Title

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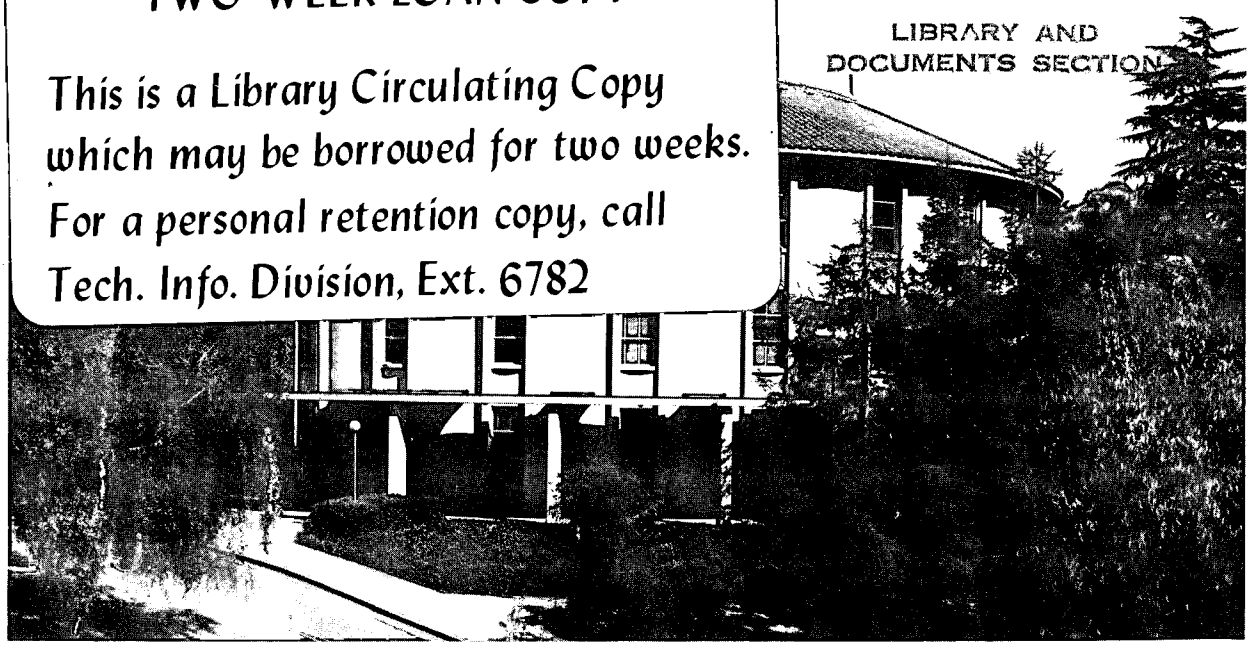

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DISCLAIMER

STIMULATION OF DARK CO_2 FIXATION BY AMMONIA IN ISOLATED MESOPHYLL CELLS OF *PAPAVER SOMNIFERUM* L

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ABSTRACT

Addition of 2 mM ammonium ion to isolated mesophyll cells of Papaver somniferum resulted in a 3-fold or greater increase in their rate of dark ^{14}C fixation, while even 0.1 mM NH_4^+ nearly doubled that rate. The most rapid increase in the labeling of a metabolite occurred is aspartate, and was accompanied by a decrease in the steady-state level of labeled phosphoenolpyruvate. No change in labeled pyruvate level occurred, and alanine labeling declined. Ammonium ion addition had no effect on the respiratory rate of these cells in the dark. We conclude that NH_4^+ stimulates phosphoenolpyruvate carboxylation in this system, but has no detectable effect on the pyruvate kinase reaction in the dark. Our results are compared with earlier findings and the possible regulatory function of ammonia is discussed.

KEY WORDS: Ammonia
Dark CO_2 fixation
Mesophyll
Papaver
Phosphoenolpyruvate carboxylase

ABBREVIATIONS:

C	carbon
Chl	chlorophyll
µE	micro-einstein
PEP	phosphoenolpyruvate
PEPC	phosphoenolpyruvate carboxylase

INTRODUCTION

In photosynthesizing leaves which reduce nitrate, disposal of the potentially toxic ammonia produced requires a supply of carbon skeletons for amination. This production of amino acids, probably mediated by glutamine synthetase and glutamate synthase (13), depletes the citric acid cycle of 2-oxoglutarate, which must be replenished by acceleration of glycolysis, several citric acid cycle reactions, and dark CO_2 fixation. It is reasonable to suggest, then, that increased cellular ammonia levels might constitute the message that stimulates organic acid production.

In Chlorella, ammonium salts have been shown to stimulate carbon flow into amino acids and intermediates of the citric acid cycle, as well as the rates of dark CO_2 fixation and respiration (8,9,15). More recently, radiolabeling studies with higher plants have demonstrated increased levels of amino acids and related compounds following ammonia addition in leaf discs (21) and isolated mesophyll cells (17) during photosynthesis. The effect in mesophyll cells was attributed to stimulation of the enzymes pyruvate kinase (EC 2.7.1.40) and phosphoenolpyruvate carboxylase (EC 4.1.1.31) (17,21), but increased dark CO_2 fixation was not shown directly.

We report here continued work with isolated mseophyll cells of Papaver somniferum. We have taken advantage of their high quality and suitability for rapid sampling (16) to study fluctuations in radiocarbon flow immediately following ammonia addition in the dark. These experiments present evidence that ammonia stimulates phosphoenolpyruvate carboxylation in mesophyll cells and suggest that this effect is independent of ammonia effects on glycolytic and respiratory rates.

MATERIALS AND METHODS

Cell isolation: Mesophyll cells from Papaver somniferum were isolated as previously described using a commercial preparation of pectinase (Macerase, Calbiochem) (16). After 24 hr in assay medium lacking inorganic nitrogen (12 hr dark, 4°C; 12 hr light, 24°C), cell suspensions containing 20-40 μg chlorophyll·ml^{-1} were prepared for ^{14}C-uptake experiments. The cells used appeared intact by light microscopy, showed no evidence of plasmolysis, and exhibited high rates of photosynthetic CO_2 fixation (ca. 100 μg atoms carbon·mg chlorophyll^{-1}·hr^{-1}).

Dark CO_2 fixation: 1 ml samples of cell suspension in assay medium, pH 8, were placed in serum-stoppered 5 ml Erlenmeyer flasks, enclosed in black bags, and placed on the rotary shaker-water bath described previously (7). Uptake was initiated by addition of 5 mM $NaH^{14}CO_3$. 100 μl samples were removed at intervals with a syringe and killed in 80% methanol. Ammonia was added as NH_4Cl after an initial rate had been established.

Photosynthetic prelabeling: 1.5 ml samples of cell suspension were placed in serum-stoppered 5 ml Erlenmeyer flasks fitted with inlet and and outlet ports, placed on the rotary shaker, and illuminated from below with fluorescent lamps (450 μE·m^{-2}·sec^{-1}). The ports were attached to a closed steady-state gas circulation system (20), and photosynthesis was initiated by the addition of 0.12% $^{14}CO_2$ in air. After 30 minutes of photosynthesis, the lights were turned off and flasks placed in black bags. 5 mM NH_4Cl was added after 20 minutes of darkness. Samples were taken as in the dark fixation experiments.

For analysis of 2-oxoacids, greater incorporation of radiolabel was required. Flasks of cells were prepared as described above and allowed to photosynthesize for 60 minutes. 3 mM NH_4Cl was added after 45 minutes of

of darkness. 100 µl samples of cell suspension were injected at intervals into 0.1% dinitrophenylhydrazine in 1 N HCl.

Analysis of labeled metabolites: Separation and identification of most ^{14}C-labeled products was done by paper chromatography and radioautography as previously described (18,21). 2-oxoacids were converted to their 2,4-dinitrophenylhydrazones by the method of Bachelard (3) and spotted on 250 micron cellulose thin-layer plates (Analtech). The plates were developed in n-butanol:ethanol:0.5 N NH_4OH:: 7:1:2 for 4 hr in the first dimension, whereupon the 2-oxoglutarate derivative was removed for counting. The pyruvate dinitrophenylhydrazone was then developed in n-butanol: propionic acid:H_2O:: 74:36:49 for 3.5 hr in the second dimension (19,21). ^{14}C incorporation was determined by liquid scintillation and expressed as µg atoms carbon incorporated per mg chlorophyll. Chlorophyll was measured according to the method of Arnon (1).

Oxygen uptake: The respiratory rate of the cells was measured in the dark using a Beckman oxygen electrode inserted into a 1 ml plexiglass cuvette. The cell suspension was stirred with a magnetic bar, and the cuvette was maintained at 25°C using a recirculating water bath.

RESULTS

Addition of 2 mM ammonium chloride caused a rapid three- to four-fold increase in the rate of dark CO_2 fixation in isolated mesophyll cells of P. somniferum. Even 0.1 mM NH_4Cl nearly doubled the rate (Table 1). The earliest and most marked change in the rate of labeling occurred in aspartate, while increases also occurred in malate, citrate, and glutamate (Fig. 1-2). Labeling of alanine and phosphoenolpyruvate was negligible in

dark fixation experiments, as would be expected for PEP carboxylation, which labels only C4 of oxaloacetate and malate. Alanine or PEP might be produced in these cells by the decarboxylation of malate or oxaloacetate, but would contain ^{14}C only to the extent that C1 and C4 equilibrate via fumarase.

In cells prelabeled in the light and subsequently placed in the dark, ammonia caused a large transient rise in aspartate labeling and a rapid drop in the steady-state level of phosphoenolpyruvate (Fig. 3). The pyruvate level was unaffected by ammonia, while alanine labeling declined (Fig.4). Reproducible results were not obtained for oxaloacetate, probably because of spontaneous decarboxylation of its dinitrophenylhydrazone derivative (2). Ammonia consistently caused a drop in glutamate labeling, while 2-oxoglutarate and glutamine labeling increased (not shown). These data concur with the view that glutamine synthesis in photosynthetic cells can occur at the expense of glutamate and ATP in the dark, while net production of glutamate from 2-oxoglutarate and glutamine is a light-dependent reaction requiring reduced ferredoxin in the chloroplast (6,13).

Experiments with the oxygen electrode (not shown) indicated that isolated P. somniferum mesophyll cells respire in the dark at a rate of approximately 20 μmoles $O_2 \cdot mg\ chlorophyll^{-1} \cdot hr^{-1}$. This rate was unaffected by concentrations of NH_4Cl up to 20 mM.

DISCUSSION

If catalytic levels of citric acid cycle intermediates are to be maintained, net amino acid synthesis must be accompanied by anapleurotic reactions to supply the necessary carbon skeletons. Other experiments have demonstrated the stimulatory effect of ammonia on glycolysis at the

step of pyruvate synthesis (8,9,17,21). Our results show directly that low levels of ammonium ion stimulate dark CO_2 fixation in isolated mesophyll cells. Two findings suggest that this stimulation is due to increased phosphoenolpyruvate carboxylase activity: In dark experiments, the most abrupt change in the rate of ^{14}C uptake occurred in aspartate (Fig. 1), thought to be in rapid equilibrium with oxaloacetate, the primary product of phosphoenolpyruvate carboxylation (17). Also, in prelabeling experiments, we observed that the rapid rise in aspartate labeling was accompanied by a drop in steady-state phosphoenolpyruvate levels (Fig. 3). Paul et al. (17) have argued on thermodynamic grounds that PEPC fulfils the anapleurotic function in mesophyll cells.

Phosphoenolpyruvate carboxylase has been assigned a cytoplasmic location (11; Hammel and Bassham, unpublished). Ammonia produced by nitrate reduction in leaves could most easily act as a modulator of carbon flow into amino acids if changes in chloroplastic ammonia levels resulted in corresponding adjustments in cytoplasmic ammonia levels. The permeability of the chloroplast to ammonia (14) and the energetics of the glutamate dehydrogenase reaction, which probably favor deamination (13) support the existence of such an interaction. Furthermore, recent findings suggest that extensive cytoplasmic ammonia release and reassimilation play a major role in the nitrogen economy of green cells (10). Thus, high cytoplasmic levels of ammonia, reflecting increased nitrate reduction in the chloroplast, could stimulate the production of carbon skeletons by phosphoenolpyruvate carboxylase as required for amino acid synthesis.

It is noteworthy that NH_4^+ causes no apparent increase in the level of pyruvate in these cells in the dark, as opposed to the results obtained

in the light (17). This conclusion is supported by the lack of change in the level of pyruvate labeling and by the drop in labeled alanine induced by ammonia (Fig. 4). Furthermore, no increase in respiratory rate was observed in the presence of NH_4^+. An increase in that rate would be expected if increased pyruvate utilization via acetyl CoA had occurred. Thus, we attribute the observed drop in phosphoenolpyruvate level to increased dark CO_2 fixation and not to a stimulation of pyruvate kinase. Our results also differ from those obtained with Chlorella, in which ammonia enhanced both pyruvate labeling (9) and respiratory rate (15) in the dark. While ammonia effects on pyruvate kinase have been ascribed to decreased ATP levels (5,9,17), the effect on phosphoenolpyruvate carboxylase appears to occur independently in mesophyll cells, and is presumably due to some other mechanism.

Nitrate supplied to the roots of whole plants stimulates organic acid accumulation in the leaves, attributed to increased dark CO_2 fixation (12), and also causes decreased phosphoenolpyruvate levels in pea roots (22). These effects may result from increased cytoplasmic ammonia levels during nitrate reduction. We cannot propose a definitive mechanism for PEPC stimulation by ammonia, however, it is possible that an explanation lies in the basic pH optimum of the enzyme. Davies (4) has proposed that, because of this property, PEPC operates as part of a cytoplasmic pH-stat. Thus, the disposal of excess cellular NH_3 produced by nitrate reduction could be related to cellular pH regulation. According to this model, an increase in cytoplasmic pH caused by ammonia accumulation would stimulate PEPC activity, resulting in neutralization of the ammonia and increased availability of 2-oxoacids for glutamate production and subsequent transamination.

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Table 1 Stimulation of dark CO_2 fixation in isolated mesophyll cells of P. somniferum by different concentrations of NH_4^{+a}

NH_4Cl (mM)	µg atoms C·mg $chl^{-1}\cdot hr^{-1}$
0	0.7
0.1	1.2
0.7	1.8
1.3	2.1
2.7	2.4
6.7	2.5
13.3	2.5

[a]Incorporation was initiated by the addition of 5 mM $NaH^{14}CO_3$. Samples were taken at intervals, acidified and blown dry under N_2, and counted by liquid scintillation. The rate of dark fixation was determined from linear plots of incorporation vs. time.

LEGENDS FOR FIGURES

Fig. 1. Effect of NH_4^+ on dark $^{14}CO_2$ incorporation into aspartate and glutamate in isolated mesophyll cells of P. somniferum. 2 mM NH_4Cl was added after 16 minutes of incorporation (○): control; (△): 2 mM NH_4^+.

Fig. 2. Effect of NH_4^+ on dark $^{14}CO_2$ incorporation into citrate and malate in isolated mesophyll cells of P. somniferum under the conditions described in Fig. 1. (○): control; (△): 2 mM NH_4^+.

Fig. 3. Effect of NH_4^+ on ^{14}C labeling of phosphoenolpyruvate and aspartate in isolated P. somniferum mesophyll cells in the dark. The cells were first prelabeled with $^{14}CO_2$ in the light. The lights were extinguished (31 min) and 5 mM NH_4Cl added (51 min).

Fig. 4. Effect of NH_4^+ on ^{14}C labeling of alanine and pyruvate in isolated P. somniferum cells in the dark. The cells were first prelabeled with $^{14}CO_2$ in the light. Alanine: Lights extinguished at 31 min and 5 mM NH_4Cl added at 51 min. Pyruvate: Lights extinguished at 60 min and 3 mM NH_4Cl added at 105 min.

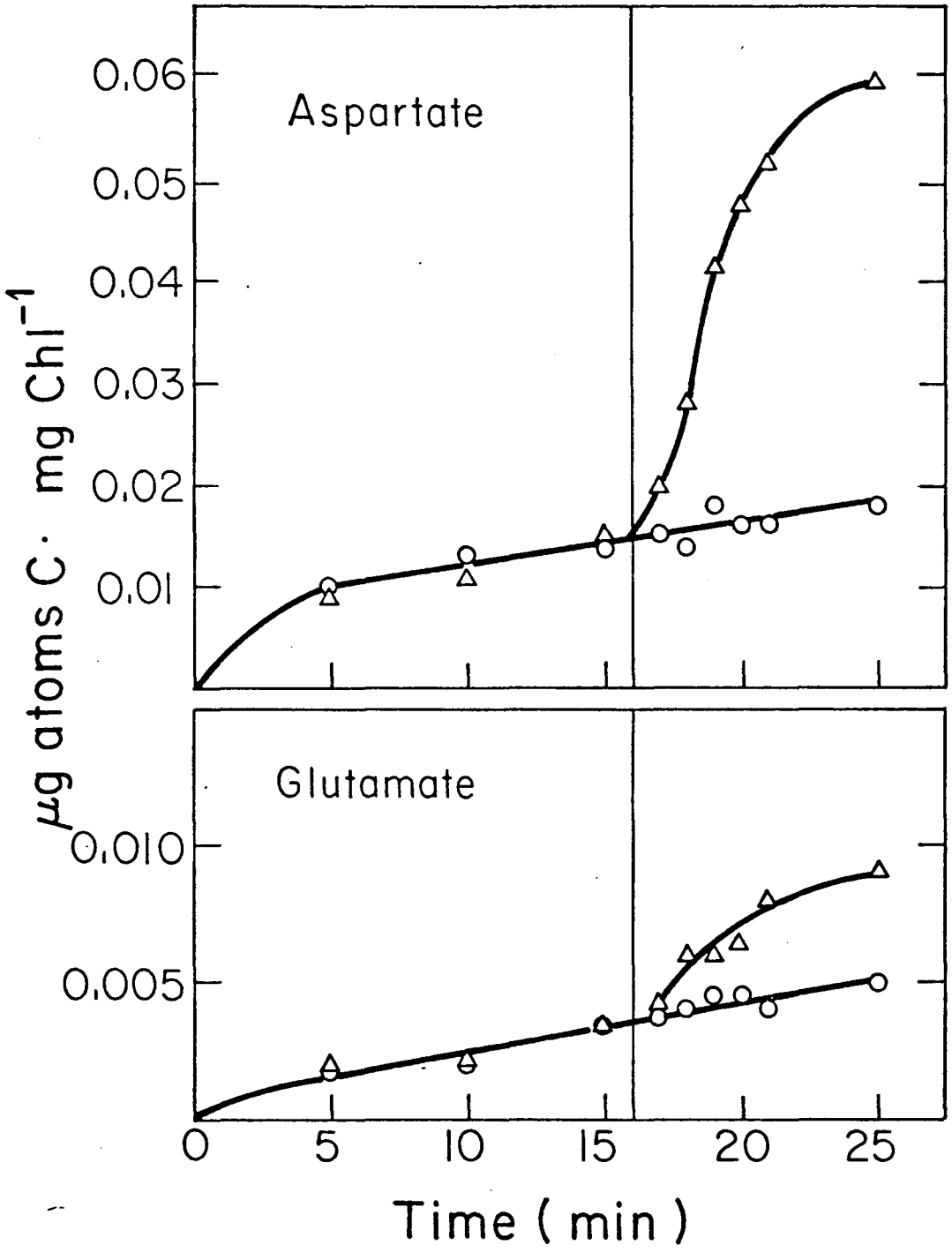

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FIG. 1

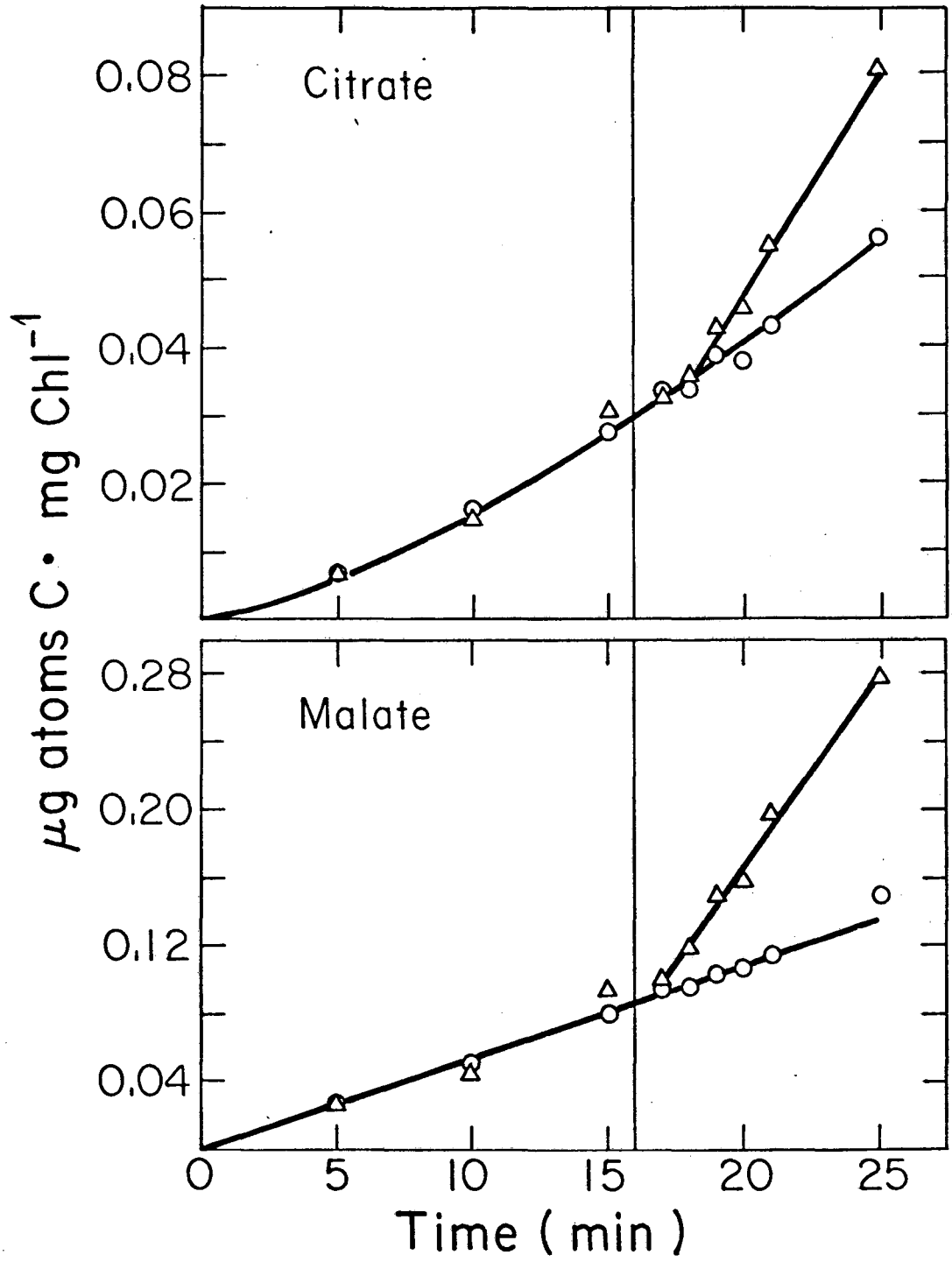

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FIG. 2

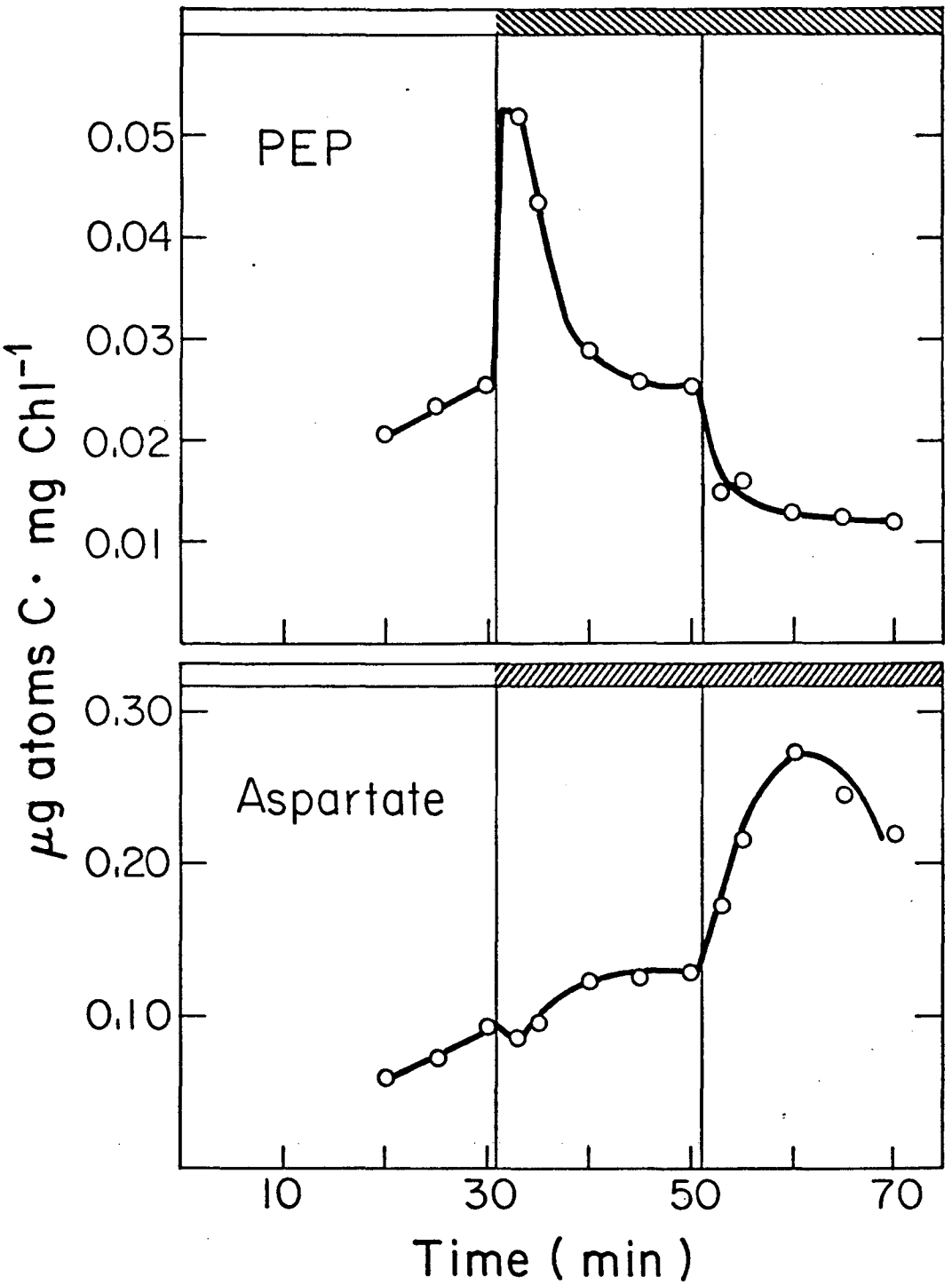

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FIG. 3

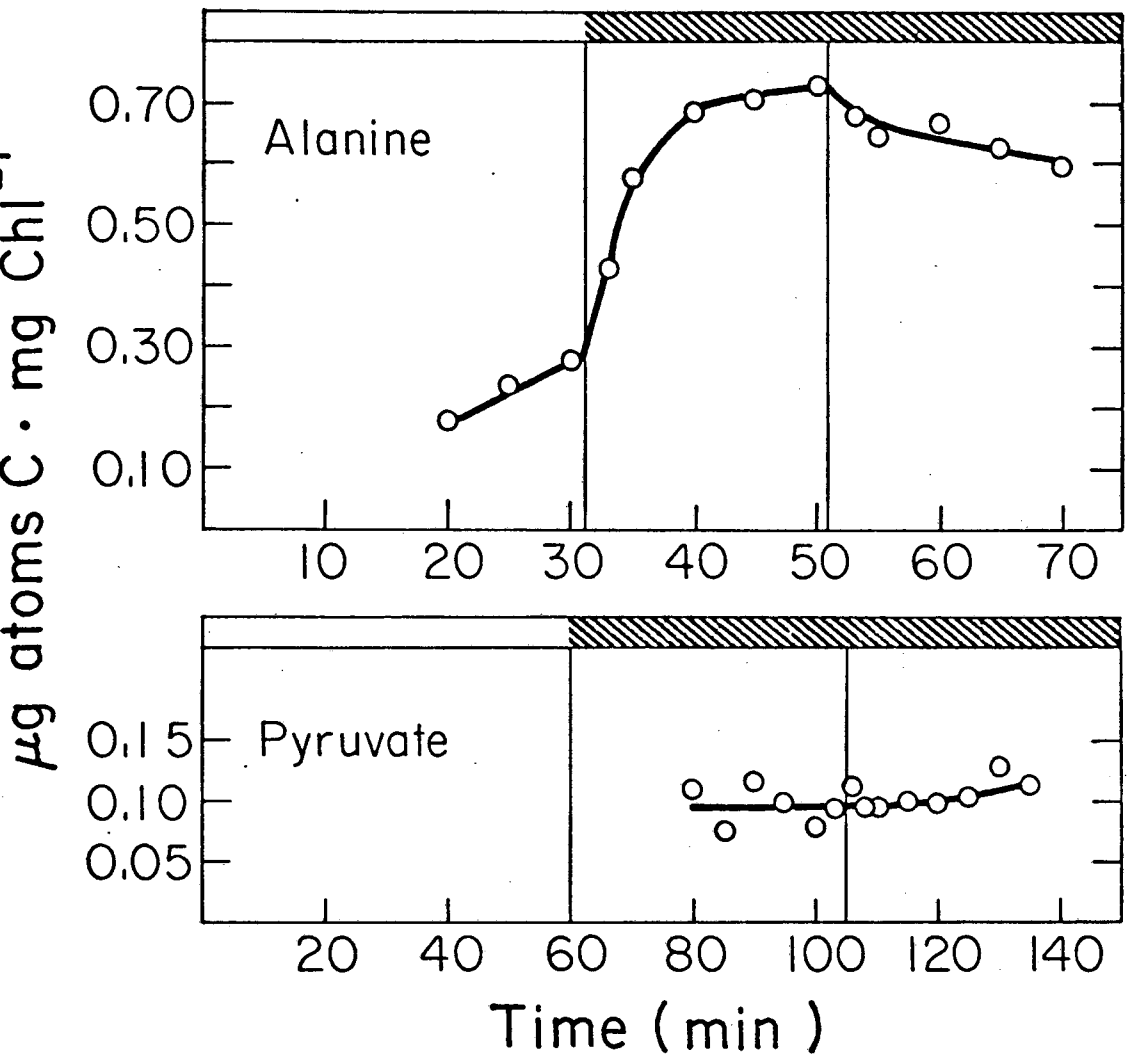

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FIG. 4

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