11. Official Methods of Analysis, Association of official Analytical Chemists, Washington, D. C., 1984, 14th Edn. p. 220.

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Increase in NADH-glutamate synthase activity in bean leaf segments by light

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Light increased NADH-glutamate synthase activity in excised leaf segments from dark grown bean seedlings both in the absence as well as presence of ammonium nitrate. The enzyme activity in excised leaves in the presence of ammonium nitrate increased linearly after a lag of 1 h upto 12 h in light, although there was only a slight increase in enzyme activity in dark. The light-induced increase in enzyme activity was inhibited by cycloheximide and DNP and was unaffected by lincomycin and DCMU. Sucrose and glucose supply had differential effects on dark and light-induced enzymes. It is suggested that light induces *de novo* synthesis of the enzyme in leaf segments.

PYRIDINE nucleotide dependent glutamate synthase (glutamate: NAD^+ oxidoreductase (transaminating) EC 1.4.1.14) plays an important role in ammonium assimilation in young seedlings[1,2], and it coexists with the ferredoxin-dependent enzyme in non-green tissues[3]. The effect of light on glutamate synthase activity has been studied only in a few cases and the results seem to be variable. Exposure of etiolated pea and barley shoots to light increases ferredoxin-dependent glutamate synthase, but has no effect on NADH-enzyme[4,5]. Further, upon greening of tobacco cultured cells and callus of *Bovardia ternifolia*, an increased Fd-glutamate synthase activity and unaltered NAD(P)H-dependent activities have been reported[6,7]. However, Fd-glutamate synthase does not change in etiolated maize leaves during their greening[8]. On the other hand, NADH-glutamate synthase is described as a light-regulated enzyme in spinach leaves[9]. In the present investigation, the effect of light/dark changes on levels of NADH-glutamate synthase was studied with a view to find out the possible mechanism.

Seeds of *Phaseolus vulgaris* L.cv. Rajmah were surface sterilized with 0.1% $HgCl_2$ for 30 sec and then washed thoroughly with distilled water. Seedlings were raised in plastic pots containing washed sand in darkness at $25 \pm 2°C$. They were watered on alternate days with $\frac{1}{2}$ strength Hoagland's solution containing no nitrogen. Primary leaves from 7-day old uniformly grown seedlings were used for the described treatments at pH 6.0 either in continuous light of about 65 Wm^{-2} radiant flux density or in darkness at $25 \pm 2°C$.

All the operations for enzymie extraction were carried out at 1–4°C. The enzyme was extracted from the fresh material with pestle and mortar using a pinch of acid washed sand in an extraction medium consisting of 0.2 M phosphate buffer pH 7.5, 2 mM EDTA, 100 mM KCl, 12.8 mM mercaptoethanol and 0.5% Triton X-100. The ratio of leaf material to extraction medium was 1:4 (g:ml). The extract was centrifuged at 20,000 × g for 10 min. The supernatant obtained was saturated to 30% by adding solid ammonium sulphate. The precipitated protein was removed by centrifugation and solid ammonium sulphate was added to the supernatant to bring it upto 55% saturation. The precipitated protein was separated again by centrifugation at 20,000 × g for 10 min and dissolved in one ml of 0.025 M phosphate buffer (pH 7.5) containing 1 mM EDTA, 50 mM KCl, and 12.8 mM mercaptoethanol. The protein solution was then filtered through a sephadex G-75 column (25 × 1 cm) and eluted with the same buffer. Two ml fractions were collected and 4th and 5th fractions with maximum activity were pooled for enzyme assay.

The enzyme activity was assayed spectrophotometrically by observing decrease in absorbance at 340 nm, by a method slightly modified from that of Matoh *et al*[3]. The assay mixture consisted of 10 mM phosphate buffer (pH 7.5) containing 0.4 mM EDTA and 20 mM KCl, 0.2 mM NADH, 0.4 ml enzyme preparation, 0.67 mM 2-oxoglutarate and 2.67 mM glutamine in a total volume of 3.0 ml. The reaction was started by the addition of glutamine and the absorbance was noted after 5 min. Control sets did not include glutamine. One enzyme unit was expressed as nmole NADH oxidized h^{-1}.

Protein in the extract was estimated by Folin phenol reagent by Lowry's[10] method, after precipitation with TCA.

The data presented are average values of at least three replicates with ± S.E. in the tables.

Supply of 5 mM ammonium nitrate to excised leaves from dark grown seedlings increased total and specific enzyme activities both in dark as well as in light (Table 1). The enzyme activities in the leaf segments from dark grown seedlings were higher in light than in dark both in the absence as well as presence of ammonium nitrate.

In the dark, the enzyme activity in the leaf segments increased slowly upto 12 h after a considerable lag of

Table 1. The effects of light on NADH-glutamate synthase activity in leaf segments from dark grown seedlings either in the absence or presence of ammonium nitrate.

Incubation media	Enzyme activity, units g^{-1} fr. wt.		Enzyme activity, units mg^{-1} protein	
	Dark	Light	Dark	Light
−N	504 ± 24 (100)	1016 ± 259 (201)	28.0 ± 2.0 (100)	46.0 ± 11.2 (164)
+N	840 ± 130 (100)	1549 ± 156 (184)	33.2 ± 4.3 (100)	58.4 ± 4.6 (176)

Leaf segments from 7-day old bean seedlings grown in dark were floated on ¼ strength Hoagland's solution containing either no nitrogen or 5 mM NH_4NO_3 in dark or in light for 24 h. Values relative to dark are given in parentheses.

approximately 5 h and then decreased slightly at 24 h (Figure 1). In the light, the enzyme activity increased linearly upto 12 h after an initial lag of 1 h only and a steady state level was maintained upto 24 h at least.

Supply of cycloheximide to leaf segments completely inhibited the increase in enzyme activity by light, while lincomycin and DCMU did not inhibit the same (Table 2). On the other hand, chloramphenicol

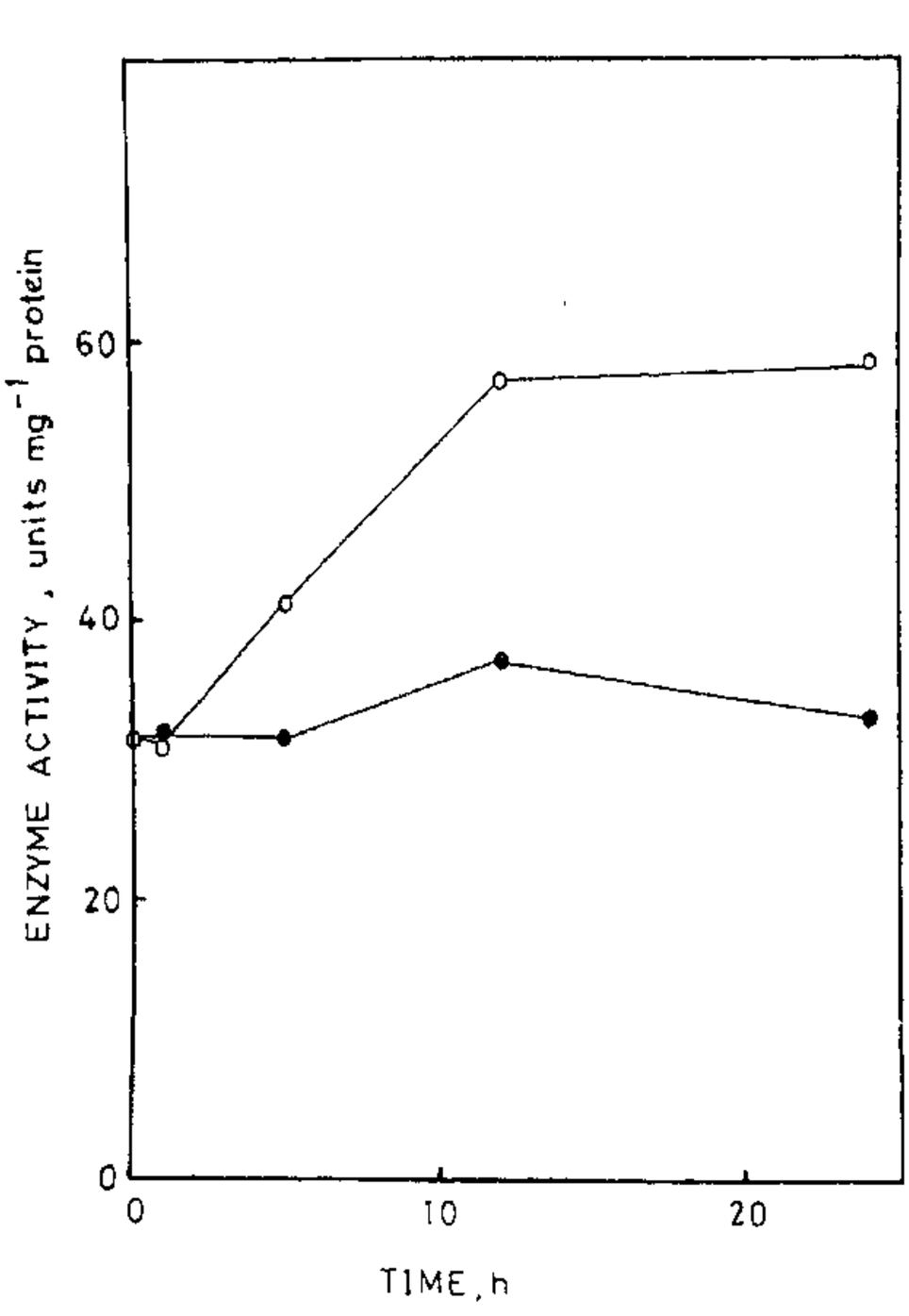

Figure 1. Time course of increase in glutamate synthase activity by ammonium nitrate in dark and light. Leaf segments from 7-day old dark grown bean seedlings were floated on ¼ strength Hoagland's solution containing 5 mM NH_4NO_3 in dark or in light for desired time period. Open circles—light; closed circles—dark.

Table 2. The effects of inhibitors on increase in NADH-glutamate synthase by light in the presence of ammonium nitrate in excised leaves from dark grown seedlings.

Treatment	Enzyme activity, units mg^{-1} protein
In dark (control)	33.2 ± 4.3 (100)
In light	
No inhibitor	58.4 ± 4.6 (176)
+ Cycloheximide, 5 μg/ml	29.0 ± 1.4 (87)
+ Chloramphenicol, 1 mg/ml	105 ± 12.0 (316)
+ Lincomycin, 1 mM	51.2 ± 2.0 (154)
+ DCMU, 0.01 mM	47.0 ± 0.4 (141)
+ DNP, 1 mM	27.9 ± 4.3 (84)

Leaf segments from 7-day old dark grown seedlings were floated on ¼ strength Hoagland's solution containing 5 mM NH_4NO_3 for 24 h either in dark (control) or in light in the presence of different inhibitors. Values relative to control are given in parentheses.

stimulated increase in enzyme activity by light considerably.

Supply of 5 mM sucrose and 10 mM glucose to excised leaves from dark grown seedlings increased enzyme activity both in light and dark, although the inducing effect of light in the presence of glucose was marginal (Table 3).

When enzyme preparations from light or dark induced leaf segments were stored at 25°C for 1 h in dark the enzyme activity increased considerably; the increase being higher for dark than for light-induced enzyme (Table 4). However, the increase in the presence of sucrose was lowered for light induced enzyme and the activity was almost completely inhibited in dark-induced enzyme. Addition of glucose prevented the increase in light-induced enzyme and lowered a bit in case of dark-induced enzyme.

The experiments demonstrate an increase in glutamate synthase activity by ammonium nitrate in excised bean leaf segments, which is higher in light than in dark. On the other hand, the enzyme activity in greening pea shoots does not change much, although Fd-glutamate synthase increases substantially[4]. The effect of light on

Table 3. Effects of *in vivo* supply of sucrose and glucose on increase in NADH-glutamate synthase activity by light in the presence of ammonium nitrate in excised leaves from dark grown seedlings.

Treatment	Enzyme activity, units mg^{-1} protein		Percent increase by light
	In dark	In light	
Control	33 ± 4.3	58 ± 4.6	76
+ Sucrose (5 mM)	48 ± 2.9	91 ± 4.6	90
+ Glucose (10 mM)	55 ± 2.0	65 ± 3.1	18

Leaf segments from 7-day old dark grown seedlings were floated on ¼ strength Hoagland's solution containing 5 mM NH_4NO_3 either in light or in dark for 24 h. Carbohydrates were included in the incubation medium as indicated.

Table 4. The effects of *in vitro* addition of sucrose and glucose on the stability of glutamate synthase from light and dark treated bean leaves.

Incubation time, h	Carbohydrate added	Enzyme activity, relative to control	
		Dark-induced	Light-induced
At 0 h (control)	One	100	100
After 1 h	None	187	136
	Sucrose (5 mM)	7	110
	Glucose (10 mM)	140	96

The enzyme preparations from light or dark treated leaves of dark grown seedlings were stored at 25°C for one h in dark in the absence or presence of sucrose and glucose. The values relative to 0 h control are given in the table. The control value was the same as in Table 3.

enzyme activity in the present case does not seem to be related to its possible effects on nitrogen uptake, as the same is observed both in the absence as well as presence of ammonium nitrate in the nutrient medium.

Increase in enzyme activity during incubation of excised leaf segments in light seems to involve *de novo* synthesis of the enzyme, as there is a considerable time lag. (Figure 1) and the increase is inhibited by cycloheximide (Table 2). Apparently, the site of enzyme synthesis is cytoplasmic as inhibitors of protein synthesis on 70 S ribosomes such as lincomycin, has no effect on the increase. On the other hand, chloramphenicol increases enzyme activity considerably. The mechanism of this increase is not understood at the moment. The enzyme induction by light appears to be dependent on mitochondrial activities related to ATP generation as well, as supply of DNP inhibits the same.

The insensitivity of light-induced increase in NADH-glutamate synthase activity to DCMU, excludes any direct participation of photosynthesis in the process. However, differential effects of carbohydrates glucose and sucrose on dark- and light-induced enzyme activities are observed both during *in vivo* as well as *in vitro* supply of carbohydrates (Tables 3 and 4). Further, the degree of activation during storage at 25°C is also different for dark- and light-induced enzyme. We believe therefore, that a new isozyme of glutamate synthase is synthesized during light treatment and that this isozyme is more active than the one synthesized in the dark. Further experiments are in progress to separate the two enzymes.

1. Beevers, L. and Storey, R., *Plant Physiol.*, 1976, **57**, 862.
2. Matoh, T. and Takahashi, E., *Planta*, 1982, **154**, 289.
3. Matoh, T., Ida, S. and Takahashi, E., *Plant Cell Physiol.*, 1980, **21**, 1461.
4. Matoh, T. and Takahashi, E., *Plant Cell Physiol.*, 1981, **22**, 727.
5. Wallsgrove, R. M., Lea, P. J. and Miflin, B. J., *Planta*, 1982, **154**, 473.
6. Suzuki, A., Nato, A. and Gadal, P., *Plant Sci. Lett.*, 1984, **33**, 93.
7. Suzuki, A., Audet, C. and Oaks, A., *Plant Physiol.*, 1987, **84**, 578.
8. Harel, E., Lea, P. J. and Miflin, B. J., *Planta*, 1977, **134**, 195.
9. Ito, O., Yoneyama, T. and Kumazawa, K., *Plant Cell Physiol.*, 1978, **19**, 1109.
10. Lowry, O. H., Roseborough, N. J., Farr, A. L. and Randall, R. J., *J. Biol. Chem.*, 1951, **193**, 265.

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Drug targeting to a fast growing rat histiocytoma

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AK-5 is a rat macrophage-like tumor line and the effect of daunomycin was tested on this cell line. Daunomycin alone is not highly effective against this tumor, however, when conjugated to cytotoxic anti-AK-5 antibodies, kills the tumor cells thereby regressing tumors in 100% animals. Anti-AK-5 acts as a carrier for daunomycin to reach the target cells.

In effective cancer chemotherapy there is always the risk of toxicity to the normal proliferating cells. This limitation could be improved by the specific targeting of antineoplastic drugs to the tumor cells, using specific carriers as conjugates of the drugs. Polyclonal and monoclonal antibodies against tumor-associated antigens have been used as carriers for various drugs and toxins, and their use in regression of the tumors have been demonstrated[1-5]. We illustrate one such example of antibody conjugation to the antitumor drug daunomycin. AK-5 is a rat histiocytic tumor line which possesses several typical characteristics of a macrophage-like cell such as Fc-receptors, Ia-determinant, non-specific estrase, lysozyme and phagocytosis[6]. AK-5 is highly immunogenic[6] and induces the production of cytotoxic antitumor antibodies in the host when injected subcutaneously. These antibodies are directed against a surface antigen, which has been purified and characterized to be specific to AK-5 cells. When the tumor cells are injected intraperitoneally into rats, they kill all the injected animals. However, the tumor gets completely regressed when a combination of immunomodulators and antineoplastic drug treatment is given[7]. The drug daunomycin by itself is not completely effective in regressing the AK-5 tumor as ascites. We have raised anti AK-5 antibodies in rats by injecting 5×10^6 AK-5 cells subcutaneously. After about 25 days, the animals are injected 5×10^6 tumor cells i.p. and bled 5 days later to get the antisera. Immunoglobulin fraction was precipitated with ammonium sulphate and passed through a column of Sephadex G-150. The partially purified antitumor antibodies were conjugated to daunomycin using the glutaraldehyde method[8] and the