AN NADP-DEPENDENT L-GLUTAMATE DEHYDROGENASE FROM CHLOROPLASTS OF VICIA FABA L.

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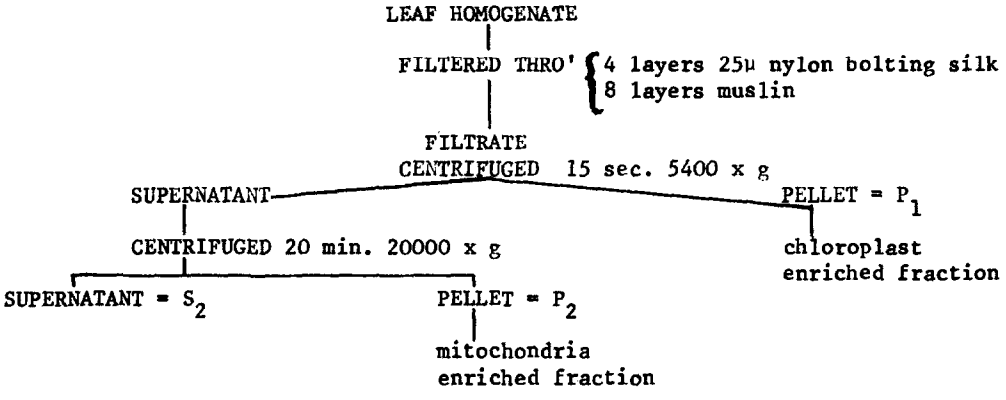
Amino-acids become rapidly labelled during CO_2-fixation in whole Chlorella cells and may account for 32% of the total C fixed during steady-state photosynthesis. (Smith, Bassham and Kirk, 1960). Formation of amino-acids during in vitro photosynthesis has also been demonstrated in isolated chloroplasts (Rosenberg, Capindale and Whatley, 1958) but the mode of incorporation of nitrogen into the photosynthetically produced carbon skeleton has been little investigated. Holm-Hansen (1959) and Moses et al (1959) have reported that ammonium ions will stimulate the rate of incorporation of $^{14}CO_2$ into glutamate in whole Chlorella cells. A likely enzymic incorporation would be by amination of 2-oxoglutarate by NAD-dependent L-glutamate dehydrogenase but Ritenour et al (1967) concluded that in the leaves of Zea mays and Setaria faberii this enzyme was confined to the mitochondria. We were also unable to detect significant activity of this enzyme in chloroplast fractions from leaves of Vicia faba. This paper reports the evidence for an NADP-dependent L-glutamate dehydrogenase which appears to be localised exclusively in the chloroplast lamellar system. The synthetic activity of this enzyme is six times greater in the direction of glutamate formation than in the production of 2-oxoglutarate and it would seem likely to be responsible for in vivo ammonium incorporation in chloroplasts.

Abbreviations: NAD - nicotinamide-adenine dinucleotide (free acid); NADP - nicotinamide adenine dinucleotide phosphate (disodium salt); $NADH_2$ - nicotinamide adenine dinucleotide reduced (disodium salt); $NADPH_2$ - nicotinamide adenine dinucleotide phosphate reduced (tetrasodium salt); Tricine - Tris (hydroxymethyl) methyl glycine.

MATERIALS AND METHODS: Plants of Vicia faba L. var. 'The Sutton' were grown as previously described (Ridley and Leech, 1968) under warm white fluorescent Phillips Reflectalites (colour 29), 2500 foot candles with a 16 hour-day. Freshly-harvested leaves were homogenised in phosphate buffer (KH_2PO_4/Na_2HPO_4) 0.1M, pH 6.8 containing:- sorbitol (0.33M), NaCl (0.1%), $MgCl_2$ (0.1%) and sodium isoascorbate (0.1%). The ratio of medium to leaves was 2.5:1 by weight. A leaf homogenate was prepared according to the method of Walker (1964) and fractionated by differential centrifugation as follows:



All operations were carried out at 0°C and as rapidly as possible. The pellets P1 and P2 were separately resuspended in 10 ml. tricine-NaOH buffer (0.02M, pH 8.0) and homogenised in a Ten Broek ground glass homogeniser. Examination by phase contrast microscopy showed that this hypotonic medium broke virtually all the chloroplasts.

Enzyme assays given under Tables I and III; chlorophyll determined by the method of Arnon (1949).

RESULTS: The total activities of the NAD and NADP-dependent L-glutamate dehydrogenase in (P1), (P2) and the supernatant S2 fractions are shown in Table I, Assay I. The NADP-dependent activity (A) I, is greater in the chloroplast-enriched fraction (P1) than in the mitochondria-enriched fraction (P2) while the activity of the NAD-enzyme is greater in the mitochondria-enriched fraction. Assay II gives the corresponding values for the spectrophotometric assay i.e. the proportion of the total enzyme activity of each

cell fraction which can be solubilized by osmotic breakage of the organelles. Virtually no NADP-dependent activity can be removed by this treatment: in contrast the NAD-enzyme in the mitochondria-enriched fraction appears to be very readily solubilized. These results suggested that the NADP-dependent L-glutamate dehydrogenase may be firmly bound to the chloroplast lamellar system.

Table I: Distribution of NAD-dependent and NADP-dependent L-glutamate dehydrogenase activity in cell fractions from leaves of Vicia faba L.

Cell fraction	NADP-dependent enzyme		NAD-dependent enzyme	
(A) nano-moles 2-oxoglutarate aminated/20 minutes	ASSAY I	ASSAY II	ASSAY I	ASSAY II
P1 (chloroplast enriched)	749	47	336	115
P2 (mitochondria enriched)	392	17	506	763
S2	-	0	-	221
Ratio P1/P2	1.91	2.76	0.66	0.15
(B) nano-moles keto-acid aminated/mg. chlorophyll/20 minutes.	ASSAY I	ASSAY II	ASSAY I	ASSAY II
P1	1810	53	837	131
P2	1960	22	2530	987

ASSAY I - colorimetric (Friedeman, 1943). Details given in Table III. ASSAY II - spectrophotometric (Bulen, 1956). The resuspended pellets P1 and P2 and the supernatant S2 were centrifuged at 50000 x g for 30 min. and aliquots of the supernatants assayed. The oxidation of reduced nicotinamide coenzyme was measured at 340 nm at 25°C. Reaction medium (in μ moles): $NADH_2$ or $NADPH_2$, 0.5; potassium 2-oxoglutarate (pH 7.0), 40: $(NH_4)_2SO_4$, 300; tricine NaOH buffer (pH 8.0), 420. 0.5 ml supernatant containing the enzyme added at zero time. Total volume 3 ml. The control cuvette contained the enzyme and all reagents except 2-oxoglutarate.

This suggestion is firmly supported by a comparison of the total activities of the NADP-enzyme in P1 and P2 expressed in terms of mg. chlorophyll in the fraction (Table IB): the values for the chloroplast-enriched fraction and the mitochondria-enriched fraction are virtually identical. It would seem likely that the activity associated with P2 is located in the fragments of lamellae present in this fraction. The NAD-dependent L-glutamate dehydrogenase activity shows no constant ratio with chlorophyll and its presence in the chloroplast-enriched fraction could be accounted for by the presence of contaminating mitochondria.

To check more rigorously that the NADP-dependent glutamic dehydrogenase is located in the chloroplast lamellae, intact chloroplasts (Leech, 1964) and naked lamellar systems (James and Leech, 1964), both known to be virtually

completely freed from cytoplasmic contamination, were prepared and assayed. The intact chloroplasts were osmotically ruptured before the assay.

Table II: NADP-dependent L-glutamate dehydrogenase activity in (1) Intact chloroplasts (2) Chloroplast lamellar systems from Vicia faba L

Cell fraction	Chlorophyll (mg.)	Change in 2-oxoglutarate (n moles/10')	n moles/mg chlorophyll/10'
(1) Intact chloroplasts	1.06	818	770
(2) Lamellar systems	3.70	2910	790

Chloroplast and lamellar systems prepared as described above. Incubation conditions as for reaction medium A in Table III.

As seen from the results in Table II, the enzyme is present in both intact chloroplasts and in naked lamellar systems and the rate of decrease of 2-oxoglutarate per mg. chlorophyll is the same for both types of suspension. The levels of activity for both intact chloroplasts and lamellae were also very close to the values already found for P1 and P2 (Table I B). The NADP-dependent L-glutamate dehydrogenase is clearly restricted to a location in the chloroplast lamellar system.

The enzymic amination of 2-oxoglutarate and the reductive deamination of glutamate were studied in more detail in the chloroplast-enriched P1 fraction. On incubation of P1 with 2-oxoglutarate and $NADH_2$ or $NADPH_2$, the reaction was found to be linear over the first ten minutes, some activity was found in the absence of coenzymes (Table III) but none in the absence of ammonium ions, in the absence of chloroplasts or with boiled chloroplasts.

The small activity in the absence of added reduced coenzyme is probably a measure of the endogeneous coenzyme isolated with the fraction. Recently Heber and Santarius (1965) and Harvey and Brown (1968) have recorded endogeneous $NADH_2$ and $NADPH_2$ in isolated chloroplast fractions.

When the reaction mixture was chromatographed on thin layers of kieselguhr G, with n. butanol-acetic acid-water (4:1:1) (v:v:v) as solvent, the product of the reaction was ninhydrin positive, had the same R_f as authentic glutamate and when eluted and cochromatographed with glutamate gave a single spot in the butanol solvent system and also with saturated phenolic H_2O as the solvent.

Table III: Activity of L-glutamate dehydrogenase in a chloroplast-enriched fraction (P1) from Vicia faba leaves measured by A. amination of 2-oxoglutarate: B. reductive deamination of glutamate.

Conditions of Assay	Change in 2-oxoglutarate (n moles/mg. chlorophyll/10'		
	No coenzyme	NADP-dependent	NAD-dependent
A. Decrease in 2-oxoglutarate	82	442	109
B. Increase in 2-oxoglutarate	64	80	240

Colorimetric assay of the enzyme. 0.5 to 2.0 ml. of resuspended pellets P1 and P2 or the supernatant S2 were incubated and the conversion of 2-oxoglutarate to glutamate (Reaction medium A) or of glutamate to 2-oxoglutarate (Reaction medium B) measured by the change in dicarboxyclic keto-acid concentration. The enzyme reaction was stopped by the addition of 4 ml. 50% TCA to 6 ml reaction mixture and 2-oxoglutarate concentration measured by the method of Friedeman (1943). With each 1 ml. sample half the quantity of each reagent recommended by Friedeman for 3 ml samples was used and oxygenation effected by swirling the samples on a 'Whirlimix' vortex stirrer. Aliquots were taken at each separation stage and the concentration of the 2,4 dinitrophenylhydrazone derivative assayed by optical density measurements at 420 nm. O.D. was shown to be linear with concentration over the range 0 - 150µg keto acid.

Reaction medium A contained (µmoles): potassium 2-oxoglutarate (pH 7.0), 20; $NADH_2$ or $NADPH_2$, 5; $(NH_4)_2SO_4$, 250; tricine-NaOH buffer (pH 8.0), 800. Final volume 6.0 ml. containing 2.4 mg. chlorophyll incubated at 25° in a shaking waterbath for 10'.

Reaction medium B contained (µmoles): sodium L-glutamate (pH 7.0), 100; NAD or NADP, 5; tricine-NaOH buffer (pH 8.0), 900. Final volume 6.0 ml. containing 1.4 mg. chlorophyll. Other conditions as A.

The rate of glutamate synthesis with $NADPH_2$ was six times the rate of 2-oxoglutarate synthesis under similar conditions (Table III). A completely reversed pattern was found for the NAD-dependent enzyme where the rate of 2-oxoglutarate synthesis was twice as rapid as the rate of glutamate synthesis. This suggests a situation similar to that found in yeast (Holzer, 1966) where there appear to be two separate functional enzymes, one responsible for glutamic acid oxidation using NAD and another which catalyses the synthesis of glutamate. The present results would suggest the possibility that in leaves of Vicia faba the former activity is localized in the mitochondria and the latter in the chloroplast lamellar system.

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