Purification and General Properties of δ -Aminolaevulate Dehydratase from *Nicotiana tabacum* L.

By A. S. SHETTY AND G. W. MILLER

Department of Botany, Utah State University, Logan, Utah 84321, U.S.A.

(Received 17 February 1969)

1. δ -Aminolaevulate dehydratase (EC 4.2.1.24) was purified 80-fold from tobacco leaves and its properties were studied. 2. The enzyme had optimum pH 7.4 in potassium phosphate buffer, K_m 6.25×10^{-4} M at 37° and pH 7.4, optimum temperature 45° and an activation energy of 11100 cal./mole. 3. The enzyme lost activity when prepared in the absence of cysteine, and this activity was only partly restored by the later addition of thiols. Reagents for thiol groups inactivated the enzyme. 4. Mg^{2+} was essential for activity, and EDTA and Fe^{2+} were inhibitory; Mn^{2+} was an activator or an inhibitor depending on the concentration.

The condensation of two molecules of δ -aminolaevulinic acid to form a pyrrole compound, porphobilinogen, by δ -aminolaevulate dehydratase (δ -aminolaevulate hydro-lyase, EC 4.2.1.24) has long been established (Shemin & Russell, 1953; Neuberger & Scott, 1953). The enzyme is widely distributed in Nature, and there have been numerous reports on its isolation and characterization from various sources.

Whereas δ -aminolaevulate dehydratase is generally known to behave like a typical thiol enzyme, the effect of EDTA and various cations on the enzyme appears to differ with enzyme sources. Activation by EDTA was observed in preparations from mice (Coleman, 1966), but inhibition was noted in certain other preparations (Gibson, 1955; Gibson, Neuberger & Scott, 1955; Batlle, Ferramola & Grinstein, 1967; DeBarreiro, 1967). Nandi & Waygood (1967) reported that the inhibition by EDTA of the enzyme from wheat leaves was reversed by addition of Mn^{2+} . Burnham & Lascelles (1963) observed no inhibition by EDTA of the enzyme from *Rhodospseudomonas spheroides*. Fe^{2+} was reported to activate the enzyme from mice (Coleman, 1966), but to inhibit that from ox liver (Gibson *et al.* 1955). Nandi & Waygood (1967) reported neither an activation nor an inhibition of wheat-leaf enzyme by 10 mM- Fe^{2+} . This enzyme was activated by both Mn^{2+} and Mg^{2+} over a wide range of concentrations (Nandi & Waygood, 1967; Nandi, Cohen & Shemin, 1968), but with the enzyme from *R. spheroides*, Mn^{2+} activated only at low concentrations, and at high concentrations it became inhibitory (Nandi *et al.* 1968). Burnham & Lascelles (1963) reported a requirement for K^+ by the enzyme from *R. spheroides*. Wheat-leaf en-

zyme, however, was not activated by K^+ (Nandi & Waygood, 1967).

Although δ -aminolaevulate dehydratase is an enzyme required in the pathway leading to the formation of porphyrins, haem compounds and chlorophyll, reports on the isolation and purification of the enzyme from plant sources are limited. Tigier, Batlle & Locascio (1968) reported a sevenfold purification of the enzyme from soya-bean callus tissue, and Nandi & Waygood (1967) obtained a 16-fold purification from wheat-leaf extract. The present paper reports an 80-fold purification of the enzyme from *Nicotiana tabacum* L.

MATERIALS AND METHODS

Chemicals. Calcium phosphate gel was prepared by the method of Keilin & Hartree (1951). Porphobilinogen used as standard was a generous gift from Dr B. F. Burnham, Chemistry Department of this University. All other chemicals used were of analytical grade, obtained from various commercial sources in the U.S.A.

Plant material. Tobacco (*Nicotiana tabacum* var. Havana-38) plants were grown in full strength Hoagland's nutrient solution (Hoagland & Arnon, 1938) in growth chambers with temperature (night 18°, daytime 24°), light intensity (2600 foot-candles) and humidity being controlled. Fully expanded leaves from 5–8-week-old plants were used in these studies. For the survey of plant species leaves from 3–4-week-old plants were used.

Extraction and purification of the enzyme. All operations were conducted in the cold (0–4°). (1) Preparation of the leaf homogenate. A 25 g. batch of the leaves (midribs removed) was homogenized for 2 min. in a prechilled Waring Blender with 50 mM-potassium phosphate buffer, pH 6.93, containing 10 mM-cysteine. The homogenate was strained through four layers of cheesecloth and made up to 100 ml. with the same buffer.

(2) Ammonium sulphate fractionation. The leaf homogenate from step (1) was centrifuged at 20000g for 15 min. The supernatant was brought to 35% saturation with ammonium sulphate (205 g./l.) and the precipitate removed after 10 min. by centrifuging at 12000g for 10 min. The supernatant was brought to 40%, 45% and 50% (by addition of 30 g./l., 30 g./l. and 35 g./l. respectively) saturation with ammonium sulphate successively and the process of centrifugation repeated at each stage as described above. The precipitate formed between 45% and 50% saturation was suspended in water. Both yield and specific activity of the enzyme were higher when such a successive centrifugation procedure was adopted, as compared with only two centrifugations at 45% and 50% saturation with ammonium sulphate.

(3) Calcium phosphate gel adsorption. The step (2) enzyme was divided into two equal parts and diluted to 10 ml. with water. To each of these were added 0.2 ml. of 3M-sodium acetate buffer, pH 5.6, and 1.5 ml. of calcium phosphate gel (40 mg./ml.) that had been aged for over a year. After the mixture had stood for 10 min., it was centrifuged at 12000g for 10 min. The precipitate was suspended in 11 ml. of 10 mM-potassium phosphate buffer, pH 7.4, and recentrifuged. It was re-extracted in the same way, in 11 ml. of 60 mM-potassium phosphate buffer at the same pH. The eluate was the step (3) enzyme.

(4) Continuous-flow electrophoresis. The step (3) enzyme was subjected to continuous-flow electrophoresis in the Elphor model FF electrophoretic separator (Brinkman Instruments Inc., Westbury, L.I., N.Y., U.S.A.) operated at 200 mA and 900 v. Sample was injected at the rate of about 5 ml./hr. and the background buffer in the separating chamber was allowed to flow at the rate of 250 ml./hr. Potassium phosphate buffer (25 mM) was used for the separation at pH 7.4 and the electrode-rinse buffer was 75 mM-potassium phosphate buffer, pH 7.4. The results of purification are summarized in Table 1.

Enzyme assay. The enzyme activity was determined by measuring the product, porphobilinogen, by the method of Mauzerall & Granick (1956). A standard reaction mixture contained 10 mM-GSH, 10 mM- δ -aminolaevulinic acid hydrochloride (neutralized with NaOH) and 10 mM-Mg²⁺ in addition to the enzyme extract and 50 mM-potassium phosphate buffer at pH 7.4, in a final volume of 1 ml. Incubation was at 37° for 30 min. The reaction was stopped by adding an equal volume of trichloroacetic acid containing HgCl₂ (10% aq. trichloroacetic acid-0.1 M-HgCl₂, 4:1, v/v). After centrifugation an equal volume of Ehrlich's reagent (*p*-dimethylaminobenzaldehyde solution in acetic acid and HClO₄) was added to a measured sample of the supernatant.

The E_{555} was measured. The amount of porphobilinogen formed was calculated from the molar extinction coefficient 6.2×10^4 . All results given are averages of three experiments.

Preliminary experiments with crude extract indicated that there was no net synthesis of porphyrins during the incubation period. Samples from incubation mixtures were mixed with an equal volume of 5M-HCl and the E_{405} was measured to determine porphyrins. The molar extinction coefficient used for the determination of porphyrins was 5.3×10^5 (Shemin, 1962). The amounts of porphyrin present at zero time and at the end of the incubation period were the same and therefore no correction for porphobilinogen seemed necessary.

Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) on a 10%-trichloroacetic acid precipitate of the enzyme extract, with dry casein as standard. A Perkin-Elmer model 303 atomic absorption spectrophotometer was used for the determination of metals.

Enzyme unit. A unit of enzyme activity is defined as the amount of enzyme producing 1 nmole of porphobilinogen in 60 min. Specific activity is the number of units of enzyme activity/mg. of protein.

Identification of porphobilinogen. Porphobilinogen was identified by paper chromatography by the method of Berlin, Neuberger & Scott (1956). The assay mixture, adjusted to pH 2.0, was evaporated to dryness in a rotary evaporator. The residue was extracted in a minimum amount of aq. 90% (w/v) ethanol and spotted on Whatman no. 1 chromatography paper. An authentic sample of porphobilinogen was spotted alongside as a marker. Chromatograms were developed in a chamber saturated with vapours of the solvent mixture butan-1-ol-water-acetic acid (63:27:10, by vol.) by using the descending technique. Porphobilinogen was detected by spraying Ehrlich's reagent.

As an alternative method, a sample containing porphobilinogen (pH 5-6) was applied to a column (0.7 cm. $\times$ 2 cm.) of the strong base resin Dowex 2 (X8; acetate form). The column was washed with water, and porphobilinogen was eluted with 1M-acetic acid followed by 0.2M-acetic acid (Mauzerall & Granick, 1956).

RESULTS

Survey of plant species. Six different plant species, namely corn, tobacco, spinach, soya bean, wheat and pea, were tested for δ -aminolaevulate dehydra-

Table 1. *Purification of δ -aminolaevulate dehydratase from leaves of Nicotiana tabacum*

Incubation conditions were as described in the Materials and Methods section except that the pH of incubation mixtures was 7.3.

Step no.	Purification step	Total vol. (ml.)	Activity (total units)	Total protein (mg.)	Sp. activity (units/mg. of protein)	Yield (overall) (%)	Purification
1	Leaf homogenate	100	7403	1112	7	100	1
2	(NH ₄) ₂ SO ₄ precipitation	46	6586	41	173	89	25
3	Calcium phosphate eluate	22	3598	10	360	47	50
4	Continuous-flow electrophoresis	60	1675	3	558	23	80

Table 2. *δ -Aminolaevalate dehydratase activity from different plant extracts*

The reaction mixture contained 0.2 ml. of enzyme extract (35–50% saturation $(\text{NH}_4)_2\text{SO}_4$ precipitate), 10 mM-GSH, 10 mM- δ -aminolaevalate, 10 mM- Mg^{2+} and 50 mM- KH_2PO_4 - K_2HPO_4 buffer, pH 7.4, in a final volume of 1 ml. Incubation was for 30 min. at 37°.

Plant species	Activity (units/g. fresh wt. of leaf)	Protein content (mg./g. fresh wt. of leaf)	Sp. activity (units/mg. of protein)
Corn	859	4.8	179
Tobacco	976	9.9	99
Spinach	503	9.3	54
Soya bean	271	8.2	33
Wheat	128	12.1	11
Pea	54	6.1	9

tase activity. A 35–50% saturation ammonium sulphate precipitate of the leaf homogenate was used. As shown in Table 2, the enzyme preparation from tobacco leaves had the highest total activity and that from corn had the highest specific activity. Wheat and pea were the lowest in both total activity and specific activity. Subsequent experiments were carried out exclusively with tobacco-leaf extracts.

Porphobilinogen formation. The enzyme extracts from different purification steps were studied, but the results reported here are with the calcium phosphate-gel eluate (50-fold purification) unless otherwise indicated.

Evidence for porphobilinogen formation from δ -aminolaevalic acid was obtained by paper chromatography. The biosynthesized porphobilinogen and the authentic marker had an identical R_F (0.47). Adsorption on Dowex 2 (X8) and elution with 1M-acetic acid confirmed this identification.

Effects of time, protein concentration, temperature and pH. Under standard assay conditions the amount of porphobilinogen formed was linear with time at least up to 3 hr., by which time approximately 10% of the substrate was converted into porphobilinogen. The enzyme activity was proportional to the enzyme concentration from 23 to 276 μg . of protein/ml. Activity increased with temperature over the range 25–45° under the experimental conditions (Fig. 1). There was a marked decrease in the activity above 45°, followed by complete inactivation at 60°. Fig. 2 is a plot of the reciprocal of absolute temperature against the logarithm of the initial velocity. The activation energy was determined to be 11 100 cal./mole, with a temperature coefficient, Q_{10} , of about 2. The pH optimum in potassium phosphate buffer is about 7.4, as shown in Fig. 3. A sharp decrease on the acid side and a more gradual decrease on the alkaline side was found.

Fig. 1. Tobacco-leaf δ -aminolaevalate dehydratase activity and incubation temperature. Reaction mixture with 10 mM- δ -aminolaevalic acid, 10 mM-GSH, 10 mM- Mg^{2+} , 58 mM- K_2HPO_4 - KH_2PO_4 buffer, pH 7.4, and 0.3 ml. of step (3) enzyme (0.46 mg. of protein/ml.) was incubated for 30 min. at the temperatures indicated.

Fig. 2. Plot of initial velocity of tobacco-leaf δ -aminolaevalate dehydratase against reciprocal of incubation temperature. The reaction mixture with 10 mM- δ -aminolaevalic acid, 10 mM-GSH, 10 mM- Mg^{2+} , 58 mM- K_2HPO_4 - KH_2PO_4 buffer, pH 7.4, and 0.3 ml. of step (3) enzyme (0.46 mg. of protein/ml.) was incubated for 30 min. at the temperatures indicated.

Substrate concentration and initial velocity. Substrate saturation was reached at about 5–10 mM- δ -aminolaevalic acid. A K_m value of 6.25×10^{-4} M was calculated from a Lineweaver & Burk (1934) plot (Fig. 4). Nandi *et al.* (1968) reported that K^+ ,

Fig. 3. pH-activity curve for tobacco-leaf δ -aminolaevulate dehydratase. The reaction mixture, with 10 mM- δ -aminolaevulic acid, 10 mM-GSH, 10 mM-Mg²⁺, 80 mM-KH₂PO₄-K₂HPO₄ buffer, pH as indicated, and 0.2 ml. of step (3) enzyme (0.43 mg. of protein/ml.) was incubated for 30 min. at 37°.

Fig. 4. Reciprocal plot of initial velocity of tobacco-leaf δ -aminolaevulate dehydratase and substrate concentration. The reaction mixture containing 10 mM-GSH, 10 mM-Mg²⁺, δ -aminolaevulic acid as indicated, 58 mM-KH₂PO₄-K₂HPO₄ buffer, pH 7.4, and 0.3 ml. of step (3) enzyme (0.43 mg. of protein/ml.) was incubated for 30 min. at 37°.

Li⁺, Rb⁺ and NH₄⁺, but not Na⁺, are allosteric effectors for δ -aminolaevulate dehydratase from *R. spheroides*. In the light of this observation the

Table 3. Activation of tobacco-leaf δ -aminolaevulate dehydratase by thiol compounds

The reaction mixtures, containing different thiols as indicated, 10 mM-Mg²⁺, 90 mM-KH₂PO₄-K₂HPO₄ buffer, pH 7.4, and 0.3 ml. of step (2) enzyme (0.9 mg. of protein/ml.), were preincubated at 37° for 30 min. δ -Aminolaevulate (final concn., 10 mM) was added and incubated for another 30 min.

Amount added Thiol compound	Increase in activity over control (%)		
	10 mM	5 mM	2.5 mM
GSH	81	42	—
Cysteine	56	46	—
Dithiothreitol	—	44	23
2-Mercaptoethanol	37	21	—
Thioglycollate	24	18	—

Table 4. Effect of thiol inhibitors on tobacco-leaf δ -aminolaevulate dehydratase

The reaction mixture contained 10 mM- δ -aminolaevulic acid, 10 mM-Mg²⁺, 68 mM-K₂HPO₄-KH₂PO₄ buffer, pH 7.4, 0.3 ml. of step (3) enzyme (0.43 mg. of protein/ml.) and GSH, *p*-chloromercuribenzoate and iodoacetamide as indicated. Incubation was for 30 min. at 37°.

Treatment				
GSH (mM)	<i>p</i> -Chloro- mercuri- benzoate (mM)	Iodoaceta- mide (mM)	Activity (units)	Inhibition (%)
5	—	—	45.8	—
—	—	—	45.4	0
5	1	—	44.5	3
—	1	—	28.7	37
5	—	1	43.9	4
—	—	1	41.1	9

substrate-saturation experiment was conducted with either sodium phosphate or potassium phosphate buffers on the dialysed step (3) enzyme. Similar results were obtained with both buffers.

Effects of thiol activators and inhibitors. Addition of thiol compounds such as cysteine to the extraction medium was essential for enzyme activity. Without the addition of cysteine, step (2) enzyme retained only about 10% of its activity whereas no activity was observed with step (3) enzyme. The step (2) enzyme prepared in the absence of added cysteine regained only a part of the activity on subsequent preincubation for 30 min. with thiols. Table 3 shows the effect of different thiol compounds on step (3) enzyme. GSH appeared to be the best activator under these conditions.

The effect of thiol-group inhibitors was studied on the step (3) enzyme prepared by the standard method. *p*-Chloromercuribenzoate gave 37% inhi-

Fig. 5. Effects of Mg^{2+} and Mn^{2+} ions on tobacco-leaf δ -aminolaevalate dehydratase activity. The reaction mixture contained 10mm- δ -aminolaevalic acid, 10mm-GSH, 40mm- K_2HPO_4 - KH_2PO_4 buffer, pH 7.4, 0.3 ml. of step (3) enzyme (0.35 mg. of protein/ml.) and Mg^{2+} (●) or Mn^{2+} (○) as indicated. The plots are not directly comparable since the Mn^{2+} and Mg^{2+} experiments were conducted on different enzyme preparations. Incubation was for 30 min. at 37° .

Fig. 6. Effects of Mn^{2+} and Fe^{2+} on tobacco-leaf δ -aminolaevalate dehydratase activity in mixtures containing added Mg^{2+} . A 0.5 ml. sample of step (3) dialysed enzyme (0.25 mg. of protein/ml.) was incubated for 30 min. at 37° with 40mm- K_2HPO_4 - KH_2PO_4 buffer, pH 7.4, 10mm- δ -aminolaevalic acid, 10mm-GSH, 10mm- Mg^{2+} , and Mn^{2+} (○) or Fe^{2+} (●) added at the concentrations indicated.

bition at 1mm concentration (Table 4). In separate experiments Cu^{2+} and Hg^{2+} completely inactivated the enzyme at 5mm concentration.

Effects of metal ions and EDTA. Step (3) enzyme

dialysed against 2mm-tris-hydrochloric acid buffer containing 1mm-cysteine was incubated in a standard assay system with various concentrations of Mg^{2+} or Mn^{2+} . As shown in Fig. 5, the initial velocity increased with increasing Mg^{2+} concentrations up to 10mm. Mn^{2+} at concentrations up to 2.5mm activated the enzyme. Higher concentrations were strongly inhibitory. Fig. 6 illustrates the effect of Mn^{2+} and Fe^{2+} at various concentrations in reaction mixtures containing 10mm- Mg^{2+} . Both Mn^{2+} and Fe^{2+} were inhibitory. At 2.5mm- Mn^{2+} and 2.5mm- Fe^{2+} the inhibition was 8% and 54% respectively.

Since absolute dependence of activity on Mg^{2+} or Mn^{2+} was not observed even with the dialysed enzyme, the dialysis residue was analysed for metal ions. The endogenous contributions of Mg^{2+} , Mn^{2+} and Fe^{2+} to the reaction mixture were 0.02mm, 0.01mm and 0.01mm respectively. Step (3) enzyme was therefore dialysed against EDTA to remove more endogenous metals and then redialysed against buffer to remove the excess of EDTA. Enzyme activity of the dialysed extract was very low in the absence of added metal-ion activators, but increased when either Mg^{2+} or Mn^{2+} was added. The activation by Mg^{2+} was much greater than that by Mn^{2+} .

At 1mm, 5mm and 10mm concentrations EDTA inhibited the tobacco-leaf δ -aminolaevalate dehydratase by 59%, 72% and 88% respectively. Both Mg^{2+} and Mn^{2+} partially reversed the EDTA inhibition at low concentrations, but at higher concentrations (above 2.5mm) only Mg^{2+} was an activator.

Requirement for Mg^{2+} was found to be absolute for the tobacco-leaf enzyme. Freshly prepared step (3) enzyme was passed through a Sephadex G-25 column (2.5cm. $\times$ 25cm.) equilibrated with deionized water and collected in 5ml. fractions. Two of the most active (adjacent) fractions were pooled together and the enzyme activity was measured in the presence and absence of Mg^{2+} . No activity was observed in the absence of Mg^{2+} .

ATP and sodium pyrophosphate, each at 20mm, inhibited the step (3) enzyme by 29% and 22% respectively. Sodium fluoride and monofluoroacetate were without effect at the above concentration. Haemin chloride, protoporphyrin IX, twice-crystallized bovine haemoglobin and cytochrome *c* were not inhibitory up to 100 μ M.

DISCUSSION

Extensive purification of δ -aminolaevalate dehydratase has been reported in micro-organisms (Nandi *et al.* 1968) and in animals (Batlle *et al.* 1967; Calissano, Bonsignore & Cartasegna, 1966; Calissano, Cartasegna & Matteini, 1966). Information

on the enzyme from plant tissues, however, is incomplete. The step (3) enzyme in the present study is apparently the purest δ -aminolaevulate dehydratase (50-fold purification) from plant sources. Our preliminary survey of plant species indicated that tobacco and corn leaves are the best source for this enzyme (Table 2). Wheat and pea leaves appeared to be poor sources. This may be attributed to the presence of some native inhibitors. The presence of such inhibitors was reported by other workers in wheat leaves (Nandi & Waygood, 1967). It is also possible that the enzyme in these cases was not completely precipitated at 35–50% saturation with ammonium sulphate.

Kinetic studies indicated that the enzyme was active over a wide range of temperatures (25–45°) above which a drastic decrease in activity occurred, presumably owing to denaturation of enzyme protein. DeBarreiro (1967) reported an optimum temperature 50–55° for yeast δ -aminolaevulate dehydratase when incubated for 30 min. The maximum activity of this enzyme from cow liver was at 65° (Batlle *et al.* 1967) with a 1 hr. incubation. The tobacco-leaf enzyme appeared more temperature-sensitive than that from yeast or cow liver. Energy of activation for the tobacco-leaf enzyme was somewhat lower than that reported for the enzyme from mouse (Coleman, 1966).

The optimum pH 7.4 in phosphate buffer observed in our experiment agrees with the reported value for wheat-leaf enzyme (Nandi & Waygood, 1967). The optimum pH for the plant enzyme in general appears to be higher than that for the enzyme from animal sources (Gibson *et al.* 1955; Batlle *et al.* 1967; Coleman, 1966) and is lower than that for the enzyme from micro-organisms (Burnham & Lascelles, 1963; Nandi *et al.* 1968; De Barreiro, 1967).

The K_m value obtained in this study ($6.25 \times 10^{-4} M$) is lower than those reported by Nandi & Waygood (1967) for wheat-leaf enzyme ($1 \times 10^{-3} M$) and by De Barreiro (1967) for yeast enzyme ($1.5 \times 10^{-3} M$).

The experiments with thiol activators and inhibitors show the thiol nature of the tobacco-leaf δ -aminolaevulate dehydratase. Enzyme activity decreased markedly when cysteine was not present in the extraction medium. Cu^{2+} , Hg^{2+} , *p*-chloromercuribenzoate and iodoacetamide inhibited the enzyme, suggesting not only that the enzyme has thiol groups but that these are essential for activity. The thiol-containing reagents may also have a stabilizing effect on the enzyme, since it retained activity only when stored in the presence of cysteine. Thioglycollate activated the tobacco enzyme by about 25%, an observation contrary to that for wheat-leaf enzyme (Nandi & Waygood, 1967).

Burnham & Lascelles (1963) and Nandi *et al.*

(1968) indicated a requirement for K^+ by the bacterial enzyme. K^+ was reported not essential for the plant enzyme (Nandi & Waygood, 1967). It was further shown that univalent cations other than Na^+ are allosteric activators of the bacterial enzyme (Nandi *et al.* 1968). Results obtained in the present study confirm the observation made with wheat-leaf enzyme that K^+ is not essential for activity.

The observations that EDTA inhibited the enzyme and that the enzyme dialysed against EDTA regained part of its activity on addition of Mg^{2+} suggest that Mg^{2+} is essential for activity and that EDTA chelates the endogenous Mg^{2+} . Activation by Mn^{2+} , unlike that by Mg^{2+} , occurred only at low concentrations. Mn^{2+} and Mg^{2+} probably act on the same site, since Mn^{2+} did not cause any activation when Mg^{2+} was present at optimum concentrations. Nandi & Waygood (1967) reported that Mn^{2+} and Fe^{2+} did not inhibit the wheat-leaf enzyme. With the tobacco enzyme, however, both Fe^{2+} and Mn^{2+} proved to be inhibitors in the presence of Mg^{2+} . This may indicate the presence of a regulatory mechanism. The fact that Mn^{2+} at the same concentration activated the enzyme either with or without dialysis against EDTA (Fig. 5) suggests that Mn^{2+} did not reverse the inhibition by EDTA of tobacco-leaf enzyme, a fact contrary to that reported for wheat-leaf enzyme (Nandi & Waygood, 1967).

This investigation was supported in part by United States Atomic Energy Commission grant AT(11-1)-1287. The authors thank Dr Ming-Ho Yu for his helpful suggestions.

REFERENCES

- Batlle, A. M. C., Ferramola, A. M. & Grinstein, M. (1967). *Biochem. J.* **104**, 244.
- Berlin, N. I., Neuberger, A. & Scott, J. J. (1956). *Biochem. J.* **64**, 80.
- Burnham, B. F. & Lascelles, J. (1963). *Biochem. J.* **87**, 462.
- Calissano, P., Bonsignore, D. & Cartasegna, C. (1966). *Biochem. J.* **101**, 550.
- Calissano, P., Cartasegna, C. & Matteini, M. (1966). *Ital. J. Biochem.* **15**, 18.
- Coleman, D. L. (1966). *J. biol. Chem.* **241**, 5511.
- DeBarreiro, O. L. C. (1967). *Biochem. biophys. Acta*, **139**, 479.
- Hoagland, D. R. & Arnon, D. I. (1938). *Circ. Colo. agric. Exp. Sta.* 347.
- Gibson, K. D. (1955). In *Ciba Found. Symp.: Porphyrin Biosynthesis and Metabolism*, p. 27. Ed. by Wolstenholme, G. E. & Millar, E. C. P. London: J. and A. Churchill Ltd.
- Gibson, K. D., Neuberger, A. & Scott, J. J. (1955). *Biochem. J.* **61**, 618.
- Keilin, D. & Hartree, E. F. (1951). *Biochem. J.* **49**, 88.
- Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.

- Mauzerall, D. & Granick, S. (1956). *J. biol. Chem.* **219**, 435.
- Nandi, D. L., Cohen, K. F. B. & Shemin, D. (1968). *J. biol. Chem.* **243**, 1224.
- Nandi, D. L. & Waygood, E. R. (1967). *Canad. J. Biochem.* **45**, 327.
- Neuberger, A. & Scott, J. J. (1953). *Nature, Lond.*, **170**, 1093.
- Shemin, D. (1962). In *Methods in Enzymology*, vol. 5, p. 883, Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Shemin, D. & Russell, C. S. (1953). *J. Amer. chem. Soc.* **75**, 4873.
- Tigier, H. A., Batlle, A. M. C. & Locascio, G. (1968). *Biochim. biophys. Acta*, **151**, 300.