ASPARTATE-DEPENDENT ALANINE PRODUCTION BY LEAF DISCS OF *AMARANTHUS PANICULATUS*, AN ASPARTATE UTILIZING NAD-MALIC ENZYME TYPE C_4 PLANT

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SUMMARY

Illuminated leaf discs of *Amaranthus paniculatus* formed alanine rapidly after infiltration with aspartate. α-oxoglutarate and glutamate accelerated aspartate-dependent alanine production. Alanine was also formed by *A. paniculatus* leaf discs infiltrated with oxalacetate; this production was stimulated only by glutamate. Our results confirm that alanine can be formed rapidly from aspartate in C_4 plants. Aspartate-dependent alanine production was stimulated greatly by light. The stimulatory effect of light could almost be replaced by ATP in presence of sodium ions. These findings are discussed in relation to a possible light-dependent active transport of amino acids into the bundle sheath, mediated by Na^+-dependent ATPase activity.

INTRODUCTION

C_4 plants use preferentially either malate or aspartate for primary carbon fixation and subsequent transfer of radioactivity to end products via 3-phosphoglyceric acid (PGA) (Hatch, 1971b; Black, 1973). The decarboxylation of malate is mediated by NADP malic enzyme located in the bundle sheath (Hatch and Slack, 1970). Edwards *et al.* (1971) suggested that PEP carboxykinase could catalyse the decarboxylation of aspartate in some C_4 plants but the mechanism of aspartate decarboxylation in many other C_4 plants remained unclear until Hatch and Kagawa (1974) discovered NAD-malic enzyme and proposed that this enzyme decarboxylated oxalacetate formed from aspartate.

We have already reported the occurrence of the C_4-pathway of photosynthesis in *Amaranthus paniculatus* (Das and Raghavendra, 1973) and noted that it was an aspartate-utilizing NAD-malic enzyme type C_4-plant (Raghavendra, 1975). High activities of enzymes of nitrogen metabolism (such as nitrate reductase, aspartate- and alanine aminotransferases) in leaves of *A. paniculatus* (Raghavendra, 1975) indicated efficient nitrogen metabolism in them. These observations led us to study the metabolism of externally added aspartate in *A. paniculatus.*

During our preliminary investigations, when leaf discs of *A. paniculatus* infiltrated with aspartate, were illuminated, alanine appeared rapidly. Such asparate-dependent alanine production was of great interest in view of the conflicting reports on alanine labelling in 'aspartate using' C_4 plants. Earlier investigations indicated that alanine acquired radioactivity from C_4 acids via PGA and phosphoenol pyruvate (PEP) (Osmond, 1967; Johnson and Hatch, 1968; Chen, Brown and Black, 1971; Downton, 1971; Hatch, 1971a). But Kennedy

and Laetsch (1973, 1974) thought that a substantial part of fixed $^{14}CO_2$ in *Portulaca oleracea* was incorporated into alanine via pyruvate and not by routes involving PGA or C_4 acids. Hatch (1975) reiterated that in *P. oleracea* labelling of alanine was from ^{14}C initially incorporated into C_4 acids.

Hence we examined in detail aspartate-dependent alanine production by leaf discs of *Amaranthus paniculatus*. Our results indicate that alanine production from aspartate can be rapid. We also report observations on the influence of light and sodium ions on such alanine formation.

MATERIALS AND METHODS

Plant material

Plants of *Amaranthus paniculatus* L. were raised in seed pans on soil supplemented with manure (1 part soil + 3 parts farmyard manure) under an approximate 12 h photoperiod, (temperature 38°C day and 20°C night). The second to fourth leaves (starting from fully matured leaf) were taken from 3- to 4-week old seedlings.

Leaf disc incubation

Leaf discs of 1 cm diameter (1 g) were infiltrated with 30 ml of 0.02 M tris-HCl buffer (pH 7.5) containing 10 mM aspartate, 2 mM $MgCl_2$ and 2 mM cysteine hydrochloride. Control sets were infiltrated with water. The infiltrated discs were either illuminated at 4000 ft-c or maintained in darkness, at 40°C. All treatments were duplicated. At required intervals, the leaf discs were removed, quickly washed with water, blotted dry and extracted with 80% (v/v) ethanol. The alcohol extract was concentrated under reduced pressure.

Chromatography

A portion of extract was spotted onto Whatman No. 1 filter paper and the amino acids separated by one-dimensional ascending chromatography with n-butanol:acetic acid:water (100:22:50, v/v). The solvent was run twice. The amino acids were detected by spraying with 0.5% (w/v) ninhydrin in 95% (v/v) acetone. The amino acids were identified by comparing with authentic samples spotted on the same sheet. They were also eluted and observed for their behaviour in another solvent; methanol:water:pyridine (80:20:4, v/v). Their identity was established after co-chromatography.

Alanine estimation

The estimation of alanine on the chromatograms was based on the method of Giri, Radhakrishan and Vaidyanathan (1952). The alanine region, visualized after spraying with 0.5% ninhydrin in 95% (v/v) acetone, was cut out and eluted overnight with 4 ml 75% (v/v) ethanol containing 2 mg copper sulphate. The optical density of the eluate was measured at 540 nm. The concentration of the amino acid was evaluated from a standard curve prepared for alanine under similar conditions.

The rate of production of alanine was fastest during the second 30-min period after infiltration (Fig. 2). The rate of alanine production was observed, therefore, during that period and computed for 1 h. In some of the experiments 100 mM oxalacetate was used instead of aspartate. The following compounds were also sometimes included in the infiltration medium at the given final concentrations: a-oxoglutarate, 10 mM glutamate, 10 mM; ATP 10 mM and sodium chloride, 100 mM (unless otherwise specified).

Enzyme extraction and assays

Leaf material (1.5–2.0 g) was extracted at $0 \pm 2°C$ with 4 vols. of 50 mM tris-HCl buffer, pH 7.8 containing 5 mM dithiothreitol, 1 mM EDTA, 2 mM $MgCl_2$ and 10 mM 2-mercaptoethanol. The extract was filtered through four layers of cheesecloth. A portion was set aside for chlorophyll estimation, and the extract was cleared by centrifuging at 10 000 *g* for 10 min. The cleared samples were run through Sephadex G-25 columns (1 × 6 cm) which were pre-equilibrated with the above extraction medium. The enzyme preparation for assays was diluted and used so as to give linear activity with time for at least 10 min.

The enzymes were assayed spectrophotometrically at $30 \pm 1°C$ by already described procedures: NADP malic enzyme (EC 1.1.1.40) (Raghavendra and Das, 1976); NAD malic enzyme (Hatch and Kagawa, 1974); aspartate-aminotransferase (EC 2.6.1.1) (Edwards and Gutierrez, 1972); and alanine aminotransferase (EC 2.6.1.2) (Edwards and Gutierrez, 1972).

Chlorophyll

Chlorophyll was estimated after extracting into 80% (v/v) acetone (Arnon, 1949).

RESULTS

When the leaf discs of *Amaranthus paniculatus* infiltrated with aspartate were illuminated, alanine appeared rapidly. Alanine production was most rapid at pH 7.5 and at 40°C (Fig. 1a, 1b). Light stimulated alanine formation but there was no clear saturation even at a light intensity of 6000 ft-c (Fig. 1c, 2a).
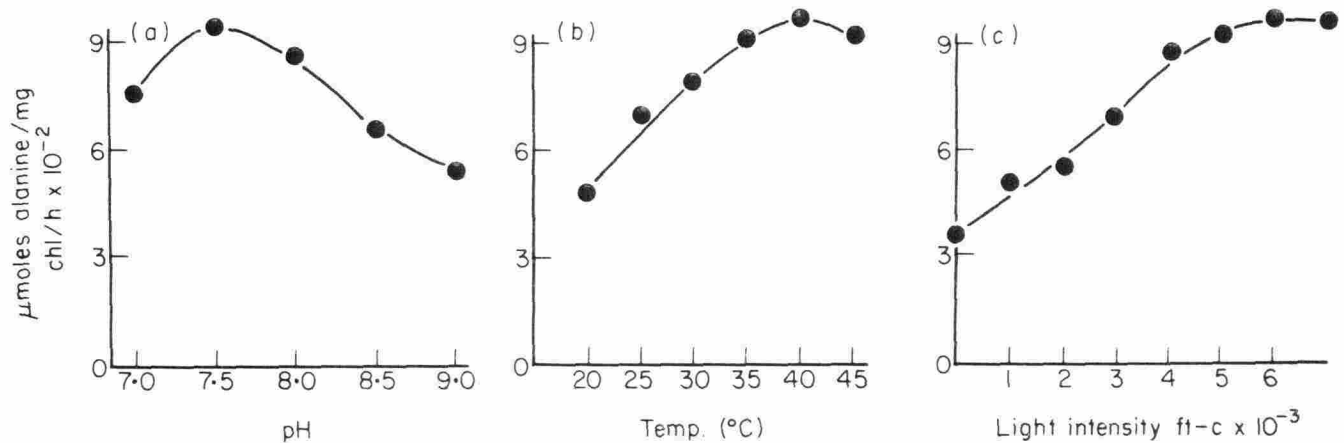

Fig. 1. Effect of pH (a), temperature of incubation (b) and light intensity during illumination (c) on aspartate-dependent alanine production by *Amaranthus paniculatus* leaf discs.

The addition of α-oxoglutarate or glutamate accelerated alanine formation (Fig. 2a). *A. paniculatus* leaf discs also produced alanine when fed with oxalacetate (Fig. 2b). Surprisingly, light exhibited only a slight stimulatory effect on alanine production in the presence of oxalacetate and α-oxoglutarate had no effect; however, glutamate again slightly enhanced alanine formation.

ATP stimulated the aspartate-dependent alanine formation both in light and in darkness (Fig. 3a). In darkness, with ATP together with sodium chloride, alanine production from aspartate was almost the same as that in light. The effects of ATP and Na^+ on alanine pro-
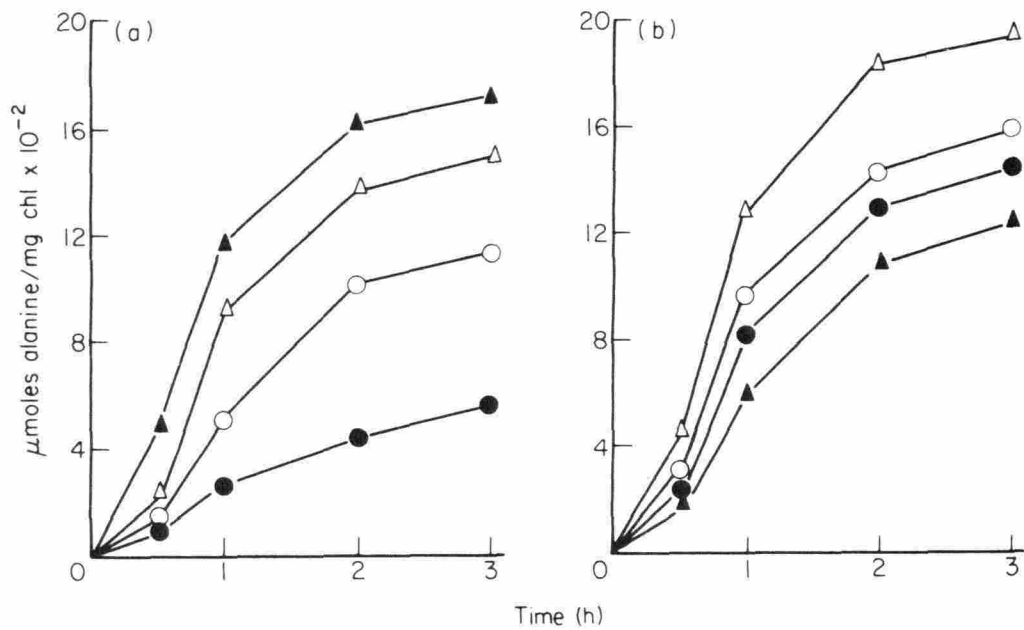
Fig. 2. Alanine production by *Amaranthus paniculatus* leaf discs infiltrated with aspartate (a) or oxalacetate (b). (●) In darkness; (○) in light; (▲) plus a-oxoglutarate in light; (△) plus glutamate in light.
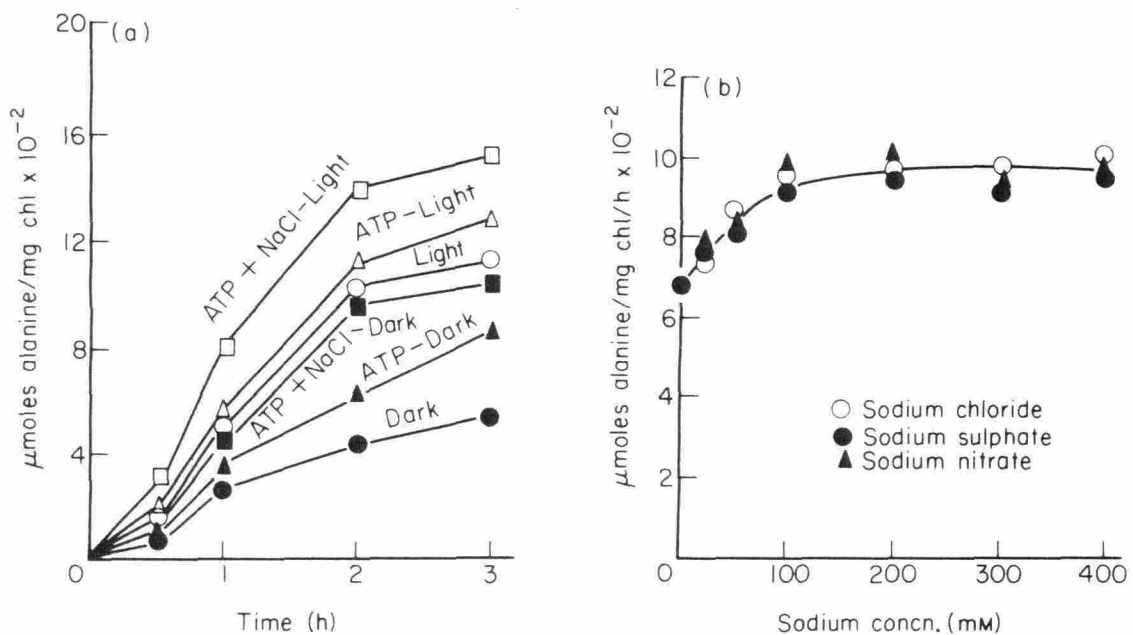

Fig. 3. Effect of ATP and sodium chloride on aspartate-dependent alanine production by *Amaranthus paniculatus* leaf discs (a) in light or darkness, (b) shows the effect of sodium concentration in relation to the accompanying anion on such alanine production in light. (a) □, ATP + NaCl—light; △, ATP—light; ○, light; ■, ATP + NaCl—dark; ▲, ATP—dark; ●, dark.

Table 1. *Effect of ATP and sodium ions on aspartate-dependent alanine formation in dark or light by the leaf discs of Amaranthus paniculatus*

Experimental condition	μmoles formed/mg chl/h	
	Dark	Light
No addition	360	720
+ ATP	480	680
+ ATP + sodium	640	1050

Table 2. *Enzyme levels in leaves of Amaranthus paniculatus*

Enzyme	Activity (μmoles/mg chl/h)
Aspartate aminotransferase	1884
Alanine aminotransferase	1626
NADP malic enzyme	36
NAD malic enzyme	235

duction are summarized in Table 1. A concentration of 100 mM sodium was optimal for its effect irrespective of the accompanying cation: chloride, sulphate or nitrate (Fig. 3b).

A. paniculatus leaves possessed high aspartate- and alanine-aminotransferase activities (Table 2). High activity of NAD-malic enzyme and low activity of NADP-malic enzyme placed *A. paniculatus* into the NAD-malic enzyme group of C_4-plants (Gutierrez, Gracen and Edwards, 1974; Hatch *et al.*, 1975; Raghavendra and Das, 1976).

DISCUSSION

Aspartate-dependent alanine formation by leaf discs of *Amaranthus paniculatus* was enhanced by the addition of a-oxoglutarate or glutamate (Fig. 1). These observations, together with highly active aspartate and alanine aminotransferases in leaves of *A. paniculatus* (Table 2), suggest the following sequence for aspartate metabolism:

$$\text{aspartate} + a\text{-oxoglutarate} \xrightarrow[\text{aminotransferase}]{\text{aspartate}} \text{oxalacetate} + \text{glutamate}$$

$$\text{oxalacetate} \xrightarrow{\text{NAD malic enzyme}} \text{pyruvate} + CO_2$$

$$\text{pyruvate} + \text{glutamate} \xrightarrow[\text{aminotransferase}]{\text{alanine}} \text{alanine} + a\text{-oxoglutarate.}$$
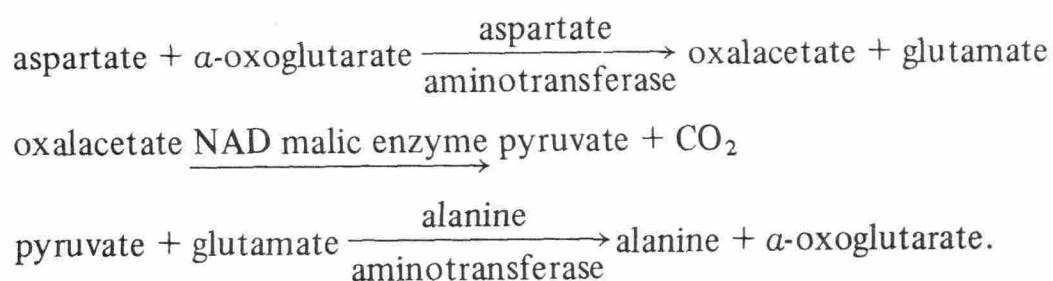
This sequence was further supported by the observation of alanine production in presence of oxalacetate at comparable rates to those with aspartate. Oxalacetate-dependent alanine production was stimulated only by glutamate and not by a-oxoglutarate. Edwards and Gutierrez (1972) noticed that the production of oxalacetate from aspartate by the bundle-sheath strands of *Panicum miliaceum* depended on a-oxoglutarate. But the rates recorded by them, in light and darkness, were similar. Unlike the bundle-sheath strands, aspartate metabolism in leaves was dependent on light. The responses of alanine production to light intensity and temperature (Fig. 1) were quite similar to those of carbon fixation by C_4 plants. However, at present we are unable to explain the significance of these factors in relation to alanine formation.

Our results help to clarify the controversy over alanine formation in C_4 plants. Alanine production from aspartate was rapid and was evidently catalysed by aminotransferases through the sequence indicated above. Hence we support the view (Hatch, 1975) that alanine can be derived from aspartate, one of the C_4 acids. The rapidity of alanine formation from aspartate might be the reason for early labelling of alanine in *Portulaca* observed by Kennedy and Laetsch (1973, 1974). Alanine labelling during carbon fixation in light was commonly observed in plants metabolising aspartate as the main C_4 acid (Osmond, 1967; Johnson and Hatch, 1968; Chen *et al.*, 1971; Downton, 1971; Hatch, 1971a).

Illumination was essential for transfer of ^{14}C from primary fixation products to end com-

pounds (Hatch and Slack, 1966; Raghavendra, 1975; Rathnam and Das, 1975). But the dependence of alanine formation from aspartate on light suggests that it is an energy requiring process. Indeed ATP (in the presence of Na^+) could replace the requirement for light. Since the conversion of aspartate to alanine requires no energy, it is possible that illumination promotes the entry of aspartate into either mesophyll or bundle-sheath cells. The present understanding of the C_4-pathway (Hatch, 1971b) is that aspartate formed during CO_2 fixation moves into the bundle-sheath and is decarboxylated to release pyruvate and CO_2. Pyruvate is converted into alanine in the bundle-sheath and alanine travels back to the mesophyll where it is reconverted into pyruvate. Thus the present results in the light of available literature suggest that light may catalyse the entry of aspartate into bundle-sheath. The absence of light stimulation of oxalacetate-dependent alanine production supports this proposal.

The stimulation by sodium ions resembles the Na^+ stimulation of ATPase activity in leaf extracts of *Setaria, Pennisetum* and *Amaranthus* (Raghavendra, 1975; these results are communicated elsewhere). Na^+-K^+ activated ATPase mediates the amino acid transport of animal systems (Smith, 1969; Smith and Lane, 1971). Ouabain, an inhibitor of Na^+-K^+ ATPase, inhibited amino acid transport in animal tissues, and also inhibited the transfer of radioactivity from C_4 acids to end products in *Setaria* and *Amaranthus* (C_4 plants) but not in *Rumex*, a C_3 plant (Raghavendra, 1975). Hence we feel that an ATPase may be catalysing the active amino acid transport into the bundle sheath of C_4 plants. The concentration of Na^+ required for maximal production of alanine (100 mM) was similar to the optimal Na^+ concentration for ATPase activity (80 mM). Karpilov, Bil and Gukasyan (1975) and Karpilov, Bil and Malyshew (1975) have recently indicated the necessity for an active transport of C_4 acids in C_4 plants, and proposed further that an ATPase might be involved.

Na^+-K^+ dependent ATPases were already reported in leaves of certain mangrove plants (Kylin and Gee, 1970), sugarbeet roots (Hansson and Kylin, 1969) and stomatal guard cells of *Commelina benghalensis* (Raghavendra, Rao and Das, 1976). We feel therefore that the role of Na^+-K^+ ATPase activity in the rapid transport of metabolites in C_4 plants needs further detailed investigations.

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