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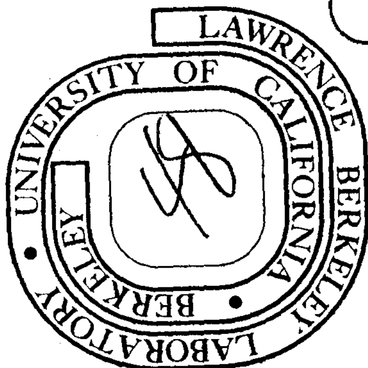

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Running Title: Ammonia Effects on Mesophyll Cells

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EFFECTS OF AMMONIA ON CARBON METABOLISM IN PHOTOSYNTHESIZING LEAF-FREE MESOPHYLL CELLS FROM *PAPAVER SOMNIFERUM*

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Abstract

Addition of ammonia to a suspension of photosynthesizing leaf-free mesophyll cells from Papaver somniferum quantitatively alters the pattern of carbon metabolism by increasing rates of certain key rate-limiting steps leading to amino acid synthesis and by decreasing rates of rate-limiting steps in alternative biosynthetic pathways. Of particular importance is the stimulation of reactions mediated by pyruvate kinase and PEP carboxylase. The increased rates of these two reactions which result in an increased flow of carbon into the TCA cycle correlate with a rapid rise in glutamine (via glutamine synthetase) which draws carbon off the TCA cycle as α-ketoglutarate. Increased flux of carbon in this direction appears to come mainly at the expense of sucrose synthesis. The net effect of addition of ammonia to mesophyll cells is thus a redistribution of newly fixed carbon away from carbohydrates and into amino acids.

Key Words

Ammonia - Carbon metabolism - Mesophyll - Modulation - *Papaver somniferum*

Introduction

Effects of ammonia on plant cell metabolism have been demonstrated in the alga Chlorella pyrenoidosa (Kanazawa et al., 1970; Kanazawa et al., 1972) as well as more recently with alfalfa leaf discs (Platt et al, 1977). Suspensions of unicellular algae offer an excellent system for observing transient effects of modulators on cell metabolism since sampling, incubation, and killing procedures are relatively simple. It is impossible to perform identical experiments with higher plant leaflets or leaf discs due to obvious problems with uptake of potential modulators as well as problems with sampling procedures. With leaf discs, preincubation with the modulator is therefore a necessary prerequisite and the resulting perturbations are generally slower and of lower magnitude than in comparable experiments with algae. Despite these difficulties, the results from exposure of leaf discs to 5 mM ammonia (Platt et al., 1977) were quite encouraging and displayed a trend indicative of the effects observed with Chlorella pyrenoidosa.

We have recently succeeded in isolating and maintaining in liquid suspension photosynthetically active cells from a higher plant, Papaver somniferum (Paul and Bassham, 1977). While retaining the biochemical features of higher plant cells, these cells may be experimentally manipulated in much the same way as with algae. These cells can be maintained in a viable state for a number of days, so that it is possible to carry out manipulations not easily achieved with whole leaves or leaf discs. For example, cells can be starved for fixed nitrogen prior to the experiment, thus enhancing the potential for metabolic responses when fixed nitrogen is provided.

The flow of carbon dioxide into intermediate metabolites in such isolated mesophyll cells was studied with respect to the modulating influence of ammonia. The results establish the capability of ammonia to act as a powerful regulatory agent in higher plants.

Materials and Methods

Cell isolation.

Mesophyll cells from the C-3 plant, Papaver somniferum, were isolated as previously described by using the enzyme pectinase (Paul and Bassham, 1977). Digestion medium (pH 5.7) and assay medium (pH 8.0) were also as described before except that bovine serum albumin was brought to 0.2% (w/v) in both and polyvinyl pyrolidine (PVP) was included at a concentration of 0.1% (w/v). Also, KNO_3 was eliminated from the media with the level of potassium ion being adjusted by the inclusion of K_2SO_4 (2.5 mM).

Photosynthetic $^{14}CO_2$ Incorporation.

After 24 h (12 h dark, 8°C; 12 h light, 24°C) in the assay medium lacking inorganic nitrogen described above, 1400 μl aliquot samples of the cell suspensions were placed in serum-stoppered micro-fernbach flasks with inlet and outlet tubes, and assayed at 22°C in a Plexiglass water bath illuminated from below with fluorescent lamps (450 $\mu\ E \cdot M^{-2} \cdot sec^{-1}$) (Jensen and Bassham, 1966). The flasks, in turn, were attached through manifolds to a closed steady-state gas circulation system (Platt et al., 1976). Cells were preincubated for a few minutes in the light in CO_2 -free air and photosynthesis was initiated with addition of 320 ppm $^{14}CO_2$ (14 μCi/μmole) in air. Samples (100 μl) were taken at regular intervals throughout the experiment and killed in 80% methanol. After prelabeling the cells for 35 min, ammonia was added as NH_4Cl (2 mM final concentration) to the cells.

Samples were withdrawn at 2 min, 5 min and thereafter at 5 min intervals following the addition. Overall incorporation rates are lower than previously described (Paul and Bassham, 1977) since the former experiments were conducted at high partial pressures of CO_2, whereas these experiments were conducted with air levels of CO_2.

Analysis of Photosynthetic Products.

Separation and identification of the soluble products of ^{14}C incorporation were accomplished by descending paper chromatography and radioautography as described previously (Paul and Bassham, 1977; Pederson et al, 1966). The α-ketoacids were analyzed as 2,4-dinitrophenyl-hydrazones (Bachelard, 1965) and separated according to the method of Platt et al (1977). All incorporation results are expressed on the basis of μgram-atoms of ^{14}C incorporated per mg Chl.

Results

Addition of 2 mM ammonia to photosynthesizing nitrogen-starved mesophyll cells induces several immediate changes in metabolism. The most notable of these is the dramatic shift in product/substrate ratios of pyruvate/phosphoenolpyruvate (PEP) and aspartate/PEP in the direction of products (Fig. 1). Ratios of pyruvate/PEP increased from 0.67 to 1.90 upon addition of NH_4^+ while the aspartate/PEP ratio shifted from 4.4 to 14.0. It should be noted that because pools of oxalacetate (OAA) (the immediate product of PEP carboxylase) were very low in control cells, an accurate value could not be obtained; therefore aspartate, which should closely reflect changes in the OAA pool, was utilized. The kinetics of glutamate (Fig. 5) further substantiates its use as an indicator of the OAA pool size (see Discussion).

Due to the increased flux of carbon into pyruvate, alanine (derived from the transamination of pyruvate) also shows a rapid rate of accumulation (Fig. 2). Concurrently, there was an immediate increase in tricarboxylic acid (TCA) intermediates (Fig. 3 and 4) while at the same time, the demand for TCA intermediates (via α-ketoglutarate) was increasing with the elevated incorporation of ammonia into glutamine (Figure 5). The glutamate level drops initially but fully recovers after 5 min (Fig. 5). Of the metabolites investigated in this study, only sucrose displays a sustained decrease in rate of synthesis (Fig. 6). After 25 min the sucrose pool in +ammonia cells accumulates 16% less carbon than in the control. Starch synthesis remains essentially unaffected (data not shown). The metabolic consequence of exposing mesophyll cells to NH_4^+ is thus increased ketoacid and amino acid biosynthesis together with inhibition of sucrose synthesis.

Discussion

Pyruvate Kinase.

The rapid response of mesophyll cell metabolism to the introduction of NH_4^+ is similar to that observed with *Chlorella* (Kanazawa et al., 1970). A rapid increase in the ratio of pyruvate/PEP is a clear indication that the well known regulatory enzyme, pyruvate kinase (Duggleby and Dennis, 1973; Nakayama et al., 1976) is subject to modulation by ammonia in plants, as has been suggested before (Kanazawa et al., 1970; Platt et al., 1977). The modulatory effect might well be an indirect one. For example, it has been shown in *Chlorella* that introduction of NH_4^+ caused an increase in the ADP/ATP ratio (Kanazawa et al., 1977) which, in turn, might modulate the activity of pyruvate kinase (Duggleby and Dennis, 1973). An increase in the affinity of pyruvate kinase for its substrate, PEP, is the most plausible explanation for the results.

Though accumulation of alanine is also quite rapid upon NH_4^+ addition, removal of pyruvate cannot account for the activation of the kinase since pyruvate also increased rapidly. In any event, the conversion of PEP to pyruvate is a rate-limiting step in metabolism, and its rate is not substantially affected by pyruvate concentration. It should also be noted that the rapid increase in the pyruvate pool occurs in the face of increased pyruvate utilization both for alanine synthesis and presumably for acetyl CoA synthesis since citrate formation increases. Increased citrate formation cannot occur as a consequence of increased PEP carboxylation alone--supplies of both acetyl-CoA and of C-4 acids must increase at the same time. There is no large pool of acetyl CoA to draw on; only an increased rate of turnover of a small acetyl CoA pool can accommodate the increased rate of citrate formation resulting in the observed growth in its pool size. Note also that citrate is being used more rapidly for the synthesis of α-ketoglutarate and glutamine.

PEP Carboxylase.

Of several conceivable C-3 carboxylation reactions whose respective enzymes have been noted in higher plants (Table 1: reactions 1-3), the one catalyzed by PEP carboxylase (E.C. 4.1.1.31) (reaction 1) is the most plausible because of favorable energetics. The standard free energy changes ($\Delta G^{\circ\prime}$) for the appropriate reactions have been determined as described in Table 1. However, the actual free energy changes (ΔG) in the cell depend on physiological concentrations of reactants and products. While we do not have accurate measurements of metabolite concentrations, reasonable approxmations can be made for calculation of the reaction free energies (see Table 1). An assumed concentration

which is in error by a factor of 10 would cause the final ΔG to vary by only 1.4 Kcal. At physiological metabolite concentrations only the anaplerotic reaction catalyzed by PEP carboxylase (reaction 1) is thermodynamically possible. Those reactions catalyzed by PEP carboxykinase (E.C. 4.1.1.32) and malate dehydrogenase (E.C. 1.1.1.40) are both highly unfavorable in the direction of carboxylation and would therefore be expected not to occur under physiological conditions. Pyruvate carboxylase, another potential anaplerotic enzyme, has not been reported in higher plants (Scrutton and Young, 1972). In this regard it is important to note that PEP and pyruvate carboxylases are apparently never found together in any single cell (Utter and Kolenbrander, 1972) probably because they fulfill the same metabolic function. The assignment of the anaplerotic function in higher plants to PEP carboxylase is in agreement with Utter and Kolenbrander (1972).

A large negative free energy change, such as that associated with the PEP carboxylase reaction, is often indicative of a regulatory step (Bassham and Krause, 1969). It is therefore not surprising that addition of ammonia to leaf mesophyll cells apparently stimulates the PEP carboxylase reaction. The ratio of aspartate to PEP, which increases more than 3-fold immediately upon addition of NH_4^+, was used to monitor PEP carboxylase since the OAA pool in the control cells was too low to be accurately measured. Use of aspartate fluctuations as an indicator of OAA fluctuations is justified on the basis of reversibility of the transamination reaction (Hatch and Shaio-Lim, 1973). If one considers the relationship of the respective metabolites in an equation for the equilibrium constant (K_{eq}), the validity of using aspartate becomes clear:

$$K_{eq} = \frac{[\text{Asp}][\alpha\text{-ketoglutarate}]}{[\text{OAA}][\text{glutamate}]}$$

Since glutamate actually decreased initially while α-ketoglutarate was increasing, OAA would have to increase initially even more rapidly than did aspartate. Such a rapid shift in the ratio of product to substrate can only occur with a sudden change in the kinetics of the reaction and must be due either directly or indirectly to modulation by ammonia.

TCA Cycle, Glutamine and Glutamate.

The increased rate of formation of oxaloacetate serves to prime the TCA cycle for its biosynthetic (anaplerotic) function. Thus the increased demand for ketoacids by glutamine synthetase and glutamate synthase (Lea and Miflin, 1974; O'Neal and Joy, 1973) is nicely correlated with the activation of PEP carboxylase. The amination of glutamate by glutamine synthetase together with the cell's higher transamination requirements cause a momentary drop in the pool of glutamate (Fig. 5). However, elevated carbon flow from PEP eventually reestablishes the glutamate pool. The proposed modulation of pyruvate kinase and PEP carboxylase may offer an explanation for the results of Kirkby and Knight (1977) who found that uptake of nitrate by tomato plants and its subsequent reduction to ammonia induced organic acid formation in leaves.

Carbohydrate Synthesis.

The diversion of carbon through PEP into pyruvate and OAA must necessarily come at the expense of other cellular metabolites since CO_2 incorporation is only slightly elevated. The partial inhibition of sucrose synthesis, which is a major product of mesophyll cell metabolism, can easily account for the increased carbon flow through PEP. A similar mechanism apparently operates in alfalfa leaf discs (Platt et al., 1977) as well as in Chlorella (Kanazawa et al., 1970). Also in agreement with

the earlier studies is the lack of immediate effect of ammonia on the other major mesophyll product, starch.

Conclusions

The principal effect of ammonia seems to be the generation of an increased flux of carbon into glutamine at the expense of sucrose synthesis. It has been suggested by O'Neal and Joy (1973) that glutamine synthetase is an especially effective sink for NH_4^+ in higher plants and may thus prevent toxic concentrations of NH_4^+ from accumulating in the chloroplast where this enzyme is located (O'Neal and Joy, 1974). An adequate supply of keto-acid is provided via the dual activation of pyruvate kinase and PEP carboxy-lase. The activation of both enzymes is required for an increased rate of noncyclic carbon flow through the TCA cycle if respiration is to remain the same.

Ammonia is known to act as an uncoupler of photophosphorylation (Krogmann et al., 1959), a fact which may account for the activation of pyruvate kinase since the latter enzyme is known to be modulated by ADP/ATP in higher plants (Duggleby and Dennis, 1973). Less is known about regulatory aspects of PEP carboxylase in plants (Utter and Kolenbrander, 1972) and it is therefore difficult to speculate how the activation may occur (i.e. whether NH_4^+ acts directly or indirectly).

The newly assimilated nitrogen in glutamine may be either immediately utilized in the formation of amino acids and, hence, protein synthesis or it may be transported to another part of the plant and utilized there (Lea and Miflin, 1977). In either case the result of adding ammonia to photosynthe-sizing leaf mesophyll cells is an increased capacity for protein synthesis by the plant as a whole.

Acknowledgements

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Table 1. Thermodynamic feasibility of proposed anaplerotic carboxylation reactions.

Reactions	$\Delta G^{\circ\prime a}$	ΔG^{b}
(1) $PEP^{-3} + CO_2 + H_2O \longrightarrow OAA^{-2} + HPO_4^{-2} + H^+$	-7.6	-5.1[c]
(2) $PEP^{-3} + CO_2 + NDP^{-3} \longrightarrow OAA^{-2} + NTP^{-4}$	0	+7.3
(3) $Pyruvate^- + CO_2 + NADPH \longrightarrow Malate^{-2} + NADP^+$	-0.3	+5.0
(4) $PEP^{-3} + H_2O \longrightarrow HPO_4^{-2} + Pyruvate^-$	-13.2	-
(5) $ATP^{-4} + H_2O \longrightarrow ADP^{-3} + HPO_4^{-2} + H^+$	-7.6	-

[a] The $\Delta G^{\circ\prime}$ values (all solute activities = 1 except $[H^+] = 10^{-7}M$) for reactions 1 and 2 are calculated from the free energies of formation from the elements (to give aqueous solutions) of CO_2 (-92.31 Kcal) and oxalacetate^{-2} (-190.53 Kcal), as tabulated in Krebs and Kornberg (1954) and from the corresponding value for PEP^{-3} of P-100.2 Kcal, where P represents the difference in free energy of formation from the elements of HPO_4^{-2} minus the corresponding value for water (Bassham and Krause, 1969). This value for PEP^{-3} results in a calculated hydrolysis energy for PEP^{-3} of -13.23 Kcal (reaction 4). Adding reactions 5 and 1 gives $\Delta G^{\circ\prime} = 0$ for reaction 2. The value for reaction 3 is from Krebs and Kornberg (1957).

[b] For reactions 1 and 2 we need only assume that OAA concentration $\leq$ [PEP] that $P_i = 10^{-3}$ M and CO_2 dissolved = 1.4×10^{-5} M when in equilibrium with atmospheric CO_2 (0.033%) at pH 7. For reaction 2 we assume $[NTP^{-4}]/[NDP^{-3}] = 3$, and for reaction 3 we assume $[pyruvate^-] = [malate^{-2}]$ and $[NADPH]/[NADP^+] = 10$. T = 298° K. With these assumptions ΔG for reaction 1 becomes $-7.6 - RT \ln(1.4 \times 10^{-5}/10^{-3}) = -5.1$ Kcal; ΔG for reaction 2 is $0 - RT \ln(1.4 \times 10^{-5}/3) = +7.3$ Kcal and ΔG for reaction 3 becomes $-0.3 - RT \ln(1.4 \times 10^{-5} \times 10) = +5.0$ Kcal.

[c] Though HCO_3^- is the actual substrate for PEP carboxylase (reaction 1), the free energy of the reaction will not be affected by using CO_2 dissolved in the calculation since it is in rapid equilibrium with HCO_3^-.

Legends to Figures

Figure 1. Short-term kinetic responses of (a) aspartate:PEP and (b) pyruvate:PEP ratios after addition of NH_4Cl to photosynthesizing leaf-free mesophyll cells. Cells were starved for nitrogen for 24 h prior to commencing the assay. (a): (○) aspartate:PEP ratio in control cells and (●) in cells to which NH_4^+ was added after 35 min prelabeling with $^{14}CO_2$; (b): (□) pyruvate:PEP ratio in control cells and (■) in +ammonia cells.

Figure 2. Perturbation by ammonia of carbon flow into pyruvate and alanine. (○) pyruvate labeling in control cells; (●) pyruvate labeling in cells perturbed with ammonia; (□) alanine labeling in control cells; (■) alanine labeling in +ammonia cells.

Figure 3. Perturbation by ammonia of carbon flow into the TCA intermediates malate and citrate. (○) malate labeling in control cells; (●) malate labeling in cells perturbed with ammonia; (Δ) citrate labeling incontrol cells; (▲) citrate labeling in +ammonia cells.

Figure 4. Perturbation by ammonia of carbon flow into α-ketoglutarate (2-oxoglutarate) and aspartate. (□) α-ketoglutarate labeling in control cells; (■) labeling of α-ketoglutarate in cells perturbed with ammonia; (□) aspartate labeling in control cells; (■) aspartate labeling in +ammonia cells.

Figure 5. Perturbation by ammonia of carbon flow into glutamine and glutamate. (□) glutamine labeling in control cells; (□) glutamine labeling in cells perturbed with ammonia; (○) glutamate labeling in control cells; (●) glutamate labeling in +ammonia cells.

Figure 6. Effect of ammonia on sucrose synthesis and gross CO_2 incorporation in the isolated mesophyll cells. (○) sucrose labeling in control cells; (●) sucrose labeling in cells perturbed with ammonia; (○) CO_2 incorporation in control cells; (●) CO_2 incorporation in +ammonia cells.

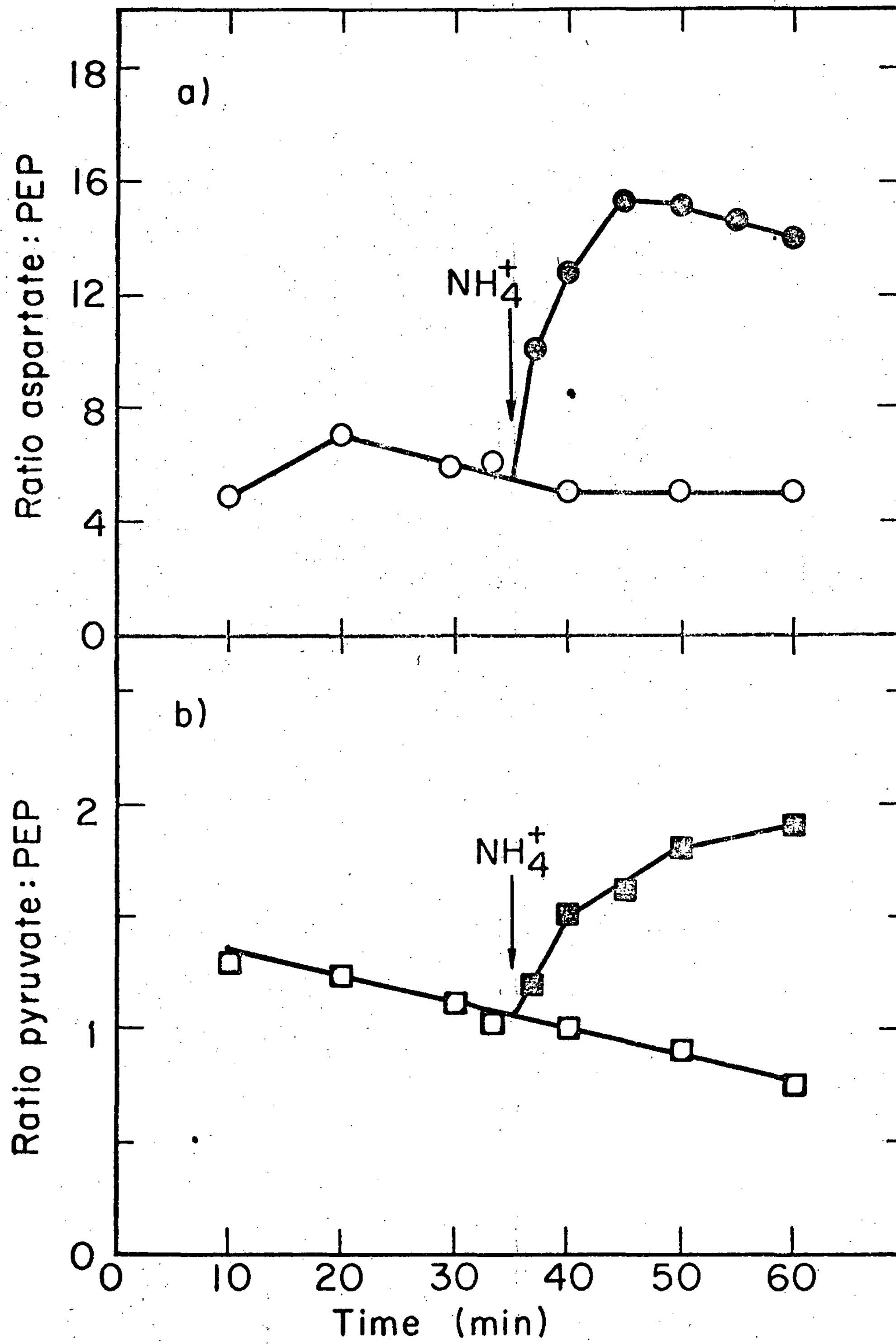

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Figure 1

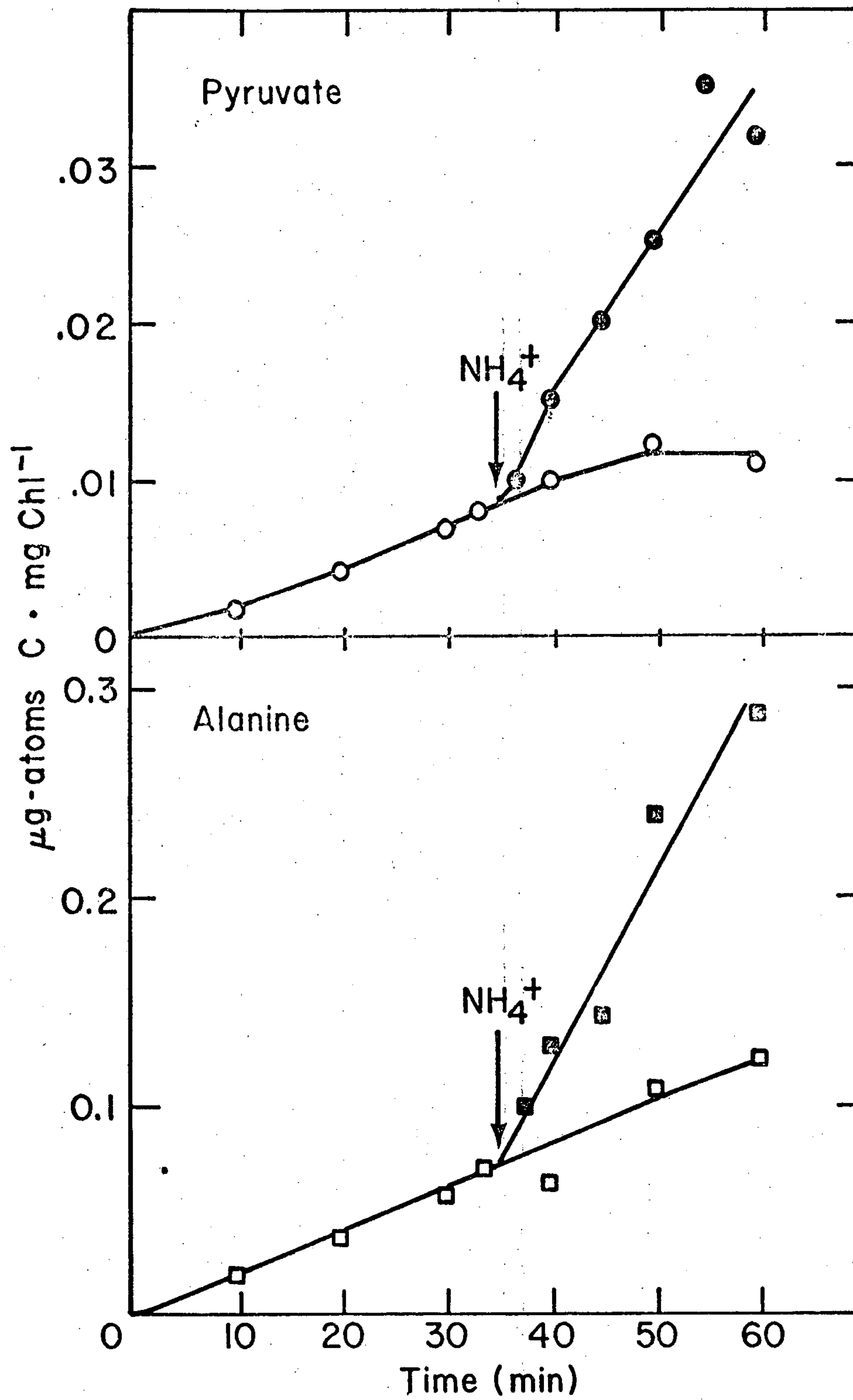

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Figure 2

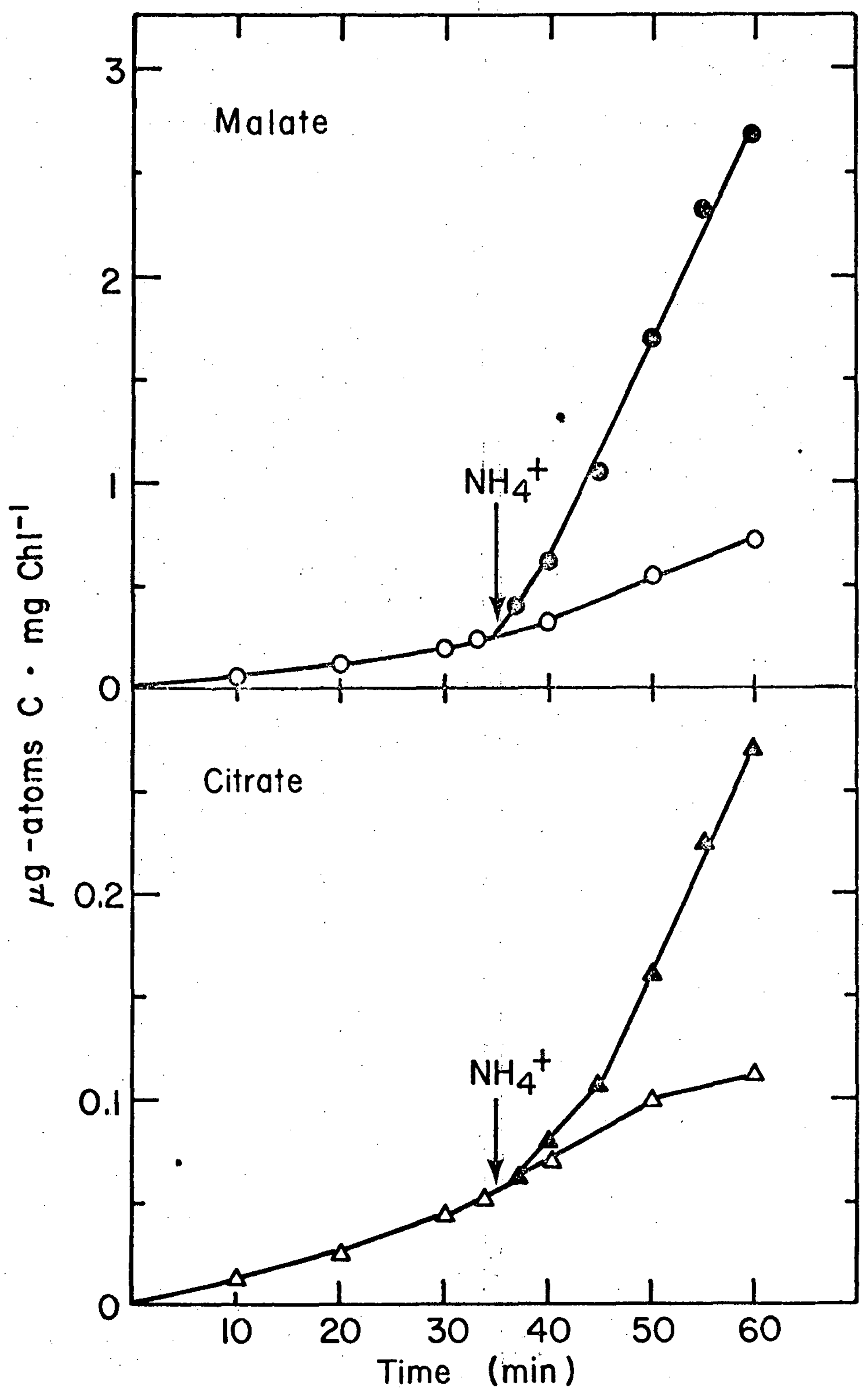

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Figure 3

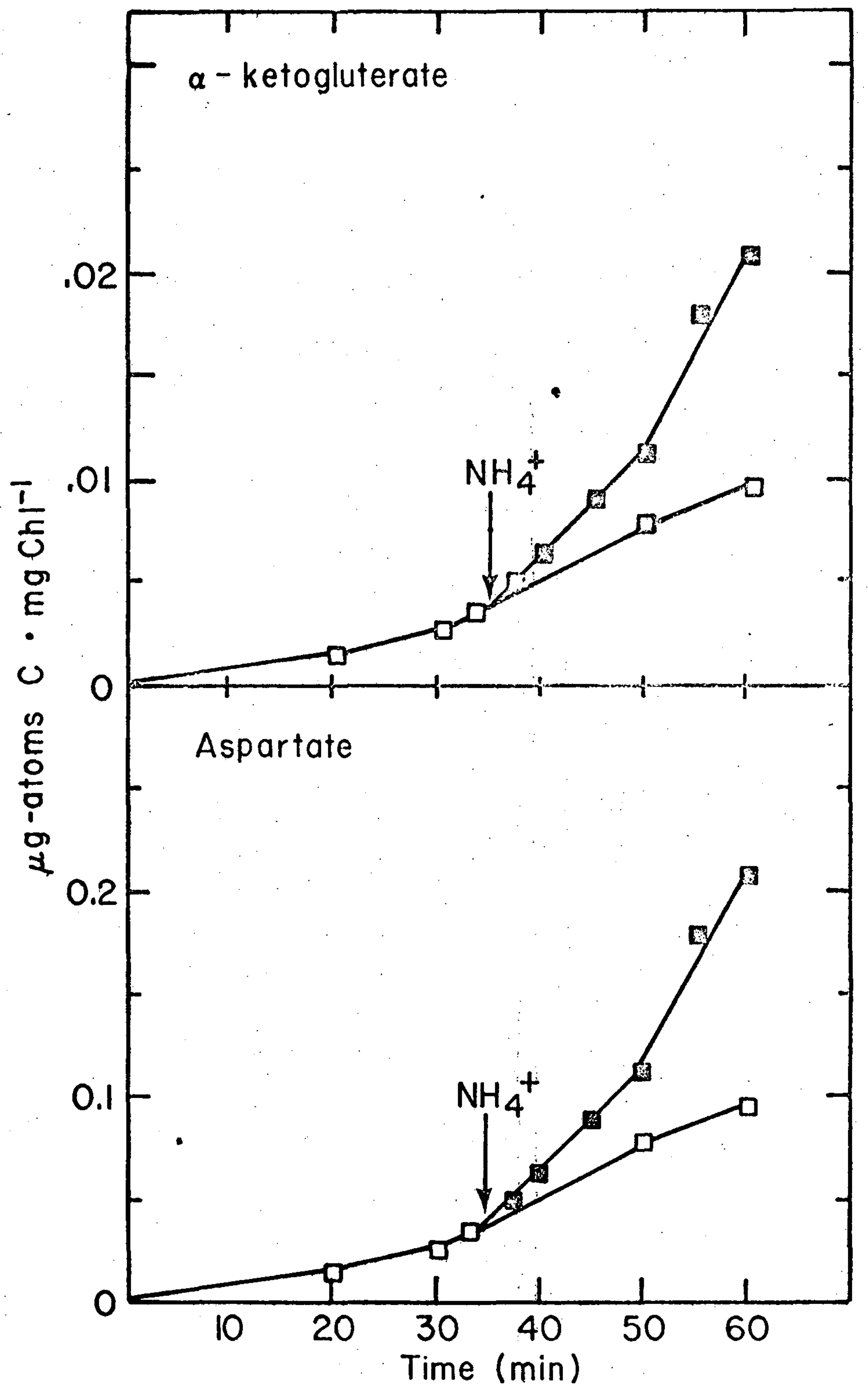

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Figure 4

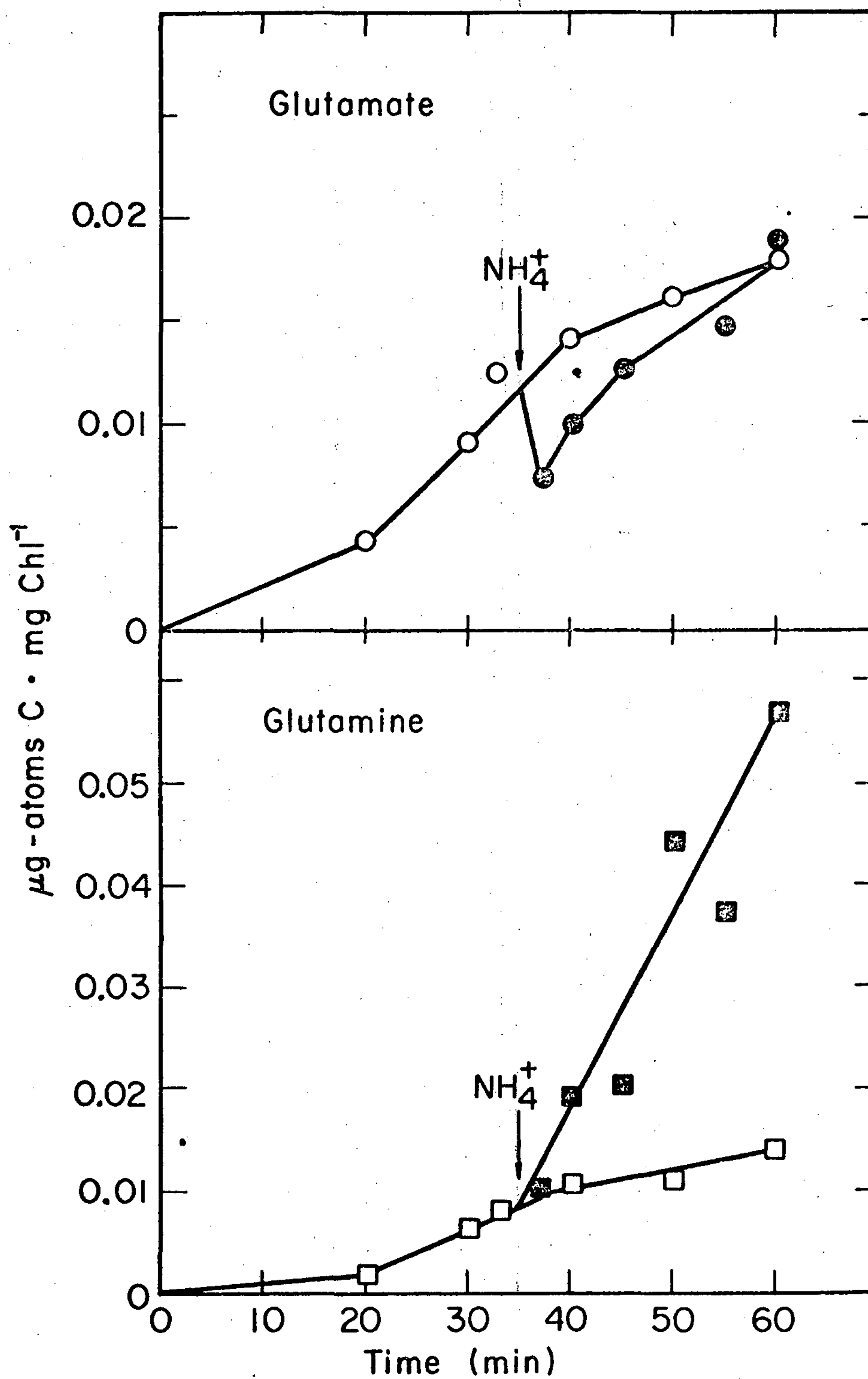

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Figure 5

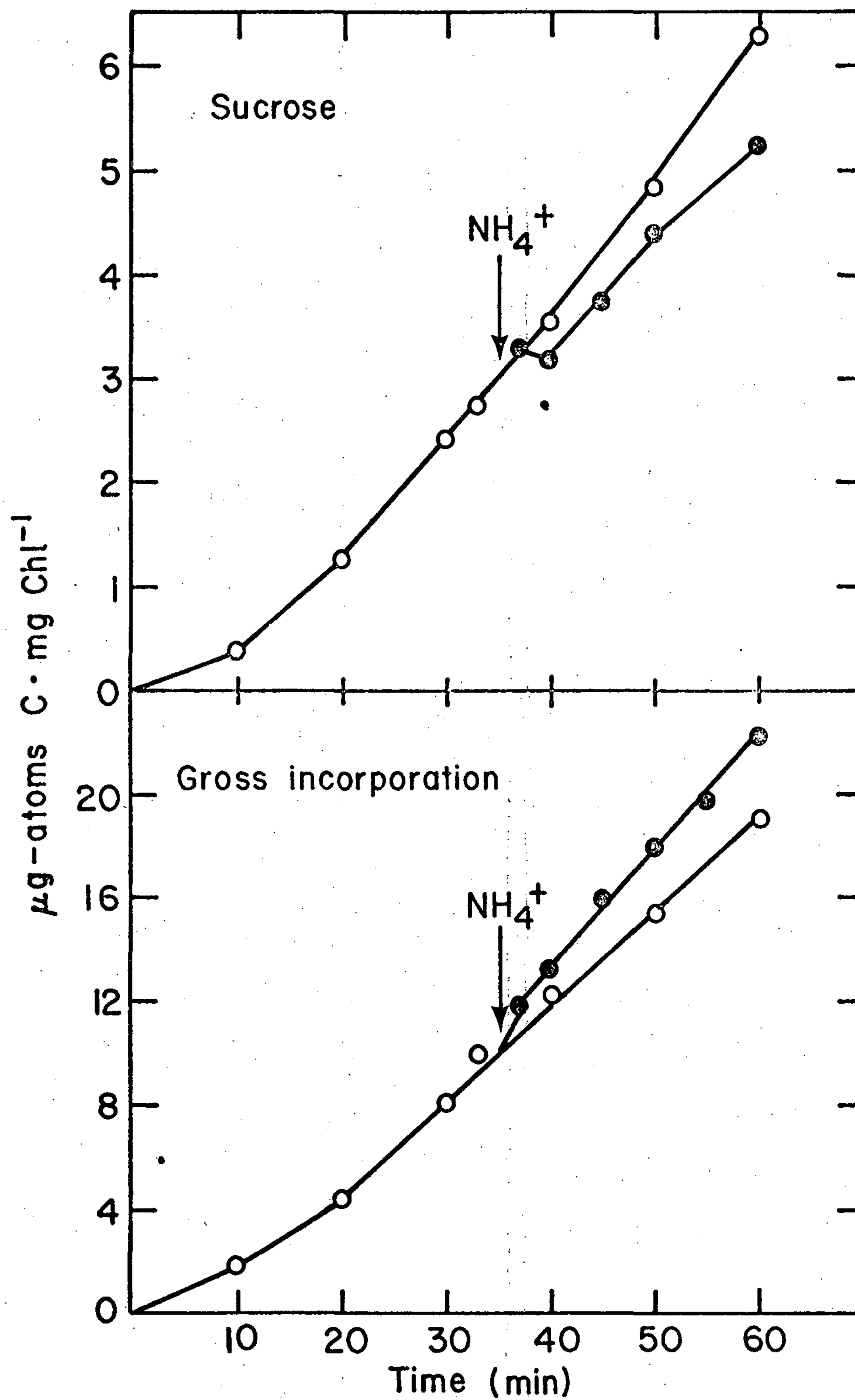

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Figure 6

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