was established. When the crop reached 10% bloom, all leaves and stems above 5 cm were removed.

\(\text{CO}_2\) levels within the chamber were monitored and maintained by an infra-red \(\text{CO}_2\) analyzer (Horiba APBA 200E) controlling a \(\text{CO}_2\) source. This system was designed to allow a drop in \(\text{CO}_2\) levels (through photosynthesis) of 10% before supplementary \(\text{CO}_2\) was added.

Growth measurements. At weekly intervals, twelve plants were removed from the chamber and divided into leaf, stem, tap root, and fibrous fractions. Following fresh weight determination, the fractions were dried for 48 hours in a 70\(^{\circ}\)C oven for dry weight measurements. Leaf area was measured using photocopies of leaves.

Photosynthetic rate measurements. Canopy photosynthesis was measured in the controlled environment chamber using recorder tracings of the output from the \(\text{CO}_2\) analyzer. This design permitted
continuous measurement of the photosynthetic rate throughout the experiment. Chamber leakage was measured periodically and never exceeded 15% of photosynthesis. Contributions due to leakage have been subtracted from all rate data.

**Metabolite labelling and measurement.** An exposure chamber capable of holding five plants in individual compartments was used for $^{14}$C studies. Each compartment was connected to a gas exchange apparatus (19) that maintained steady state concentrations of $^{12}$CO$_2$ and $^{14}$CO$_2$ throughout the experiment. Environmental conditions were the same as in the growth chamber. Plants were allowed to photosynthesize for 30 min in either 350 or 1000 ul l$^{-1}$ $^{12}$CO$_2$ before the addition of $^{14}$CO$_2$. After varying lengths of time in $^{14}$CO$_2$, plants were quickly removed from their compartments and plunged into liquid nitrogen. Plant parts were then separated and extracted (3), and the labelled metabolites were analyzed. Neutral sugars, phosphorylated sugars, and organic acids were analyzed by paper chromatography (13), amino acids by HPLC (20), and starch by enzymatic degradation followed by glucose determination (9).
Results

Photosynthetic and Growth Measurements To determine the effect of long term CO₂ enrichment on crop photosynthesis, net CO₂ uptake per plant per day was measured for both high and ambient CO₂ treatments. The results (Fig. 1) show that CO₂ enrichment increased the photosynthetic rate per plant throughout the experimental period. However, the slopes of the plots are similar for both treatments. If photosynthetic rates are expressed per unit leaf area (Fig. 2) a reduction in rate with CO₂ enrichment is observed. Again, however, the slopes of the plots are similar. These results suggest that CO₂ enrichment causes an acceleration of the seedling (< 5 weeks) growth rate, but is without effect on latter stages of growth. The high CO₂ plants thus appear to have a 'head start' of about a week. Plant growth measurements (Fig. 3) support this interpretation. High CO₂ grown plants are larger than ambient plants with the growth curves offset by about a week.

The results of the photosynthetic and growth rate measurements, when adjusted for the difference in initial growth rate, show that long term CO₂ enrichment did not enhance crop photosynthesis or growth rate beyond the seedling stage. During the seedling stage, however, growth enhancement by CO₂ is apparent.

Metabolite Labelling. In the mature alfalfa crop, long term CO₂ enrichment does not increase the crop photosynthetic rate. To determine what effect, if any, long term CO₂ enrichment has on
Fig 1. Photosynthetic rate per plant of alfalfa grown at 350 ul l⁻¹ (●) and 1000 ul l⁻¹ (○) CO₂. Arrows indicate partial defoliation of the crop.
Fig 2. Photosynthetic rate per unit leaf area of alfalfa grown at 350 ul l⁻¹ (●) and 1000 ul l⁻¹ (○) CO₂. Arrows indicate partial defoliation of the crop. Dashed lines indicate that photosynthetic rate measurements taken two days after defoliation are less precise due to interplant variability in the remaining leaf area.
Fig 3. Dry weight per plant of alfalfa grown at 350 ul l⁻¹ (●) and 1000 ul l⁻¹ (○) CO₂. Arrows indicate partial defoliation of the crop. Inset shows growth of seedlings. Note expanded vertical scale.
plant metabolism, $^{14}$CO$_2$ feeding was employed. One week after partial defoliation, plants from each treatment were exposed to $^{14}$CO$_2$ (specific activity = 9.5) at the same concentration at which they were grown. After varying lengths of time, plants were harvested and metabolite labelling kinetics determined. The results show little difference between treatments in total incorporation (Fig. 4a) and in labelling kinetics for all compounds tested (data not shown) with the exception of glycolate (Fig. 4b). Glycolate labelling rate and pool size were substantially reduced by the high CO$_2$ treatment. Thus, with long term CO$_2$ enrichment, a considerable reduction in photorespiration occurs without a concommitment increase in photosynthetic rate.

**Short Term CO$_2$ Enrichment.** Several explanations are possible for the lack of response to long term CO$_2$ enrichment by the mature crop. If photosynthate requirements are adequately met at ambient CO$_2$ levels, any increase in supply as a result of CO$_2$ enrichment might be eventually damped down by the action of the regulatory system. Alternatively, in the mature crop the supply of photosynthate may be limited by some factor other than the level of CO$_2$. In this case, increasing the CO$_2$ levels would not result in an increase in photosynthesis.

To test these alternatives, plants were removed from the ambient CO$_2$ chamber at intervals of two weeks. Photosynthetic rates, before and after the addition of 1000 ul l$^{-1}$ CO$_2$, were measured using the steady state apparatus. The results (Table 1) show that short term CO$_2$ enrichment increases the rate of
Fig 4. Total photosynthetic $^{14}$C incorporation (A) and $^{14}$C incorporation into glycolate (B) in alfalfa grown at 350 ul l$^{-1}$ (●) and 1000 ul l$^{-1}$ (○) CO$_2$. 
Table 1. Effect of 1000 ul l⁻¹ CO₂ on photosynthetic rate of plants grown at 350 ul l⁻¹

Results are expressed as percentage increase in photosynthetic rate (mg CO₂ dm⁻² hr⁻¹) after one hour at 1000 ul l⁻¹ CO₂.

Values are the mean of five plants per time point.

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photosynthesis at all stages of plant growth, with seedling plants being the most responsive. These results suggest that, for short term exposures, CO₂ and not some other factor limits photosynthesis. That brief CO₂ exposures increase photosynthesis while long term enrichment does not is consistent with the interpretation that, at ambient CO₂ levels, the demand for photosynthate is adequately met by mature crop photosynthetic rates.

Defoliation Experiments. At ambient CO₂ levels, the highest attainable (maximal) photosynthetic rate may be equal to or greater than the rate required to meet the demand for photosynthate. If the maximal photosynthetic rate is greater than the required rate, increasing the demand for photosynthate should increase the rate of photosynthesis. Partial defoliation increases the demand for photosynthate from the remaining leaves, hence their photosynthetic rate should increase.

Plants from both treatments were partially defoliated when the crop reached the 10% bloom stage. Photosynthetic rate measurements (Fig. 2) show that, following defoliation, photosynthesis increased dramatically then gradually declined over a period of weeks to its initial value. The drop in photosynthesis per unit leaf area could be solely the result of shading by the upper leaves. However, the low leaf area index of the crop (under 1.5 for all but the last week) argues against this. A more plausible explanation is that defoliation induces a large increase in photosynthetic rate which gradually declines, at least in part, due to a reduction in demand
on individual leaves. The absence of any rate enhancement by high CO₂, even during the period immediately following defoliation, is also consistent with this interpretation. These results suggest that, over much of the life cycle of the crop, photosynthesis is occurring at less than maximal rates.

**Seedlings.** The young alfalfa crop appears to respond differently to CO₂ enrichment than do latter stages. Photosynthetic rates are enhanced to a greater degree in seedlings with a short term pulse of CO₂ (Table 1) as is dry matter accumulation under long term CO₂ enrichment (Fig. 3 inset). The increased dry weight of two week old high CO₂ grown seedlings is due almost exclusively to an increase in leaf starch content (data not shown). Analysis of leaves (at the beginning of the photoperiod) shows a very high level of leaf starch in high CO₂ plants that gradually declines as the plants mature (Fig. 5a). The tap root starch levels (Fig. 5b), in contrast, are similar for both treatments. Alfalfa seedlings, unlike mature plants, therefore appear to increase their photosynthetic rate in response to elevated CO₂ levels, the additional photosynthate being deposited as starch in the leaves.
Fig 5. Leaf (A) and tap root (B) starch content of alfalfa grown at 350 ul l⁻¹ (●) and 1000 ul l⁻¹ (○) CO₂. Arrows indicate partial defoliation of the crop.
Discussion

The question of whether maximum photosynthetic capacity (source) or photosynthate demand (sinks) limits crop photosynthesis was investigated in a simulated alfalfa crop using long and short term CO₂ enrichment and partial defoliation as probes. Nodulated alfalfa is a good model system for this type of study because nitrogen fixation is energetically expensive (18), resulting in plants with a large demand for photosynthate (22,23). A high photosynthate demand per leaf, resulting from nitrogen fixation and partial defoliation, might be expected to induce high and possibly maximal crop photosynthetic rates.

CO₂ enrichment is a useful probe for investigating source or sink limitation of dry matter accumulation. If an increase in photosynthetic rate with long term CO₂ enrichment occurs, this would imply, first, that photosynthate supply is inadequate at ambient CO₂ levels and, second, that CO₂ and not some other factor limits photosynthesis. In contrast, if no increase in photosynthesis with CO₂ enrichment is observed, this would suggest that either the demand is adequately meet at ambient CO₂ levels or that some factor other than CO₂ is limiting the photosynthetic rate.

In the experiments reported here, long term CO₂ enrichment did not increase the rate of photosynthesis (Figs. 1,2,5A) or the rate of dry matter accumulation (Fig. 3) in a mature (> 5 weeks) alfalfa
crop. This lack of effect occurred in spite of a substantial reduction in photorespiration (Fig. 4B). However, short term CO$_2$ enrichment did result in enhanced photosynthesis (Table 1). Similar results have occasionally been reported for other species (2,11,12,16,21).

In the mature alfalfa crop, therefore, instantaneous photosynthesis is limited by CO$_2$ and not by the capacity of the photosynthetic apparatus. That these increased rates are not maintained over the long term in spite of a reduction in photorespiration suggests that supply exceeds demand under these conditions.

Partial defoliation provides a second, independent, probe for source or sink limitation of photosynthesis. Removing most of the leaves from a plant should increase photosynthesis by the remaining leaves if they were not already photosynthesizing at maximal rates (sink limited). If maximal photosynthesis was occurring prior to defoliation, however, an increase in rate would not be expected (source limited). In general positive responses to defoliation have been reported in the literature (summarized in 8).

In the mature alfalfa crop partial defoliation dramatically increased photosynthesis by the remaining leaves (Fig. 2). These rates gradually declined as the canopy regrew. Although shading by the upper leaves could account for part of this response, it is likely that a significant proportion of the rate increase was due to the increased demand on the remaining leaves.

The results of the CO$_2$ enrichment and defoliation experiments support the conclusion that photosynthesis in the mature alfalfa
crop is sink limited. This does not appear to be the case for seedling crop however. Two weeks after planting high CO₂ grown seedlings are already larger than ambient grown plants (Fig. 3, inset) with increased starch storage in the leaves accounting for most of this increase (Fig. 5A). Although the difference in the levels of starch disappears by the seventh week, the growth advantage of the high CO₂ seedlings is maintained throughout the experimental period.

Seedlings are generally more responsive to short term CO₂ enrichment than are mature plants (12,16 and Table 1). The increase in dry weight observed in this study suggests that long term CO₂ enrichment produces a sustained increase in photosynthetic rate as well. These results imply that alfalfa seedlings are photosynthesizing at their maximal rate in ambient CO₂. Seedling growth may therefore be source limited.

A fundamental difference exists in the control of photosynthesis in seedling and mature alfalfa. In the mature plant under optimal conditions, photosynthesis is limited by the demand for photosynthate. This seemingly inefficient mechanism may, however, be advantageous for non-cultivated plants. Catastrophic loss of photosynthetic capacity as a result of grazing or disease is a common occurrence in the wild. The ability to maintain a constant supply of photosynthate under these conditions, by increasing the rate of photosynthesis, may be more important than the occasionally higher but more variable supply that would result from photosynthesizing at maximal rates. This is especially true during the reproductive stage where, in many species, the number of
seeds is determined by the pre-anthesis photosynthetic rate while the size of the seeds is a function of post-anthesis photosynthesis (4,6). Lack of photosynthetic flexibility could produce a situation where a pre-anthesis loss of photosynthetic capacity results in few seeds being produced. A post-anthesis loss might result in the production of many small but non-viable seeds. In contrast, the ability to increase photosynthesis over normal rates would insure that sufficient numbers of viable seeds are produced following a catastrophe.

For seedlings, however, the demand for photosynthate outstrips the supply, resulting in maximal rates of photosynthesis. The necessity for very rapid seedling growth, as a result of intense interspecies competition at this stage, may outweigh any disadvantage caused by the lack of photosynthetic flexibility.

The results presented above suggest that attempting to reduce the rate of photorespiration may not be the only way to increase crop yields. In many agricultural environments the yield of a plant is primarily determined in the reproductive stage (4,6,15). At this stage it is the demand for photosynthate, rather than the supply, that limits growth. Under these conditions, therefore, increasing photosynthate demand appears to offer the best hope for increasing yields.

In summary, CO₂ enrichment and defoliation were used to investigate the relationship between the supply and demand of photosynthate in a simulated alfalfa crop. Under optimal conditions for plant growth, seedling photosynthesis occurs at maximal rates. Once beyond the seedling stage, however, alfalfa does not
photosynthesize at its maximum attainable rate. These results suggest that the demand rather than the supply of photosynthate may be the major factor limiting the growth of the mature crop. The growth of seedlings, in contrast, may be limited by the supply of photosynthate.
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CHAPTER 11

SPINACH PYRUVATE KINASE ISOFORMS

PARTIAL PURIFICATION AND REGULATORY PROPERTIES
INTRODUCTION

The flow of carbon through plant pyruvate kinase is reported to vary with changes in the rates of mitochondrial respiration (24), photorespiration (13), and ammonia assimilation (10,12,18,20). Since these are essential processes in leaf metabolism it is important to define the regulatory mechanisms responsible for the resulting changes in enzyme activity.

Very little is known, however, about the regulatory properties of leaf pyruvate kinase. The only leaf enzyme that has been characterized with respect to regulatory effectors is that from tomato (1). This enzyme is strongly inhibited by ATP and slightly activated by AMP. In contrast, more information is available on the regulatory properties of the seed enzyme (4,5,8,15,17,23). As a consequence speculation on the nature of leaf pyruvate kinase regulation has of necessity relied on the regulatory properties identified for the seed enzyme.

Seed pyruvate kinase is responsive to changes in adenine nucleotide and citrate levels (4,8,19,23). Respiratory control of this enzyme is therefore possible via changes in the cytoplasmic energy charge and citrate levels (4,5). Less is known about the mechanisms of ammonia induced changes in enzyme activity.

The increased activity of pyruvate kinase during active NH₄⁺ assimilation or photorespiration may be due to an increase in the level of the enzyme substrate, ADP, as a consequence of enhanced ATP consumption (6,19). However, when ADP levels were measured
following addition of ammonia, the increase was slight and
temporary (10). Regulation of pyruvate kinase by NH₄⁺ or one of its
assimilation products is also a possibility. However, a direct
regulatory effect of ammonia on the enzyme is now considered
unlikely (6,19) and glutamate, glutamine, aspartate or asparagine
do not affect the activity of castor bean endosperm (17) or soybean
nodule (19) pyruvate kinase. At present we are therefore unable to
explain the activation of pyruvate kinase by increased rates of
photorespiration or ammonia assimilation based on the known
regulatory properties of the seed enzyme.

The objective of this study was to determine if leaf pyruvate
kinase has regulatory properties different from those of the seed
enzyme and, in particular, if these properties can explain the
regulatory effects of NH₄⁺ addition or photorespiratory conditions
on enzyme activity.
MATERIALS AND METHODS

Plant Materials. Spinach (Spinacea oleracea L. cv America) plants were raised in Vermiculite in a controlled environment chamber. The chamber provided a radiant flux density of 250 uE m\(^{-2}\) s\(^{-1}\) with a day length of eight hours and a temperature of 15\(^{\circ}\) C. The plants were watered three times weekly with modified one-half strength Hoagland solution. When the plants were six to eight weeks old, expanding leaves between one and three centimeters in length were harvested.

Sugarbeet (Betavulgaris L. cv Giant Western), alfalfa (Medicago sativa L. cv Moapa 69), and Zeadipperennis Ultis. plants were raised under conditions similar to those described for spinach. Chlorella pyrenoidosa was cultured essentially as in (10).

Preparation of crude extracts. Leaves (one gram) were homogenized in a ground glass tissue grinder in 3 ml of 50 mM Tris-HCL (pH 7.6) containing 15 mM MgSO\(_4\), 20% (v/v) ethylene glycol, and 5 mM DTT (buffer A). To this was added 2% (w/v) insoluble polyvinylpyrrolidone. The homogenate was centrifuged at 20,000g for 20 min. This step and all subsequent steps were performed at room temperature due to the cold lability of the enzymes.

Chlorella cells were pelleted by centrifugation at 100g for two min. One ml packed pellet was mixed with 3 ml buffer A plus PVP and sonicated for 5 min. The homogenate was centrifuged at 20,000g for 20 min.

Partial Purification. The supernatant (3 ml) from the crude extract was applied to a 0.5 X 40 cm column of blue agarose
(Reactive Blue Agarose, Affigel Blue) that had been equilibrated with buffer A. The column was washed with 15 ml of the same buffer. Pyruvate kinase was then eluted by a linear KCl gradient (0.0 to 0.5M) at a flow rate of 0.2ml/min. The most active fractions were used immediately for kinetic and effector studies. Fresh enzyme preparations were prepared daily. Enzyme preparations used for determination of Mg\(^{2+}\) and K\(^+\) kinetic constants were desalted in a 1 X 10 cm Sephadex G-25 column using buffer A minus MgSO\(_4\) for equilibration and elution.

**Enzyme Assays.** The standard pyruvate kinase assay mixture contained 50 mM Mops-KOH (pH 7.1), 1.6 mM ADP, 1.0 mM PEP, 0.1 mM NADH, 15 mM MgSO\(_4\), 50 mM KCl, and four units of lactate dehydrogenase in a total volume of 1.0 ml. Activity was measured as a decrease in absorption at 340 nm in a Cary 118 spectrophotometer. One unit of enzyme activity is defined as the amount of enzyme that causes the formation of 1 umole of pyruvate per minute at 25\(^0\)C. Kinetic constants were calculated according to the procedure of Wilkinson (28) using a FORTRAN program developed for the VAX-11/780 computer. PEP carboxylase (19) and adenylate kinase (2) were assayed according to published procedures. PEP 'phosphatase' activity was monitored by omitting ADP from the pyruvate kinase assay mixture.

**Molecular Weight Determination.** Three ml of PK\(_I\) or PK\(_{II}\) samples from the blue agarose preparation were applied to a 5 x 20cm column of Sephacryl S-400 that had been equilibrated with buffer A. Elution was with the same buffer at a flow rate of 3ml/min. The column was calibrated with cytochrome C, gamma globulin, ferritin,
and thyroglobulin.

Protein Determination. Protein concentrations in the crude extract and in the blue agarose fractions were determined by the method of Bradford (3) using gamma globulin as a standard.

Chemicals and Equipment. Ethylene glycol (99%) was obtained from Aldrich. All other chemicals were purchased from Sigma. KCL concentrations were measured using a YSI model 31 conductivity meter.
RESULTS

Partial Purification. Blue agarose chromatography of the crude extract supernatant yields two peaks of PK activity (PK\textsubscript{I} and PK\textsubscript{II}) (Fig. 1). The possibility that one of the peaks might be an artifact was investigated by adding 10 uM leupeptin, 10% (w/v) polyvinylpyrrolidone, or 2% (w/v) gamma globulin to the extraction buffer or by running the column at 4 °C. The relative amount of each isofrom was not altered by these treatments. In addition, desalted PK\textsubscript{I} and PK\textsubscript{II} fractions were re-chromatographed on blue agarose after one week of storage. The enzyme from each fraction eluted as a single peak at the KCl concentration characteristic for that isofrom. These results support the conclusion that the two peaks represent isofroms rather than artifacts or degradation products.

In the preparation shown in Fig. 1, the specific activities of the most active fractions are 0.93 and 1.57 units/mg protein for PK\textsubscript{I} and PK\textsubscript{II} respectively. In the crude extract, total pyruvate kinase specific activity is 0.018 units/mg protein. The ratio of PK\textsubscript{I} to PK\textsubscript{II} following chromatography is about 1:1 in this preparation. Assuming that this ratio is not altered by the chromatography step, the specific activity of each isofrom in the crude extract is roughly 0.009 units/mg protein. Blue agarose chromatography therefore results in an approximate purification of 100 fold for PK\textsubscript{I} and 175 fold for PK\textsubscript{II}.

PK\textsubscript{I} and PK\textsubscript{II} fractions were assayed for enzymes which might
Fig. 1 Gradient elution of spinach leaf pyruvate kinase isoforms on blue agarose.
interfere with the the pyruvate kinase reaction. In both fractions the specific activities of PEP carboxylase, PEP 'phosphatase', and adenylate kinase are less than 0.020 units/mg protein. Based on the absence of contaminating enzyme activity, the PK preparations obtained after blue agarose chromatography, although not homogeneous on SDS-PAGE, were judged suitable for kinetic and effector studies.

Stability. PK_I is stable following blue agarose chromatography, losing less than 20% of initial activity after one month at 80°C. The addition of ADP results in a 50% loss of activity after one month. The addition of PEP has no effect. The enzyme is unstable to cold. In the absence of substrates, 50% of the activity is lost after one month at 4°C, 70% at -20°C, and greater than 95% at -70°C. The addition of PEP reduces this loss somewhat but with added ADP, the loss of activity increases.

PK_II is unstable following blue agarose chromatography, losing 50% of its activity after five days at 80°C. PEP stabilizes the enzyme, 75% of the activity remaining after one month at 80°C. ADP is less effective in stabilizing activity, 40% remaining after one month. PK_II is also unstable to cold, in the presence of PEP the rates of loss are similar to those found for PK_I.

Effect of pH. The pH profiles are broad, with 80% of maximal activity occurring between pH 5.5 and 7.5 for both isoforms (data not shown). Broad profiles centered between pH 6 and 7 have been reported for pyruvate kinase from a number of species (5,17,23) and appear to be characteristic of the cytoplasmic isoenzyme. The plastid isoenzyme, in contrast, exhibits a narrow profile with an
optimum of about pH 8.0 (7,8).

**Molecular Weight.** Gel filtration chromatography on Sephacryl S-400 indicated a molecular weight of 215,000 + 15,000 for PK\textsubscript{I} and 220,000 + 15,000 for PK\textsubscript{II}. Similar values have been reported for animal PK (16).

**Substrate dependence and metal requirements.** \(K_m\) values for PEP, ADP, \(K^+\), and \(Mg^{++}\) are presented in Table 1. In all cases hyperbolic kinetics were observed. The kinetic constants are similar for both isoforms and fall within the range reported in the literature: 20 to 85 \(\mu\)M for PEP (4,8,14,17,23), 20 to 150 \(\mu\)M for ADP (4,8,14,17,23), 0.5 to 1.5 mM for \(Mg^{++}\) (8,14,23), and 0.5 to 7 mM for \(K^+\) (1,8,14,27).

**Alternate Nucleotides.** \(K_m\) values for ADP, UDP, GDP, and CDP are presented in Table I. There is little difference between PK\textsubscript{I} and PK\textsubscript{II} in nucleotide specificity, and ADP is clearly the preferred substrate. Nucleotide specificity has also been investigated for the castor bean endosperm (8) and leaf (7) enzymes. In castor bean the plastid isozyme had \(K_m\) values for all nucleotides approximately an order of magnitude higher than the cytoplasmic form.

**Effectors.** The following compounds had little (less than 10%) or no effect on the activity of either isoform when tested at \(K_m\) concentrations of substrates (PEP, 40 \(\mu\)M; ADP, 100 \(\mu\)M) with metabolite concentrations at 5 mM unless otherwise noted: Alanine, glycine, serine, threonine, asparagine, glutamine, phenylalanine, tyrosine, histidine, arginine, lysine, methionine, isoleucine, leucine, valine, proline, glucose-6-phosphate, dihydroxyacetone phosphate, 3-phosphoglyceric acid, glyceraldehyde-3-phosphate,
Table 1. Michaelis Constants for Spinach
Pyruvate Kinase Isoforms.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Michaelis Constant $^a$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pyruvate Kinase I</td>
</tr>
<tr>
<td>PEP</td>
<td>0.038 ± 0.004(5)</td>
</tr>
<tr>
<td>ADP</td>
<td>0.101 ± 0.019(5)</td>
</tr>
<tr>
<td>Mg++</td>
<td>0.20 ± 0.05(2)</td>
</tr>
<tr>
<td>K+</td>
<td>1.1 ± 0.2(2)</td>
</tr>
<tr>
<td>UDP</td>
<td>0.72 ± 0.04(2)</td>
</tr>
<tr>
<td>GDP</td>
<td>1.1 ± 0.1(2)</td>
</tr>
<tr>
<td>CDP</td>
<td>9.0 ± 1.7(2)</td>
</tr>
</tbody>
</table>

$^a$Standard errors are shown with the number of enzyme preparations in parenthesis.
uridine diphosphoglucose, adenine diphosphoglucose, fructose-1,6-bisphosphate, fructose-2,6-bisphosphate (100 uM), fructose, glucose, sucrose, inorganic phosphate (20 mM), pyrophosphate, glutathione_red, glutathione_ox, acetyl CoA (1 mM), CoA (1 mM), NAD, NADH, NADP, NADPH, NH₄Cl, ascorbate, fumarate, tartrate, acetate, malate, succinate, isocitrate.

A lack of response to fructose 1,6 bisphosphate is found in most (4,17,19,23) but not all (8) studies of higher plant pyruvate kinase. In contrast, the enzyme from animal liver (11,16) is sensitive to this compound.

Several compounds either activated or inhibited one or both of the isoforms. These compounds are listed in Table II together with the % activation or inhibition. Of these, oxalate, ATP, AMP, citrate, glutamate, and aspartate were selected for further characterization.

**Oxalate.** Oxalate is a competitive inhibitor with respect to PEP for both PK₁(Fig. 2a) and PK₁(data not shown) but is without effect when ADP is the varied substrate. The inhibitor constants of 300 uM for PK₁ and 270 uM for PK₁₁ are similar to the value of 400 uM reported for the enzyme from mung bean seeds (15), but much higher than the 6 uM reported for rabbit muscle PK (21).

**ATP.** ATP at a concentration of 5 mM is a competitive inhibitor of PK₁₁ (Fig. 2b) and PK₁(data not shown) with respect to PEP. Increasing the magnesium concentration from 15 mM to 20 mM did not alter the extent of inhibition. With ADP as the varied substrate, ATP causes less than a 10% reduction in rate for either isoform.
Table II. Effect of Various Metabolites on the Activity of Spinach Pyruvate Kinase Isoforms a

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Pyruvate Kinase I</th>
<th>Pyruvate Kinase II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxalate</td>
<td>17</td>
<td>10</td>
</tr>
<tr>
<td>Citrate</td>
<td>51</td>
<td>93</td>
</tr>
<tr>
<td>α-Ketoglutarate</td>
<td>87</td>
<td>90</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>82</td>
<td>73</td>
</tr>
<tr>
<td>Glutamate</td>
<td>94</td>
<td>8</td>
</tr>
<tr>
<td>Aspartate</td>
<td>113</td>
<td>142</td>
</tr>
<tr>
<td>ATP</td>
<td>89</td>
<td>86</td>
</tr>
<tr>
<td>AMP</td>
<td>111</td>
<td>133</td>
</tr>
<tr>
<td>Adenine</td>
<td>89</td>
<td>80</td>
</tr>
</tbody>
</table>

Substrate concentrations were 40 μM for PEP and 100 μM for ADP. Metabolite concentrations were 5 mM. Assay conditions as described in Materials and Methods.
Fig. 2 Double reciprocal plots of the inhibition of pyruvate kinase II by oxalate (A) and ATP (B) and activation by AMP (C,D). Different enzyme preparations were used for each experiment.
PKII is slightly more sensitive to ATP inhibition than is PKI but at physiological levels of ATP, 0.3 mM (29), the effect of ATP on either isoform is minimal.

AMP. Activation by AMP was found for both isoforms. The data for PKII is shown in Fig. 2c,d. Similar plots were obtained for PKI (data not shown). AMP at 10 mM increased enzyme activity with either PEP (Fig. 2c) or ADP (Fig. 2d) as the varied substrate. The activation appears to be uncompetitive with respect to either substrate. At concentrations of AMP reported to occur in vivo, 0.1 to 0.3 mM (29), AMP activation of either isoform is not significant.

Citrate. Citrate inhibition of PKI is competitive with respect to PEP (Fig. 3), with ADP as the varied substrate only a slight inhibition was observed. Inhibition is not reversed by 5 mM malate, isocitrate, succinate, or acetate or 100uM AMP or 20 mM magnesium. A replot of the slope of the reciprocal plot against citrate concentration (Fig. 3, inset) shows that inhibition plateaus above 2 mM citrate without completely inactivating the enzyme. The $K_i$ for citrate, calculated according to Segel (22) is 1.2 mM. This type of inhibition kinetics, partial competitive, has not been previously reported for pyruvate kinase.

Partial inhibition kinetics could result if the preparation contained a mixture of enzymes, one of which is insensitive to citrate inhibition. This possibility was investigated by rechromatographing the enzyme preparation on DEAE sephadex. One peak of pyruvate kinase activity was observed (data not shown). This result is consistent with the presence of a single enzyme in the
Fig 3. Double reciprocal plot for pyruvate kinase 1 with respect to PEP in the presence of several fixed concentrations of citrate. The inset is a replot of the slopes of the primary plot against citrate concentration.
original preparation.

Glutamate. Glutamate acts as a mixed-type inhibitor of PKII with respect to PEP (Fig. 4a). A replot of the slope of the reciprocal plot verses glutamate concentration (Fig. 4a, inset) shows this inhibition to be partial, with a $K_i$ of 680 uM. Glutamate inhibition of PKII with respect to ADP is competitive (data not shown) with a $K_i$ of 5.0 mM. A single peak of enzyme activity was observed following re-chromatography on DEAE sephadex (data not shown) suggesting that, as with PKI, partial inhibition kinetics were not due to a mixed enzyme preparation.

The ability of other amino acids to reverse glutamate inhibition was investigated. Of the twenty protein amino acids, only aspartate was effective in reversing the inhibition.

Aspartate. Fig. 4b shows the effect of varying the aspartate concentration on the activity of PKII with respect to PEP. Aspartate activates the enzyme and a replot (Fig. 4b, inset) shows the kinetics to be partial. The activation constant for aspartate is calculated to be 50 uM. With ADP as the varied substrate no activation is observed.

Aspartate and Glutamate Interaction. Fig. 5 shows the effect on PKII of varying the amount of aspartate at several fixed glutamate levels. Aspartate concentrations below 0.5 mM have a pronounced effect on enzyme activity in the presence of 0.5 or 1.0 mM glutamate. At 5.0 mM glutamate, increasing aspartate levels produce a more gradual increase in enzyme activity.
Fig 4. Double reciprocal plots for pyruvate kinase II with PEP as the varied substrate in the presence of several fixed levels of glutamate (A) or aspartate (B). Insets are replots of primary plot slopes versus glutamate (A) or aspartate (B) concentrations.
Fig 5. Effect of aspartate on the activity of pyruvate kinase II in the absence or presence of several fixed concentrations of glutamate. Percent activity is based on the rate obtained in the absence of aspartate and glutamate. Substrate concentrations for this experiment were 40 μM for PEP and 100 μM for ADP.
Presence of PK Isoforms in Different Plant Organs. Spinach leaves of different ages, stem segments, fibrous and tap roots, developing and mature seeds, and germinating seeds were tested for the presence of both isoforms. In all cases both PK\textsubscript{I} and PK\textsubscript{II} were observed.

PK Isoforms in other Species. Leaf extracts of sugarbeet, alfalfa, and Zea diploperennis; cell extracts of Chlorella; and a commercially available preparation of dog muscle pyruvate kinase were applied to blue agarose columns and eluted with a KCl gradient. The elution profiles (Fig. 6) show that sugarbeet PK is clearly composed of two isoforms. Clear separation of isoforms was not observed for the other plant samples. However, the presence of shoulders on the main PK peaks suggests that poorly resolvable isoforms are present in these species. The animal muscle preparation, in contrast, consists of a single, clearly definable peak.

Kinetic and effector constants were determined for the two isoforms present in sugarbeet leaf. Km values for PEP and ADP, pH profiles, and oxalate, glutamate, aspartate, and citrate inhibition or activation constants were essentially identical to those obtained for the spinach leaf isoforms.

For the remaining species fractions on either side of the main peak were assayed for the extent of inhibition by citrate and glutamate. The higher plant and algal samples showed greater citrate inhibition of PK activity in fractions eluting at a lower ionic strength. Fractions eluting at higher ionic strengths were preferentially inhibited by glutamate. This differential inhibition
Fig 6. Gradient elution of sugarbeet (A), alfalfa (B), Zea diploperennis (C), Chlorella pyrenoidosa (D), and dog muscle (E) pyruvate kinase from blue agarose.
pattern is consistent with the presence of two (poorly definable) isoforms in these species. The dog muscle preparation, in contrast, was not significantly inhibited by either citrate or glutamate.
DISCUSSION

Spinach leaf pyruvate kinase consists of two isoform separable by blue agarose chromatography. These isoforms are present in all plant organs. Similar isoforms are found in sugarbeet leaves and may be present in many plant (but not animal) species. At present we do not know whether PK\textsubscript{I} and PK\textsubscript{II} are the products of separate genes or are the result of post-translational modifications. Both isoforms are cold labile and share similar pH profiles, molecular weights, and substrate and alternate nucleotide $K_m$ values. In addition both isozymes are inhibited by oxalate and ATP and activated by AMP. The properties of spinach PK\textsubscript{I} and PK\textsubscript{II} are summarized in Table III.

Oxalate inhibition of pyruvate kinase has been reported previously (15,21) and is thought to result from the close structural similarity between oxalate and the enolate form of pyruvate (21). The 100 fold difference in inhibition constants between animal and plant pyruvate kinases may result from the requirement that the plant enzyme function in an environment relatively high in oxalate (15). Many plants, including spinach, contain large amounts of oxalate, some of which is localized in the cytoplasm (26). Measurements of the size of this pool are necessary in order to determine whether oxalate inhibition is physiologically significant.

ATP inhibition is widespread among plant pyruvate kinases (1,4,8,17,19,23). However, at levels of ATP reported to occur in
<table>
<thead>
<tr>
<th>Property</th>
<th>Pyruvate Kinase 1</th>
<th>Pyruvate Kinase 11</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stability</td>
<td>cold labile</td>
<td>cold labile</td>
</tr>
<tr>
<td>pH optima</td>
<td>5.5 - 7.5</td>
<td>5.5 - 7.5</td>
</tr>
<tr>
<td>Molecular Weight</td>
<td>215,000 ± 15,000</td>
<td>220,000 ± 15,000</td>
</tr>
<tr>
<td>Substrate Km values (mM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PEP</td>
<td>0.038</td>
<td>0.045</td>
</tr>
<tr>
<td>ADP</td>
<td>0.101</td>
<td>0.114</td>
</tr>
<tr>
<td>Mg&lt;sup&gt;++&lt;/sup&gt;</td>
<td>0.2</td>
<td>0.4</td>
</tr>
<tr>
<td>K&lt;sup&gt;+&lt;/sup&gt;</td>
<td>1.1</td>
<td>2.2</td>
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<tr>
<td>Alternate Nucleotide Km values (mM)</td>
<td></td>
<td></td>
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<tr>
<td>UDP</td>
<td>0.72</td>
<td>0.73</td>
</tr>
<tr>
<td>GDP</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>CDP</td>
<td>9.0</td>
<td>6.8</td>
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<tr>
<td>Effector Kinetic Constants (mM)</td>
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<tr>
<td>Oxalate</td>
<td>$K_i = 0.3$</td>
<td>$K_i = 0.4$</td>
</tr>
<tr>
<td>Citrate</td>
<td>$K_i = 1.2$</td>
<td>no effect</td>
</tr>
<tr>
<td>Glutamate</td>
<td>$K_i = 5.0$</td>
<td>$K_i = 0.68$</td>
</tr>
<tr>
<td>Aspartate</td>
<td>no effect</td>
<td>$K_a = 0.05$</td>
</tr>
</tbody>
</table>
in vivo, 0.3 mM (29), inhibition is usually less than 15%. In the present study, both spinach leaf isoforms were also inhibited by ATP but at 1.0 mM the inhibition was slight.

With the exception of cotton seed pyruvate kinase (4), AMP does not significantly activate the plant enzyme. In the cotton seed study, AMP activation occurs at physiological levels of this nucleotide, 0.1 mM (29), and is capable of partially reversing the inhibition caused by citrate. We have found the spinach leaf isoforms to be insensitive to physiological levels of AMP and, for PKI, 0.1 mM AMP does not reverse citrate inhibition.

Citrate is an effective inhibitor of PKI with a Ki of 1.2 mM. This inhibition is competitive with respect to PEP. Citrate inhibition of pyruvate kinase has been found in most (4,8,19,23) but not all (17) studies of the plant enzyme. In all cases where inhibition was reported, the kinetics were hyperbolic with no evidence for allosteric interactions. In contrast, the partial competitive inhibition of spinach leaf PKI by citrate suggests that, although the kinetics are hyperbolic, citrate may interact with a regulatory site on the enzyme.

Spinach leaf PKII is inhibited by glutamate with a Ki of 680 uM and is activated by aspartate with a Ka of 50 uM. Glutamate is a partial mixed-type inhibitor and aspartate a partial competitive activator of PKII with respect to PEP. The partial kinetics displayed by this isoform suggest that, as with PKI, PKII may possess one or more regulatory (allosteric) sites. As aspartate can reverse the inhibition by glutamate, it is the ratio of these compounds that determines the activity of the enzyme. This is the
first report of metabolites involved in NH$_4^+$ assimilation affecting the activity of plant pyruvate kinase. The negative results in previous papers (4,17,19) may be due to the absence of PK$_{II}$ in the tissues studied or to enzyme instability.

The sensitivity of PK$_{I}$ to citrate and the low ADP $K_m$ of this isoform, close to the level found in vivo (23,29), suggests that these metabolites are involved in respiratory control of pyruvate kinase activity. This conclusion is similar to that reached for the enzyme isolated from non-photosynthetic tissues (4,5).

In contrast, the regulatory properties of PK$_{II}$ suggest that this isoform is involved in supplying additional carbon skeletons for NH$_4^+$ assimilation. (shown schematically in Fig. 7). Leaf pyruvate kinase activity increases under conditions of enhanced ammonia assimilation (10,12,18,20) or photorespiration (13) and under these conditions the balanced production of carbon skeletons for use as NH$_4^+$ acceptors may require different regulatory effectors than those utilized for respiratory control. For example, ADP regulation of enzyme activity may not play the same role in carbon skeleton production as it does in respiratory control since ADP levels do not vary significantly following NH$_4^+$ addition, at least in algae (10). In higher plants several studies show no increase in ADP levels under these conditions, one study did, however, show an increase in ADP (for review see 6).

In contrast, changes in the levels of glutamate and aspartate following NH$_4^+$ addition could explain the observed activation of pyruvate kinase. In spinach cells, fifteen minutes after the addition of NH$_4^+$, the total pool size of aspartate increased 50%
Fig 7. Metabolic consequences of aspartate activation and glutamate inhibition of Pyruvate Kinase II. Open arrows represent metabolite flow under conditions of increased NH$_4^+$ assimilation. Closed arrows represent flow under control conditions. Dashed lines show activation of pyruvate kinase by aspartate and inhibition by glutamate.
while that of glutamate decreased by 35% compared to the control (12). Reciprocal changes in the rates of aspartate (10,12,18,30) and, in some studies, glutamate (10,12,18) synthesis have also been reported. Total pool size measurements include contributions from a slowly turning over (presumably vacuolar) pool containing approximately 80% of the cellular aspartate and 50% of the glutamate (12,25). Therefore the effect of ammonia addition on the metabolically active pools is likely to be closer to a 300% increase for aspartate and a 70% decrease for glutamate. Measurements of the aspartate/glutamate ratio in the cellular compartment containing PKII, both before and after NH4+ addition, are necessary before concluding that these metabolites are physiologically important regulators of pyruvate kinase. However, the magnitude of the observed pool size changes makes this a reasonable assumption.

Isozymes of pyruvate kinase have also been found in pea and castorbean leaves (7) and in the seeds of several species (8,9). These isozymes were reported to occur in different cellular compartments (cytoplasm and plastids) and could be distinguished by pH profiles and nucleotide Km values. Based on these two criteria, both isoforms found in the present study resemble the cytoplasmic isozyme.

In summary, the two forms of pyruvate kinase from spinach leaves appear to be regulated according to the needs of different metabolic pathways. The inhibition of PKI by citrate and the responsiveness of this enzyme to ambient ADP levels suggests that this enzyme is a site for respiratory control of glycolysis.
change in ADP levels could also affect the activity of PK\textsubscript{II}. The activation of PK\textsubscript{II} by aspartate and the inhibition by glutamate suggests that this isoform is, however, primarily regulated by the cellular need for carbon skeletons for use in ammonia assimilation. The reciprocal change in the pool sizes of aspartate and glutamate following NH\textsubscript{4\textsuperscript{+}} addition (12) supports this hypothesis.
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This report was done with support from the Department of Energy. Any conclusions or opinions expressed in this report represent solely those of the author(s) and not necessarily those of The Regents of the University of California, the Lawrence Berkeley Laboratory or the Department of Energy.

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