EVIDENCE FOR THE PRESENCE OF GLUTAMATE SYNTHASE IN EXTRACTS OF CARROT CELL CULTURES

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SUMMARY: Extracts of wild carrot, either dialysed or biogel treated, catalyse the oxidation of NADPH in the presence of a-ketoglutarate and L-glutamine. The oxidation does not occur with ammonia at similar concentration to L-glutamine. NADH and L-asparagine can substitute in the reaction. Glutamine synthetase is also present in these extracts. The possibility that ammonia is assimilated in plants via glutamine synthetase and glutamate synthase now exists and is discussed.

INTRODUCTION: The reaction catalysed by glutamate synthase (L-glutamate: $NADP^+$ oxido-reductase (deaminating, glutamine forming) E.C. 1.4.1.X) was first detected in extracts of A. aerogenes grown under ammonia limitation (1). The enzyme is wide spread in micro-organisms (2,3) and has been purified from E. coli (4). The enzyme has been recently demonstrated in some yeasts (5) but was not detected in the chloroplasts of Vicia faba (6). In micro-organisms glutamate synthase acting in conjunction with glutamine synthetase (L-glutamate: ammonia ligase (ADP) E.C. 6.3.1.2) seems to be responsible for the assimilation of ammonia into glutamic acid particularly when growth is limited by ammonia supply.

Two lines of evidence suggest that in plants the assimilation of ammonia into glutamic acid may not be catalysed by glutamic dehydrogenase (L-glutamate: NAD^+ oxido-reductase (deaminating) E.C. 1.4.1.2) or

(L-glutamate: $NADP^+$ oxido-reductase E.C. 1.4.1.4). The Km for ammonia for glutamic dehydrogenase (NAD^+) purified from plants sources are 25mM or greater (7, 8, 9, 10). The level of ammonia in root exudates is given as 1.4-1.6 mM for soybean and 12.1-18.6mM for sunflower (11). These levels of ammonia suggest that glutamic dehydrogenase (NAD^+) would not be functional in ammonia assimilation. Km for ammonia for glutamic dehydrogenase ($NADP^+$) from plants do not seem to be available.

The second line of evidence is from the kinetic studies of Kanazawa et al (12, 13) in which Chlorella pyrenoidosa either photosynthesizing under steady-state conditions in a nitrogen-free medium or respiring in the dark after photosynthesizing in the absence of a nitrogen source, was treated with NH_4Cl. In both cases the level of glutamine in the cells rose immediately, the level of glutamic acid declined immediately and reached its original rate of synthesis only after several minutes. These results show that glutamine is the initial product of ammonia assimilation in Chlorella.

The above evidence led us to search for glutamate synthase in extracts of plant tissues.

MATERIALS AND METHODS: Inorganic chemicals were all reagent grade and organic chemicals were the highest grade obtainable from Sigma Chemical Company.

The wild carrot culture and its maintainence has been described (14). When the effect of nitrogen source on the level of enzymes in tissue was examined, KNO_3 medium was replaced with equimolar amounts of KCl. Nitrate

was added as 40mM sodium nitrate, ammonia was added as 10mM NH_4Cl when in combination with $NaNO_3$ or with 10mM sodium succinate when added alone.

To harvest tissue, cultures were treated with β mercaptoethanol (1ml/litre of medium), vacuum filtered on "Miracloth" and the tissue washed with 500 ml of cold 5mM β mercaptoethanol/ 1 of medium.

Extracts of tissue were prepared by sonic disruption (3x1 min. periods at 0°) of tissue with Polyclar AT (10% w/w) and 0.2M Hepes[1] (pH 7.5, KOH) containing 2mM EDTA. (1 ml./g.F.W.). After centrifugation at 12,000 x g for 30 min. at 0° the supernatant was dialysed overnight against 30 volumes of 25mM Hepes (pH 7.5, KOH) containing 1mM EDTA or passed through Biogel P6 (50-100 Mesh) (2.5 x 20mM) in the same buffer.

Enzymatic activity was measured at 35° by following absorbance at 340 nm using a Gilford 240 spectrophotometer Reaction rates were constant for at least 2 minutes. Rates are given as nanomoles/min./mg protein. The reaction mixture (1ml) contained 2mM L-glutamine, 1mM α-ketoglutarate, 1mM EDTA, 25mM Hepes and 0.16mM NADPH or NADH unless indicated. Stock solutions of reagents were adjusted with KOH to pH 7.5 at room temperature unless otherwise stated. For pH optima, pH was determined at 35° on the complete reaction mixture after the measurement of activity. Protein was determined by microbiuret method using bovine serum albumin as a standard (14).

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1) Abreviation used. Hepes M-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid.

RESULTS: The oxidation of NADPH and NADH catalysed by extracts of tissue was substantially increased when both L-glutamine and α-ketoglutarate were present in the cuvettes (Table I). The linearity of the reaction rate with extract volume is shown in figure 1.

TABLE I

Rates of pyridine nucleotide oxidation (nanomoles/min./mg proteins) in the presence and absence of L-glutamine and α-ketoglutarate by dialysed extracts of wild carrot cells grown on three nitrogen sources.

Nitrogen Source	NH_4Cl		NH_4Cl & $NaNO_3$		$NaNO_3$	
	NADPH	NADH	NADPH	NADH	NADPH	NADH
Complete	14.2	13.9	14.8	16.0	11.3	12.8
" -L-glutamine	3.7	2.9	3.8	2.6	3.3	2.9
" -α-Ketoglutarate	3.3	2.7	3.5	2.8	2.7	3.6

Tissue was grown for four days and the extracts contained 6.94, 8.58, and 5.52 mg/ml protein respectively.
Other details given in text.

The inability of NH_4^+ at the same concentration as L-glutamine to support oxidation of NADPH or NADH is shown in table 2. The ability of L-asparagine to replace L-glutamine is shown in table 2.
With NADPH and Hepes buffer the rate is optimum between pH 6.6 and 7.0. With NADH and Hepes buffer the rate of the reaction increases 20% from pH 7.0 to pH 8.0 whereas in Tris-HCl buffer the reaction showed maximum rate at pH 7.0.

Glutamic dehydrogenase (NADH) but no glutamic

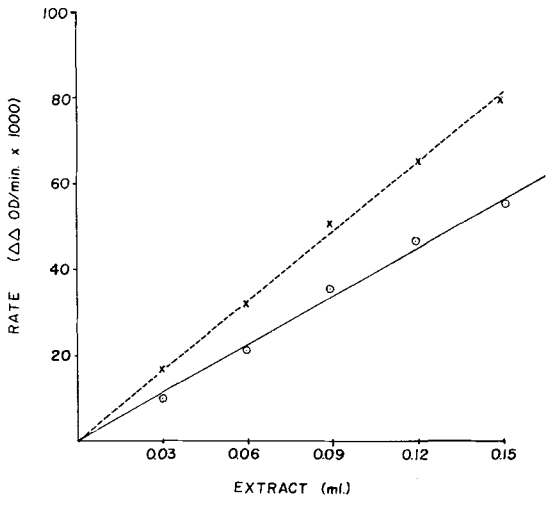

FIGURE 1

The dependance of rate of oxidation of reduced pyridine nucleotide on the amount of added extract.
Solid line - NADPH, broken line - NADH.
OD measured at 340 nm. Rate corrected for oxidation in the absence of L-glutamine. For details see text.

dehydrogenase (NADPH) could be detected in extracts when 100 mM NH_4^+ was used in the assay (Table III). When 2mM NH_4^+ (Table II) was used in the cuvettes neither glutamic dehydrogenase could be detected.

The concentration of L-glutamine giving half maximal velocity with NADPH is 0.62 mM and with NADH is 0.45mM. In both cases the plots of 1/V versus 1/S were linear over the ranges 4mM to 0.4 & 0.2mM respectively.

DISCUSSION: The data in (Table I & figure 1) show that rapid oxidation of NADPH and NADH occurs with extracts of wild carrot cells when both L-glutamine and αa-ketoglutarate are present and the rate is linearly related to the amount of extract added. The inability of NH_4^+ to replace L-glutamine shows that the observed

TABLE II

Rates of pyridine nucleotide oxidation (nanomoles/min./mg protein) in the presence and absence of L-glutamine, NH_4^+ and L-asparagine, by extracts of wild carrot cells grown on NH_4Cl + $NaNO_3$.

	NADPH	NADH
Complete	9.4	9.9
Complete -L-glutamine	4.2	3.1
Complete -L-glutamine + 2mM L-asparagine	9.1	9.0
Complete -L-glutamine + 2mM NH_4Cl	3.9	3.8

oxidation is not catalysed by a combination of a glutaminase and glutamic dehydrogenase. These extracts therefore contain an enzymatic activity (s) which is (are) analogous to glutamate synthase. This is the first report of the presence of such an activity in extracts of cells from a multicellular organism.

The activities demonstrated in these extracts have some characteristics different from those described for glutamate synthase from other sources (3, 4). Here there is: 1. approximately equal activity with either NADPH or NADH and 2. approximately equal activity with L-asparagine and L-glutamine.

Whether these differences reflect a single enzyme with a decreased specificity or several enzymes each as specific as the enzymes from micro-organisms remains to be determined.

The data presented here (Tables II & III) show that the level of NH_4^+ required in the assay mixture to demonstrate glutamic dehydrogenase (NADH) is substantial.

TABLE III

Glutamate synthase and glutamic dehydrogenase activity in dialysed extracts of wild carrot grown on different nitrogen sources.

Nitrogen Source	Glutamate synthase		Glutamic dehyrogenase	
	NADH	NADPH	NADH	NADPH
NH_4Cl	7.4	5.8	3.6	0.2
$NaNO_3$ + NH_4Cl	7.6	5.4	4.3	0.5
$NaNO_3$	7.2	5.3	2.9	0.1

Glutamic dehydrogenase assayed using 100mm NH_4^+. Tissue grown for four days, protein 6.94, 8.58 & 5.52 mg/ml respectively. Activities given as nanomoles pyridine nucleotide oxidized/min./mg protein corrected for oxidation in the absence of L-glutamine. Other details are given in text.

Thus, glutamic dehydrogenase (NADH) from wild carrot like the enzyme from other plant sources seems to have a high Km for NH_4^+. The NADPH dependent glutamic dehydrogenase is barely detectable in extracts of wild carrot (Table III). These enzymes are therefore unlikely to be involved in NH_4^+ assimilation.

The presence of glutamate synthase in these entracts suggests by analogy to micro-organisms, that this activity(s) has a role in NH_4^+ assimilation. The concentration of L-glutamine giving half maximum velocity for these reactions is also compatible with this possibility. Further glutamine synthetase is present in this tissue, (14). Glutamine synthetase from wild carrot has a Km for ammonia of 2.0mM (Caldas and Dougall, unpublished). Thus, assimilation of NH_4^+ by wild

carrot cell cultures may be via glutamine synthetase and glutamate synthase in the net reaction:

NH_4^+ α-Ketoglutarate + ATP + NADPH (NADH) $\longrightarrow$ Glutamic acid + ADP + Pi + $NADP^+$ (NAD^+).

Data in the literature suggest that these reactions also operate in Chlorella pyrenoidosa. Further because of the high Km for NH_4^+ of glutamic dehydrogenase, these reactions may be significant in NH_4^+ assimilation by a wide varety of plants.

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