Phytochemistry, 1980, Vol. 19, pp. 1919–1924. © Pergamon Press Ltd. Printed in England. 0031–9422/80/0901–1919 $02.00/0

LIGHT ACTIVATION OF NADP MALATE DEHYDROGENASE IN A RECONSTITUTED CHLOROPLASTIC SYSTEM

JEAN VIDAL, JEAN PIERRE JACQUOT and PIERRE GADAL

Laboratoire de Biologie Végétale, Université de Nancy I, C.O. No. 140, 54037 Nancy Cedex, France

(*Revised received* 25 *January* 1980)

Key Word Index—*Spinacia oleracea*; Chenopodiaceae; *Phaseolus vulgaris*; Leguminosae; NADP malate dehydrogenase; protein factors (thioredoxins); activation by dithiothreitol and by light; reconstituted chloroplastic system; *N*-ethylmaleimide.

Abstract—Isolated spinach chloroplasts exhibited a light dependent NADP-malate dehydrogenase activity. In a reconstituted chloroplastic system, it was demonstrated that the activation process requires light, chloroplastic membranes, ferredoxin, protein factors and a component which is probably a reductase. Moreover, it was shown that activation of malate dehydrogenase by dithiothreitol or by light resulted in sulfhydryl groups formation on protein factors and enzyme. Using this system, activation of the bean leaf enzyme in the presence of its own protein factors was also obtained. The influence of the different components of the system on the activation process was investigated.

INTRODUCTION

NADP malate dehydrogenase (L-malate: $NADP^+$-oxidoreductase, EC 1.1.1.82) is a chloroplastic enzyme whose activity is light dependent [1]. Wolosiuk *et al.* [2] established that the activation process performed in an illuminated reconstituted chloroplastic system requires the presence of thylakoids, ferredoxin–thioredoxin reductase, thioredoxin and the enzyme. Thioredoxin and ferredoxin–thioredoxin reductase were found to be analogous, at least in function, with the corresponding bacterial proteins, but as yet nothing is known of the chemical nature of the higher plant compounds [3–5]. On the other hand, Anderson *et al.* [6, 7] stated that NADP malate dehydrogenase activation was due to "light-effecting mediators" which are firmly bound to the photosynthetic membranes. This hypothesis rules out the possibility of the intervention of thioredoxin in the process. Previously we showed that the bean, spinach and sorghum leaf enzyme can be activated *in vitro* by dithiothreitol (DTT) and protein factors [8–10]. These factors are small MW proteins which are endogenous to the chloroplasts [11]. In this work, in order to confirm the relationship of these protein factors to the thioredoxin described by Wolosiuk *et al.* [2], we studied their efficiency in enzyme activation in a reconstituted chloroplastic system. Using this system we have been able to show that the protein factors are necessary for the photomodulation of spinach and bean leaf enzymes. We also studied the influence of the concentration of the different compounds, membranes, ferredoxin, protein factors upon the activation process and report that another substance is necessary for completion of this process. This substance, which is probably the reductase cited above, was separated from the enzyme through Sephadex G 100 gel filtration and obtained as two main peaks of activity. Moreover, a study of the inhibitory effect of *N*-ethylmaleimide (NEM) indicates that the activation process is dependent on reduction of disulfide bonds in the protein factors and the dehydrogenase.

RESULTS

Spinach leaf NADP-MDH activation by light in chloroplasts

When a suspension of chloroplasts from spinach leaves was kept in darkness, a very low NADP-MDH activity was detected (Fig. 1). Upon illumination it rose very quickly and was maximal 5–10 min after the beginning of light exposure. When returned to darkness, the activity in the

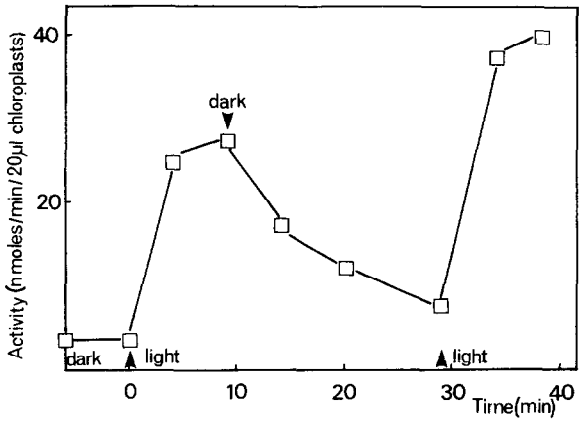

Fig. 1. Evolution of NADP-MDH activity in a suspension of chloroplasts during light–dark transitions. Chloroplasts (50–60% intact as judged by spectrophotometric control [17]) were prepared from 40 g spinach leaves as described in the Experimental. Final suspension of the organelles in 300 µl isotonic medium contained 0.18 mg chlorophyll. Illumination was performed in a small stoppered flask using the experimental device described in the Experimental. At the indicated intervals of time, aliquots (20 µl) were pipetted into 3 ml reaction medium to determine the activity of NADP-MDH released following the osmotic shock.

chloroplasts declined quite sharply but recovered following a new light exposure. This result is in agreement with the data reported by Hatch and coworkers on intact leaves of maize [1]. Moreover, it shows that the mechanism responsible for activation lies entirely in the chloroplast.

Spinach leaf NADP-MDH activation by light in a reconstituted chloroplastic system

Ten-minute illumination of a reconstituted chloroplastic system containing 10 μl of membrane suspension (12 μg chlorophyll), 10 μl of ferredoxin solution (11 μg protein), 30 μl of protein factor solution (11 μg protein) and 30 μl of an enzymatic extract (94 μg protein) purified by using Sephadex G 50, resulted in NADP-MDH activation. If one of these components was omitted, the degree of activation was greatly reduced or abolished. This result ruled out the possibility of a direct photoactivation of the dehydrogenase or an effect of the membranes alone on the enzyme; this more likely suggested a modulation through the reducing power generated by light in the photosystems and transmitted via ferredoxin and protein factors to the enzyme. It could also be concluded that the protein factors characterized here were identical, at least on the basis of their biological function, to the thioredoxins described by Wolosiuk *et al.* [2].

Influence of N-ethylmaleimide on the activity of spinach leaf protein factors and NADP-MDH

The activation of NADP-MDH by dithiothreitol (DTT) in the presence of protein factors suggested a disulfide bridge reduction. In order to demonstrate the formation of sulfhydryl groups during this process, we used a specific reagent, *N*-ethylmaleimide (NEM), known to bind them covalently.

Protein factors were incubated in the presence or absence of DTT. Then, SH-groups were blocked by NEM and this reagent was added in excess to remove the remaining DTT. Excess NEM was in turn eliminated by addition of mercaptoethanol (ME) to the medium. The experiment was based on the fact that if SH-groups were effectively formed during the DTT treatment, the reaction with NEM would result in the loss of biological activity. To verify this assumption, treated protein factors were incubated with untreated inactive NADP-MDH in the presence of DTT. The results are shown in Tables 1 and 2, and Fig. 2. In order to explain the results obtained, it should be pointed out that the activation of the enzyme by DTT without any added protein factors produced about one-third of the maximal activity, as described under experimental conditions (assay II, Table 1). When the protein factors (preincubated in the presence of DTT) were treated by NEM, they completely lost their specific activating power on the inactive enzyme (assay III, Table 1). However, if preincubation by DTT is omitted, the treatment by NEM was ineffective (assay IV, Table 1).

When NADP-MDH was treated with NEM the main difference compared to the previous results was the partial loss of activity without any preliminary reduction by DTT (assay III, Table 2). This result indicated that SH-groups were present in the inactive enzyme. However, when the enzyme was incubated in the presence of DTT without any added protein factors, activation was not observed after the NEM treatment (assay IV, Table 2). This was an unexpected result since the enzyme cannot be totally activated by DTT alone. We must conclude that DTT alone, as well as DTT plus protein factors, is able to liberate free SH-groups in NADP-MDH; in both cases the enzyme was activated, but only in the second case was the enzyme fully active.

We also tested the efficiency of NEM on the enzyme activation process in a reconstituted chloroplastic system using light as the natural donor of reducing power. It can be

Table 1. Influence of NEM on spinach leaf protein factor activity

Assay No.	Nature of the assay	Rel. act.
I	Inactive NADP-MDH + protein factors + DTT (20 mM, 20 min)	100
II	Inactive NADP-MDH + DTT (20 mM, 20 min)	32
III	Protein factors + DTT (5 mM, 40 min) + NEM (20 mM, 10 min) + ME (100 mM) + inactive NADP-MDH + DTT (20 mM, 20 min)	34
IV	Protein factors + NEM (20 mM, 10 min) + ME (100 mM), + inactive NADP-MDH + DTT (20 mM, 20 min)	93

200 μl of protein factor solution (70 μg of proteins) were incubated at 30° in the presence of DTT, 5 mM (assay III) or its absence (assay IV) for 40 min. Then 200 μl of a NEM solution in phosphate buffer 50 mM, pH 8 were added to give a final concentration of 20 mM. After 10 min of reaction, excess NEM was eliminated by the addition of 4 μl of pure ME (final concentration 100 mM). To measure the efficacy of protein factors, 200 μl of inactive NADP-MDH solution (800 μg of proteins) and DTT (20 mM) were added to the medium. The activity of the dehydrogenase was determined in 1 ml reaction medium by the spectrophotometric method after 20 min of activation. Other assays were run simultaneously using the same experimental conditions but controlling the extent of enzyme activation by the non-treated protein factors (assays I and II).

Table 2. Influence of NEM on spinach leaf NADP-MDH activity

Assay No.	Nature of the assay	Rel. act.
I	Inactive NADP-MDH + protein factors + DTT (20 mM, 20 min)	100
II	Inactive NADP-MDH + DTT (20 mM, 20 min)	31
III	Inactive NADP-MDH + NEM (20 mM, 10 min) + ME (100 mM) + protein factors + DTT (20 mM, 20 min)	57
IV	Inactive NADP-MDH + DTT (5 mM, 40 min) + NEM (20 mM, 10 min) + ME (100 mM) + protein factors + DTT (20 mM, 20 min)	30

Treatment by NEM was effected on NADP-MDH (200 μl, 800 μg of protein) previously incubated (assay III) or not (assay IV) in the presence of DTT according to the procedure used for protein factors (Table 1). Catalytic activity was then measured after addition of DTT (20 mM) in the presence of protein factors (200 μl, 70 μg of proteins). Assays I and II were carried out to control the extent of activation using the specific experimental conditions.

seen in Fig. 2 that the activity of NADP-MDH promoted by light was completely abolished by NEM treatment and that further addition of DTT did not allow recovery of the activity. In contrast, in the sample kept in darkness, NEM did not affect the activity which was revealed by DTT after elimination of NEM by ME. All these data establish that the formation of SH-groups in the protein factors and in the enzyme is crucial to the *in vivo*-activation process.

Evidence for the intervention of a reductase in the light activation of spinach leaf NADP-MDH

When the activating experiments in a reconstituted chloroplastic system were performed using an enzyme extract purified on Sephadex G 100 instead of G 50, the light activation was not observed. Activation was recovered if aliquots of fractions held back on the gel were added to the system (fraction 9 of Fig. 3). Thus another compound, distinct from ferredoxin and protein factors, appeared to be necessary for the completion of the activation process. If we accept that highly purified NADP-MDH is activated by protein factors in the presence of DTT (unpublished results) then, the only step where the novel substance can act is the reaction between ferredoxin and protein factors. As we showed above, the effect of the dithiol being a disulfide reduction, the substance could be a reductase. It is probably the enzyme recently discovered by Wolosiuk *et al.* [2] which catalyses the reduction of thioredoxin by ferredoxin. In order to

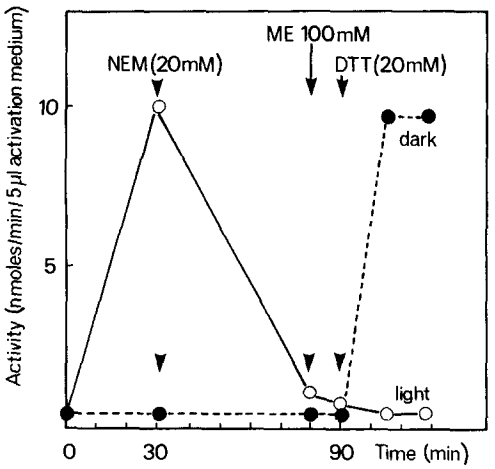

Fig. 2. Influence of NEM on light activation of spinach leaf NADP-MDH in a reconstituted chloroplastic system. Chloroplasts were prepared as described in the Experimental. The pellet of intact organelles (0.9 mg chlorophyll, 2.4 mg proteins) was resuspended in 300 µl of Tris (Cl^-) buffer 50 mM, pH 7.6. Two assays (100 µl of the suspension each) were run at room temp. (20°) in stoppered flasks; one was illuminated (20 klx), the other kept in darkness. The nature of different treatments is indicated on the figure. Final concentrations of NEM, ME and DTT in the assays are also displayed.

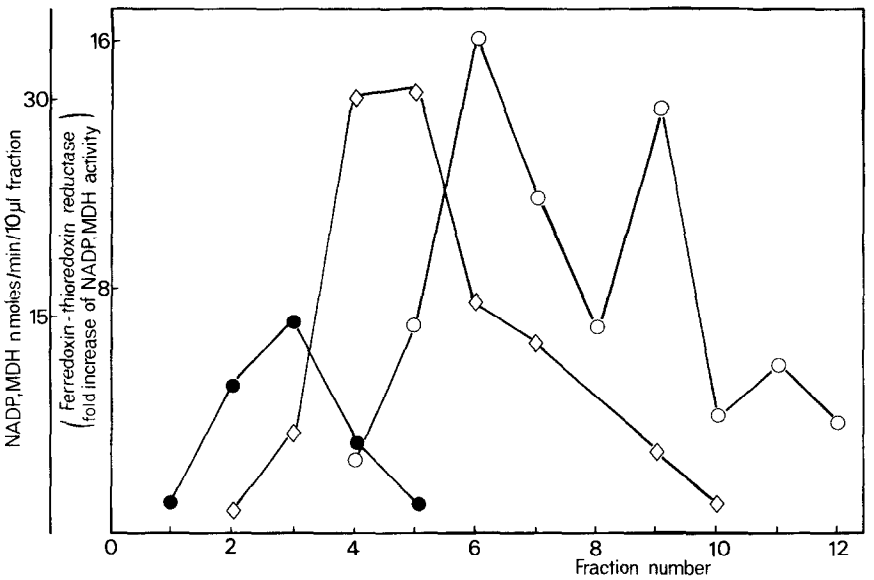

Fig. 3. Sephadex G 100 gel filtration of a chloroplastic extract from spinach leaves. A chloroplastic extract (10 ml) obtained from 40 g of spinach leaves was concentrated by Ultrafiltration (Amicon cell—membrane PM 10) to 300 µl. It was filtered on Sephadex G 100 (V_0 = 5.4 ml) equilibrated in Tris (Cl^-) buffer, 50 mM, pH 7.6; ME 14 mM. Fractions (500 µl) of the effluent were collected. NADP-MDH activity was determined on aliquots using the DTT assay in 1 ml of reaction medium (75 min activation at 30° in the presence of DTT 50 mM). Activity of the reductase was estimated in the reconstituted chloroplastic system. The complete medium contained 20 µl of fraction 4 (NADP-MDH, 100 µg of proteins) 10 µl chloroplastic membrane suspension (12 µg chlorophyll), 10 µl of ferredoxin solution (30 µg protein), 20 µl of protein factor solution (25 µg protein) and aliquots of 20 µl of the fractions. Activation was carried out for 20 min as described in Table 1. Activity in the assay placed in light is compared to a same assay kept in darkness. ●——●, Dextran blue; ◇ - - - -◇, NADP-MDH activity; ○——○, reductase activity.

better characterize this component, we have determined its chromatographic behavior in the fractions coming off the Sephadex G 100, using the reconstituted chloroplastic system. It can be seen in Fig. 3 that inactive NADP-MDH, which eluted slightly after the void volume, preceded two main peaks of the reductase. This result is in agreement with the data of Wolosiuk *et al.* [12] which indicated two fractions of ferredoxin thioredoxin reductase when chromatographed on hydroxylapatite.

Spinach leaf NADP-MDH activation: influence of the concentration of the components in the reconstituted system

In order to determine the optimal conditions for the NADP-MDH activation process, we studied the effect of different concentrations of all the components included in the reconstituted system (Fig. 4). Using the described experimental conditions, maximal activity was reached after 5 min of light exposure; this time of activation was maintained for further determinations. Ferredoxin and protein factor solutions were respectively saturating in regard to the activation process at *ca* 10 and 8 μg protein. A lag phase was seen in both cases which can be accounted by the fact that traces of ferredoxin and protein factors were probably still present. Thus the initial values, without exogenous ferredoxin or protein factors, are higher than the control in the absence of light or membranes. Membranes also gave a saturation curve for the activation of NADP-MDH, becoming most efficient at *ca* 10 μg chlorophyll in the medium.

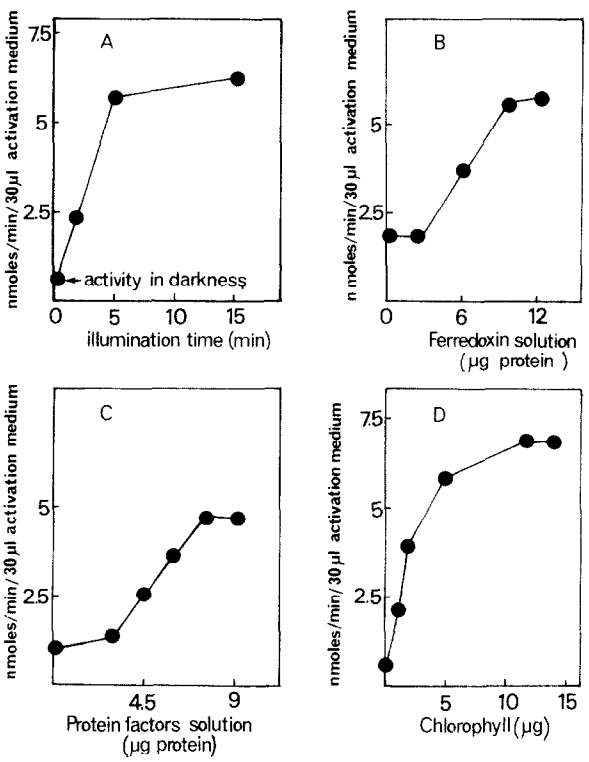

Fig. 4. Influence of ferredoxin, protein factors and chloroplastic membranes concentration on the light activation process of spinach leaf NADP-MDH. The standard activation medium contained 30 μl of NADP-MDH extract (200 μg protein), 30 μl of protein factor solution (9 μg protein), 10 μl ferredoxin solution (12 μg protein) and 10 μl chloroplastic membrane suspension (12 μg chlorophyll). When the quantity of one component varied, the volume was completed by Tris (Cl^-), 50 mM, pH 7.6; ME, 14 mM. Activation by light was carried out for 5 min as described in the text, and activity was measured in 1 ml reaction medium. A—Evolution with time of NADP-MDH activity; B—influence of different concentrations of ferredoxin solution on NADP-MDH activation; C—influence of different concentrations of protein factor solution on NADP-MDH activation; D—influence of different concentrations of chloroplastic membrane suspension on NADP-MDH activation.

Bean leaf NADP-MDH activation by light in a reconstituted chloroplastic system

Since it has been reported that in pea leaves protein factors were not necessary to promote NADP-MDH activation [7], we tested the enzyme and protein factors from another legume, French bean, in the reconstituted chloroplastic system containing spinach chloroplastic membranes and ferredoxin. As with the spinach system, all the components were required to obtain a photomodulation of the bean enzyme. Moreover we have established that protein factors from different origins (spinach, sorghum leaves) were also effective in this process (unpublished results).

DISCUSSION

Spinach leaf NADP-MDH is indirectly activated by light in a chloroplastic reconstituted system. The light effect is mediated by chloroplastic membranes, ferredoxin, protein factors and an unknown substance separated from the enzyme on Sephadex G 100, which is probably a disulfide reductase. This particular mode of regulation has also been found in the case of several enzymes from spinach leaf chloroplasts, but has never been reported in other plants [18–21]. We have now found that this type of regulation occurs also for the activation of NADP-MDH from bean leaf, suggesting its general occurrence among higher plants.

The protein factors that we have characterized seem to be identical, at least in function, to the thioredoxin reported by Wolosiuk *et al.* [2]. Like these authors, we have demonstrated previously the large distribution, the chloroplastic location and polymorphism of these small MW proteins (range 10000–30000) [8, 10, 11, 22]. Moreover, we have recently established that the activation of NADP-MDH and FDPase required specific protein factors; in addition, a non-chloroplastic form was detected in root extracts [23].

Protein factors are necessary to promote the NADP-MDH activation by light or dithiothreitol. *N*-Ethylmaleimide, a sulfhydryl reagent, mainly affected the reduced state of the factors and that of the enzyme showing that the activation resulted in the formation of sulfhydryl groups essential for biological activity. This result is in agreement with the data of Holmgren *et al.* [24] on bacterial thioredoxin, a compound implicated in DNA synthesis, which has one disulfide bridge in the inactive form and two sulfhydryl groups in the active one. However, the entire process leading to full activation of the enzyme is probably more complex than a simple disulfide bridge reduction, as shown by the necessary presence of the protein factors.

An enzyme which catalyses the reduction of thioredoxin in the presence of ferredoxin was recently discovered by Wolosiuk and Buchanan [4] and named by these authors

ferredoxin–thioredoxin reductase by analogy with the bacterial NADPH-thioredoxin reductase. In the case of spinach leaves we have obtained strong evidence that the compound responsible for the transfer of reducing power of ferredoxin to protein factors is a reductase. We detected two main peaks of this activity using Sephadex G 100 gel filtration. This result can be correlated with the fact that protein factors appear to be specific to enzyme activation and may be of physiological significance; however, at this time, nothing is known about their catalytic properties or structures.

Elsewhere, Anderson's hypothesis suggested that enzyme activation was effected by compounds bounded to thylakoids, namely 'light-effecting mediators'; this author also suggested that the reduction process was catalysed by a reshuflase rather than a reductase [6]. In this case, no net increase in sulfhydryl groups in protein factors and dehydrogenase would be detected. In contrast, our results indicated the formation of sulfhydryl groups during NADP-MDH activation which suggest the mediation of a reductase. This working hypothesis was reinforced by the fact that the protein factors probably contained only one disulfide bridge [24]; if correct, this excludes the possibility of a disulfide bridge rearrangement.

EXPERIMENTAL

Preparation of intact spinach chloroplasts. Isolation of the components necessary for the reconstituted chloroplastic system. All steps were carried out at 3°; chloroplasts were isolated from spinach leaves according to the method of ref. [13]. Leaves (40 g) were gently homogenized using a Waring blendor in 80 ml isotonic medium. The brei was filtered through gauze and centrifuged at 4000 *g* for 10 sec. For the prepn of intact organelles, the pellet was washed × 3 with 3 ml isotonic medium and finally resuspended in 0.3 ml of the same medium. Alternatively, organelles were lysed osmotically by suspension in 10 ml hypotonic medium containing Tris (Cl^-), 50 mM, pH 7.6 (pH value at 20°), mercaptoethanol (ME), 14 mM. The chloroplast membrane fragments were obtained by centrifugation (10 min, 47 000 *g*); they were washed once and resuspended in 2.5 ml Tris (Cl^-), 10 mM, pH 7.6. To the supernatant was added $(NH_4)_2SO_4$ to 80% saturation. The protein pellet collected by centrifugation (10 min, 15 000 *g*) was dissolved in 0.6 ml Tris (Cl^-), 10 mM, pH 7.6; ME, 14 mM. Filtration on Sephadex G 50 (V_0 = 7 ml) equilibrated with Tris (Cl^-), 10 mM, pH 7.6; ME, 14 mM, resulted in the separation of NADP-MDH fraction (1 ml) collected immediately after the void vol. from protein factors which eluted between 20 and 50% of the int. vol.

For a large scale prepn of protein factors the first supernatant, previously obtained by centrifugation at 4000 *g* for 10 sec, was made to 80% saturation with $(NH_4)_2SO_4$. Proteins sedimented by centrifugation (40 000 *g* for 10 min) were solubilized in 6 ml phosphate buffer, 10 mM, pH 7.6. They were filtered through Sephadex G 50 gel (void vol. = 25 ml) equilibrated with the same buffer. Protein factors collected between 20 and 50% of the int. vol. of the gel were applied to a DEAE-cellulose column (1 × 3 cm) equilibrated with phosphate buffer, 10 mM, pH 7.6. They were eluted from the column by 20 ml of buffer made up with 150 mM NaCl. This step separated protein factors from ferredoxin. The fractions containing the protein factors were concd to 500 μl by Ultrafiltration (Amicon cell membrane PM 10) then filtered through Sephadex G 25 gel equilibrated with Tris (Cl^-) buffer, 50 mM, pH 7.6, ME 14 mM. Protein factor solution (1 ml) was finally obtained.

Protein factors and enzymes were similarly prepared from 1 g lyophilized bean leaves. The culture conditions of plant material were described previously [9].

Ferredoxin was obtained according to the method of ref. [14]. After the first DEAE-cellulose step ferredoxin was filtered on Sephadex G 25 gel equilibrated with Tris (Cl^-) buffer, 50 mM, pH 7.6, then concentrated by Ultrafiltration. The ferroprotein was not completely purified but it was shown that it was not contaminated with NADP-MDH, the reductase or the protein factors.

Activation of NADP-MDH and determination of its activity. Unless otherwise indicated, the activation of NADP-MDH in a reconstituted chloroplastic system was carried out in a SMI micro-pipette (micro pettor) illuminated by an incandescent bulb (30 klx, OSRAM bulb, 150 W). Excess heat was removed by a stream of cold H_2O through a transparent Plexiglass box allowing a constant temp. of 20° for activation. The enzyme can also be activated by DTT. In this case, if protein factors were omitted, slower but significant activation was carried out by the dithiol alone. This fact was accounted for in this work to activate the enzyme in Sephadex G 100 effluent (Fig. 3) and before NEM treatment (Table 2). NADP-MDH activity was measured at 340 nm, 30° using a double beam spectrophotometer (Beckman Acta C (II) in 3 or 1 ml of medium containing phosphate buffer 50 mM, pH 8; oxaloacetate, 1.5 mM; EDTA, 0.5 mM and NADPH, 0.2 mM.

Determination of proteins and chlorophyll. Proteins were determined according to the method of ref. [15] and chlorophyll by the method of ref.]16].

Influence of N-ethylmaleimide on protein factors and enzyme activity. Spinach leaves (10 g) were homogenized in 25 ml phosphate buffer, 100 mM, pH 7, containing sodium thioglycolate 10 mM, EDTA, 1 mM and polyclar, 1 g. The brei was filtered through gauze, then centrifuged at 50 000 *g*, 20 min, $(NH_4)_2SO_4$ added to supernatant to 80% saturation. The protein pellet obtained by centrifugation 20 000 *g*, 10 min was redissolved in 4 ml phosphate buffer, 50 mM, pH 8. Half of the extract was filtered through Sephadex G 25, the other half through Sephadex G 50 previously equilibrated in the same buffer. A 4 ml aliquot of effluent from the G 25 column was immediately collected after the void vol. and was heated until protein precipitated (protein factor solution). This step, which resulted in the elimination of NADP-MDH, did not affect the activating power of the factors in the DTT assay. The G 50 effluent (4 ml) collected at the void vol. of the column contained the inactive enzyme without any contaminating protein factors (NADP-MDH solution). The experimental conditions of the activation process in the presence of *N*-ethylmaleimide are described under the figures.

Acknowledgements—We thank Miss J. Bouville for excellent technical assistance. This work was supported by a DGRST grant No. 78.7.0362.

REFERENCES

1. Johnson, M. D. and Slack, C. R. (1970) *Biochem. J.* **119**, 273.
2. Wolosiuk, R. A., Buchanan, B. B. and Crawford, D. W. A. (1977) *FEBS Letters* **81**, 253.
3. Buchanan, B. B. and Wolosiuk, R. A. (1976) *Nature* **264**, 669.
4. Wolosiuk, R. A. and Buchanan, B. B. (1977) *Nature* **266**, 565.
5. Holmgren, A., Buchanan, B. B. and Wolosiuk, R. A. (1977) *FEBS Letters* **82**, 351.
6. Anderson, L. E. and Avron, M. (1976) *Plant Physiol.* **57**, 209.
7. Anderson, L. E., Nehrlich, S. C. and Champigny, M. L. (1978) *Plant Physiol.* **61**, 601.

8. Jacquot, J. P., Vidal, J. and Gadal, P. (1976) *FEBS Letters* **71**, 223.
9. Vidal, J., Jacquot, J. P., Gadal, P. and Vidal, D. (1978) *Physiol. Plant.* **42**, 307.
10. Vidal, J., Jacquot, J. P., Membre, H. and Gadal, P. (1978) *Plant Sci. Letters* **11**, 305.
11. Jacquot, J. P., Vidal, J. and Gadal, P. (1977) *Planta* **137**, 89.
12. Wolosiuk, R. A., Crawford, N. A., Yee, B. C. and Buchanan, B. B. (1979) *J. Biol. Chem.* **254**, 1627.
13. Cockburn, W., Walker, D. A. and Baldry, C. W. (1968) *Plant Physiol.* **43**, 1415.
14. Yocum, C. F., Nelson, N. and Racker, E. (1975) *Prep. Biochem.* **5**, 305.
15. Scopes, R. K. (1974) *Analyt. Biochem.* **59**, 277.
16. Bruinsma, J. (1961) *Biochim. Biophys. Acta* **52**, 576.
17. Kartstam, B. and Albertsson, P. K. (1970) *Biochim. Biophys. Acta* **216**, 220.
18. Schürmann, P. Wolosiuk, R. A., Breazale, V. D. and Buchanan, B. B. (1976) *Nature* **263**, 257.
19. Wolosiuk, R. A. and Buchanan, B. B. (1978) *Plant Physiol.* **61**, 669.
20. Wolosiuk, R. A. and Buchanan, B. B. (1978) *Arch. Biochem. Biophys.* **189**, 97.
21. Wolosiuk, R. A., Nishizawa, A. N. and Buchanan, B. B. (1978) *Plant Physiol.* **61**, Suppl. No. 531, 97.
22. Buchanan, B. B., Wolosiuk, R. A., Crawford, N. A. and Yee, B. L. (1978) *Plant Physiol.* **61**, 38.
23. Jacquot, J. P., Vidal, J., Gadal, P. and Schürmann, P. (1979) *FEBS Letters* **96**, 243.
24. Holmgren, A., Söderberg, B. O., Eklund, H. and Bränden, C. I. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 2305.