Journal of Chromatography, 625 (1992) 13–19
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2579

Adenylate kinase from plant tissues

Influence of ribonuclease on binding properties on Mono Q

Wolfgang R. Deppert, Johannes Normann and Edgar Wagner

Institut für Biologie II, Universität Freiburg, Schänzlestrasse 1, W-7800 Freiburg i. Br. (Germany)

ABSTRACT

Adenylate kinases modulate the three adenine nucleotide pools and were found to be localized as isoenzymes in different tissues and organelles in animals and plants. For investigations of adenylate kinase isoenzymes from plant tissues different plant extracts were examined by anion-exchange chromatography. During investigations with the strong anion exchanger Mono Q, adenylate kinase activity eluted in the void volume. This void volume activity did not always occur, but depended on the age of the plants and light treatment. The nature of the factors affecting void volume activity could only be partially resolved. It could be shown that RNase treatment at the beginning of extraction led to the disappearance of void volume activity, whereas an untreated extract still showed this activity.

INTRODUCTION

Adenylate kinases (ATP:AMP phosphotransferase, E.C. 2.7.4.3) are globular, low-molecular-mass proteins with the following catalytic activity:

$$Mg \cdot ATP + AMP \rightleftharpoons Mg \cdot ADP + ADP$$

Their small size and their ubiquitous presence in all living systems made them a tool in clinical chemistry for recognizing diseases (*e.g.*, [1–3]). When Fildes and Harris (1966) [4] first showed the occurrence of specific patterns of adenylate kinase isoenzymes in human erythrocytes, investigations in this field advanced rapidly.

Because of their catalytic capability and their distribution among different tissues and organelles, the enzymes seem to play an important role in regulating energy metabolism [1,5–10]. In 1968, Atkinson [11] and Bomsel and Pradet [12] presented their hypothesis of energy charge control of metabolism by adenine nucleotides. Bomsel and Pradet showed that adenylate kinase regulates the equilibrium between adenine nucleotide pools and that the energy charge does not change even under extreme conditions. Adenylate kinase might be of great importance in regulating coupling in oxidative phosphorylation [13].

In C_3-plants, adenylate kinase levels are low in comparison with C_4-plants [14–16]. An increase in adenylate kinase activity on greening in C_4-plants and higher contents in these plants are taken as an indication of its importance in regenerating the photosynthetic primary CO_2 acceptor phosphoenolpyruvate [14–19].

In higher plants, five different isoenzymes are localized in different organelles. Most activity is localized in chloroplasts and only a small portion is cytoplasmatic or found in the nucleus and mitochondria [7,8,20,21]. Two enzymes could be isolated from chloroplasts. The major activity in these orga-

Correspondence to: W. R. Deppert, Institut für Biologie II, Universität Freiburg, Schänzlestrasse 1, W-7800 Freiburg i.Br., Germany.

0021-9673/92/$05.00

nelles results from a soluble form [22] while a small portion is bound to the envelope [23]. These two isoforms might be involved in the control of adenylate pools and adenylate translocation in plastids.

In developing seedlings of *Chenopodium rubrum*, the adenylate kinase capacity shows circadian rhythmicity and increases under phytochrome control [5,6,24]. In addition, isoenzyme patterns found after ion-exchange chromatography on DEAE-cellulose could be modulated by changes in light and temperature treatments or glucose feeding. Imposition of flower-inducing conditions led to a significant increase in mitochondrial adenylate kinase capacity, whereas the chloroplastic enzyme capacity decreased. Glucose feeding experiments led to characteristic changes in isoenzyme patterns, indicating a glucose concentration-dependent modulation of cytoplasmatic, mitochondrial and chloroplastic forms. Therefore, adenylate kinase might be involved in fine control of energy transduction [7,8].

Whereas amino acid sequences, gene loci and conformations of the enzymes from different animal and human tissues, bacteria and yeast are known (*e.g.*, [25–27]), information about the enzymes from plants is scarce.

The aim of these investigations was to purify adenylate kinase from leaves of *Chenopodium rubrum* and to separate isoenzymes from different organelles with a new and efficient chromatographic procedure [fast protein liquid chromatographic (FPLC) system].

EXPERIMENTAL

Growing conditions

Seedlings of *Chenopodium rubrum* L. (ecotype 184) were grown in moist vermiculite in small pots in a transparent container closed with a transparent lid and transferred to a growth chamber with continuous white light (270 μmol m^{-2} s^{-1}) and changing temperature conditions (12 h/12 h: 305.5 K/283 K) for 4 days and thereafter to constant conditions (297 K, white light, 70% relative humidity). After transfer to constant conditions, the plants were supplied with 40% Hoagland's nutrient solution [28]. For synchronization, plants were transferred after 20 days to a box with constant temperature (297 K) and alternating light conditions (12h/12h : dark/white light). After 4 days of synchronization the plants were kept in complete darkness for 2 days or under continuous white light for 3 days. Leaves were harvested from the second and third internodes.

Chemicals

AMP, ATP, NADH, PEP, LDH, PK and pancreatic RNase (DNase-free) were obtained from Boehringer (Mannheim, Germany), ethanolamine from Serva (Heidelberg, Germany) and 2-amino-2-methyl-1-propanol from Sigma (Deisenhofen, Germany). All other chemicals were obtained from Roth (Karlsruhe, Germany). Ready-to-use Coomassie Protein Assay Reagent was purchased from Pierce (Oud-Beijerland, Netherlands).

Instrumentation

A modular FPLC system was used, consisting of a liquid chromatographic controller (LCC-500), two pumps (P-500), four motor-driven valves (MV-8) and one motor-driven injection valve (MV-7). Absorbance at 280 nm was monitored with a UV spectrophotometer and a 10-μl flow cell. Further instruments were a mixing chamber (24 V), a fraction collector (FRAC-100), a peristaltic pump (P-1) and a superloop (50 ml) for application of large sample volumes. Small volumes were injected with a syringe by sample loops of different sizes. All instruments were obtained from Pharmacia Biosystems (Freiburg, Germany).

Chromatography

For subsequent anion-exchange chromatography a prepacked Mono Q HR 5/5 column (1 ml) was used. For prepurification a column with DEAE-Sepharose CL-6B (8 ml) was prepared. For separation the following two buffer systems were used: (I) A = 20 m*M* ethanolamine–HCl (pH 9.5)–14 m*M* 2-mercaptoethanol, B = 20 m*M* ethanolamine–HCl (pH 9.5)–1 *M* NaCl–14 m*M* 2-mercaptoethanol; and (II) A = 40 m*M* 2-amino-2-methyl-1-propanol–HCl (pH 10.25)–14 m*M* 2-mercaptoethanol, B = 40 m*M* 2-amino-2-methyl-1-propanol–HCl (pH 10.25)–1 *M* NaCl–14 m*M* 2-mercaptoethanol. All buffers for FPLC were degassed prior to use. Membranes for degassing and filtration (Millex GV, Millex GVWP) were purchased from Millipore (Eschborn, Germany). Mono Q HR 5/5, DEAE Sepharose CL-6B, Sephadex and FPLC are registered

trade-marks of Pharmacia Biosystems (Freiburg, Germany).

Extraction procedure

All preparations took place in a cold room or on ice. The centrifuge was cooled to 275 K. The extraction medium contained 250 m*M* Tris–HCl (pH 7.4), 8 m*M* $MgSO_4$, 5 m*M* EDTA and 14 m*M* 2-mercaptoethanol. Adenylate kinase was prepared according to the following procedure: 10 g of plant material were harvested and frozen with liquid nitrogen. The frozen material was transferred into PTFE cells each with a tungsten carbide ball at liquid nitrogen temperature. The material was shaken in a microdismembrator (Braun, Melsungen, Germany) for 60 s. The frozen powder was transferred into a beaker with extraction medium (1 g fresh weight per 5 ml of medium) and stirred until the solution became homogeneous. This crude extract was centrifuged for 20 min at 39 000 *g*. Solid ammonium sulphate was added to the supernatant to reach a final concentration of 75% (w/v). After 45 min of stirring the suspension was centrifuged again at 39 000 *g* for 10 min. The resulting pellet was resuspended in 10 ml of 20 m*M* ethanolamine–HCl (pH 9.5)–14 m*M* mercaptoethanol, stirred for 45 min and again centrifuged at 39 000 *g* for 10 min. The remaining ammonium sulphate was removed from the supernatant by Sephadex G-25 columns. These columns (16-ml bed volume) were especially equilibrated with the appropriate elution buffer. Between 4 and 5 ml of supernatant were applied to one of these columns and the filtrate from this step was applied to another column. The extract obtained by this procedure was filtered through a membrane of 0.22 μm pore size and thereafter applied (8–9 ml) to a column with DEAE Sepharose CL-6B for prepurification. After application of the extract to the column proteins were eluted with 30 ml of 20 m*M* ethanolamine–HCl (pH 9.5)–1 *M* NaCl–14 m*M* 2-mercaptoethanol. All combined fractions from this chromatographic step resulted in a clear protein solution without chlorophylls and phenols. Solid ammonium sulphate (75%, w/v) was added and all steps for concentrating and desalting were repeated as described above using 10 ml of the appropriate elution buffer for resuspension of precipitated pellets. The extract was filtered (0.22-μm pore size) and applied to Mono Q.

RNase treatment

In RNase-treatment the first supernatant was divided into two aliquots. To one aliquot 2 μg/ml of pancreatic RNase (DNase-free) were added while the other half was not treated. The extracts were left in the cold overnight in extraction buffer at pH 7.4. The next step was the precipitation of extract with ammonium sulphate and all other steps were as described under *Extraction procedure* using 10 ml of 40 m*M* 2-amino-2-methyl-1-propanol–HCl (pH 10.25)–14 m*M* 2-mercaptoethanol as resuspension buffer for the second precipitation step.

Adenylate kinase assay

Adenylate kinase activity was determined in the backward reaction by the formation of Mg · ADP and ADP. The reaction sequence is described elsewhere [3]. Under the chosen conditions, activity is monitored by measuring the decrease in NADH at 366 nm.

A 1-ml volume of assay mixture contained 75 m*M* Tris (pH 7.4 with HCl), 3 m*M* $MgSO_4$, 7.5 m*M* KCl, 1.8 m*M* ATP, 1.6 m*M* PEP, 2 m*M* AMP, 0.35 m*M* NADH, 260 nkat LDH and 50 nkat PK. Reactions were initiated by various amounts of sample and run at 25°C. Because of enzymes starting the reaction by bypassing adenylate kinase, all activities were tested in a double-beam spectrophotometer having a reference without AMP. Volume-dependent enzyme activities (A_v) were calculated from changes in absorbance per second.

Results for adenylate kinase activity profiles are representative for at least three independent experiments.

Determination of protein

Protein was determined according to Bradford [29] using ready-to-use Coomassie Protein Assay Reagent.

RESULTS

Anion-exchange chromatography

Prepurification on DEAE-Sepharose CL-6B with subsequent precipitation leads to clear protein extracts without chlorophylls and phenols. When leaves were harvested 2 days after transfer to continuous darkness, application of prepurified extracts to Mono Q led to elution of most activity in the void volume (Fig. 1).

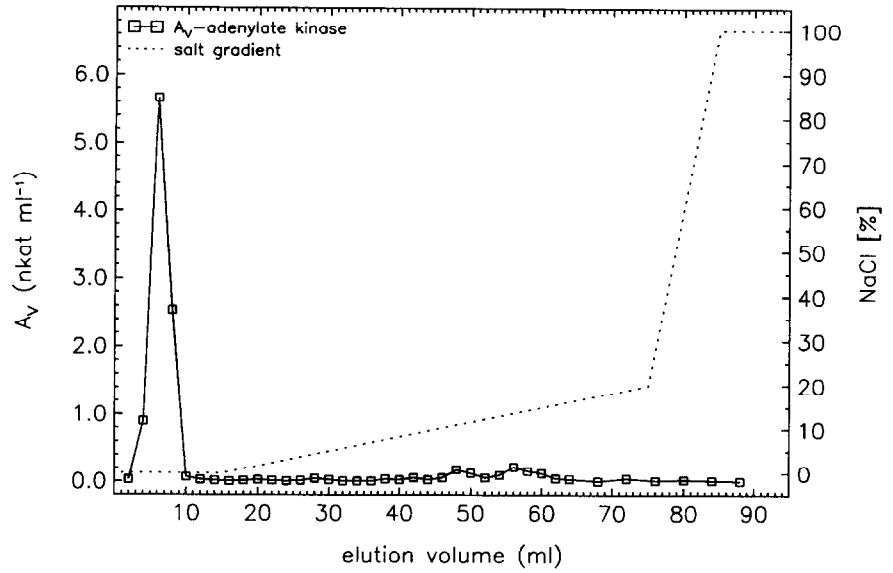

Fig. 1. Adenylate kinase activity profile on Mono Q. Leaves were harvested 2 days after transfer to continuous darkness. Buffer: A = 20 m*M* ethanolamine–HCl (pH 9.5)–14 m*M* 2-mercaptoethanol; B = 1 *M* NaCl in A. Flow-rate, 1 ml min^{-1}; protein applied, 3.7 mg; activity applied, 22 nkat; recovery, 22 nkat (100%).

For Mono Q HR 5/5 a charge of 20 mg of protein is considered to be the upper limit for the capacity of the column. To avoid overloading of the column only small amounts of protein (3–4 mg) were applied.

Collection of void volume activity and reapplication to Mono Q led to binding of *ca.* 90–100% of total activity (Fig. 2) when the column was previously washed with 1 *M* NaCl. Five activity peaks could be detected after elution with a salt gradient.

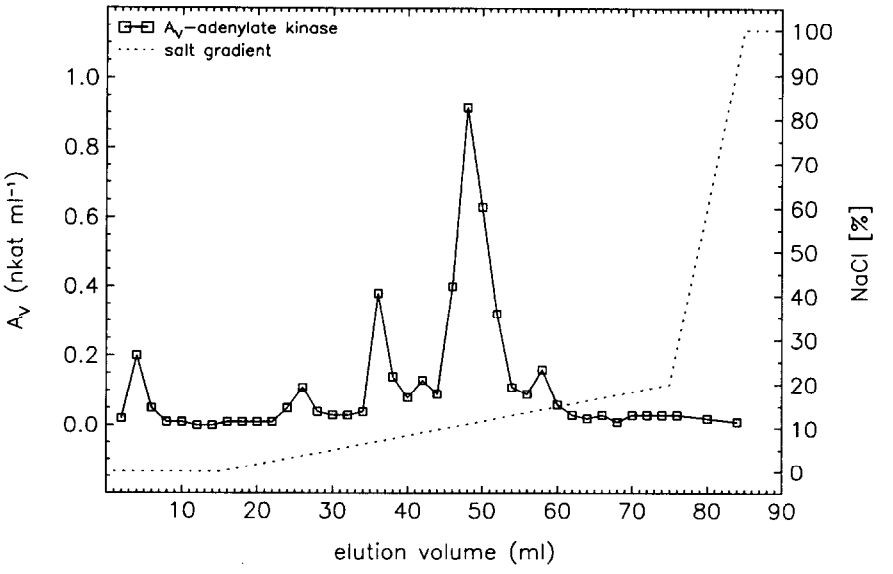

Fig. 2. Rechromatography of void volume activity. Buffer: A = 20 m*M* ethanolamine–HCl (pH 9.5)–14 m*M* 2-mercaptoethanol; B = 1 *M* NaCl in A. Flow-rate, 1 ml min^{-1}; activity applied, 10 nkat; recovery 9 nkat (90%).

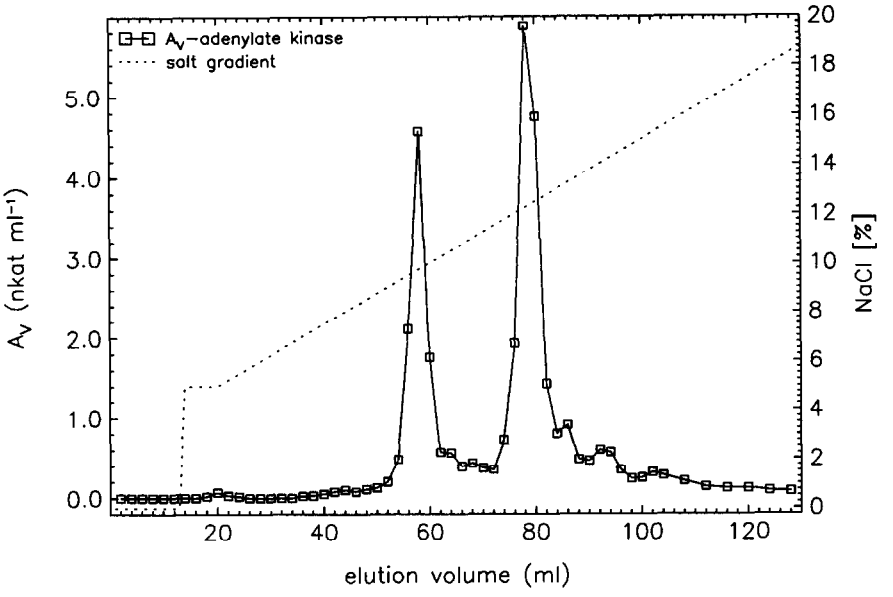

Fig. 3. Anion-exchange chromatography on Mono Q. Plants were harvested after continuous illumination for 3 days. The amount of protein applied was at capacity limit of Mono Q. Buffer: A = 20 m*M* ethanolamine–HCl (pH 9.5)–14 m*M* 2-mercaptoethanol; B = 1 *M* NaCl in A. Flow-rate, 1 ml min^{-1}; protein applied, 19.8 mg; activity applied, 72 nkat; recovery, 66 nkat (92%).

When leaves were harvested from plants that were kept in continuous white light for 3 days after synchronization, application of the prepurified extract to Mono Q led to no void volume acitivity. To probe the capacity of Mono Q for adenylate kinase, 72 nkat were applied to Mono Q. This activity corresponded to nearly 20 mg of protein, being the limit for Mono Q HR 5/5. Application of this activity and amount of protein resulted in complete binding and five activity peaks could be detected during elution with salt (Fig. 3). This result showed that overloading of Mono Q could be excluded as a reason for the appearance of void volume acitivity.

Anion-exchange chromatography: influence of RNase on binding of adenylate kinase to Mono Q

To elucidate the reason for the occurrence of void volume activity, various enzymes were added to crude extracts in order to separate adenylate kinases from lipids, DNA or sugars. Lipase, DNase, *endo*-β-N-acetylglucosaminidase F and peptide-N-glycohydrolase F were not able to remove void volume activity (data not shown). In contrast, the addition of pancreatic RNase (DNase-free) to half of the crude extract and resuspension of precipitated prepurified extract in 40 m*M* 2-amino-2-methyl-1-propanol–HCl–14 m*M* 2-mercaptoethanol led to binding of all adenylate kinase activity to Mono Q (Fig. 4, I) whereas the untreated half showed void volume activity of about 50% (Fig. 4, II). After RNase treatment, four clear activity peaks could be detected (Fig. 4, I) whereas no clear peaks of activity at the appropriate elution volumes appeared without RNase (Fig. 4, II). RNase not only prevents void volume activity but also seems to sharpen activity peaks.

DISCUSSION

Adenylate kinases have been purified from different sources (*e.g.*, [1,9,15,25,30]) and their distribution as isoenzymes in different organelles and tissues has been shown (*e.g.*, [1,4,7,8,10,31,32]). During our investigations on the distribution of adenylate kinases in plant tissues from different sources with the strong anion exchanger Mono Q, it was found that most adenylate kinase activity eluted in the void volume. The occurrence of this void volume activity depended on the age of the plants and light treatment.

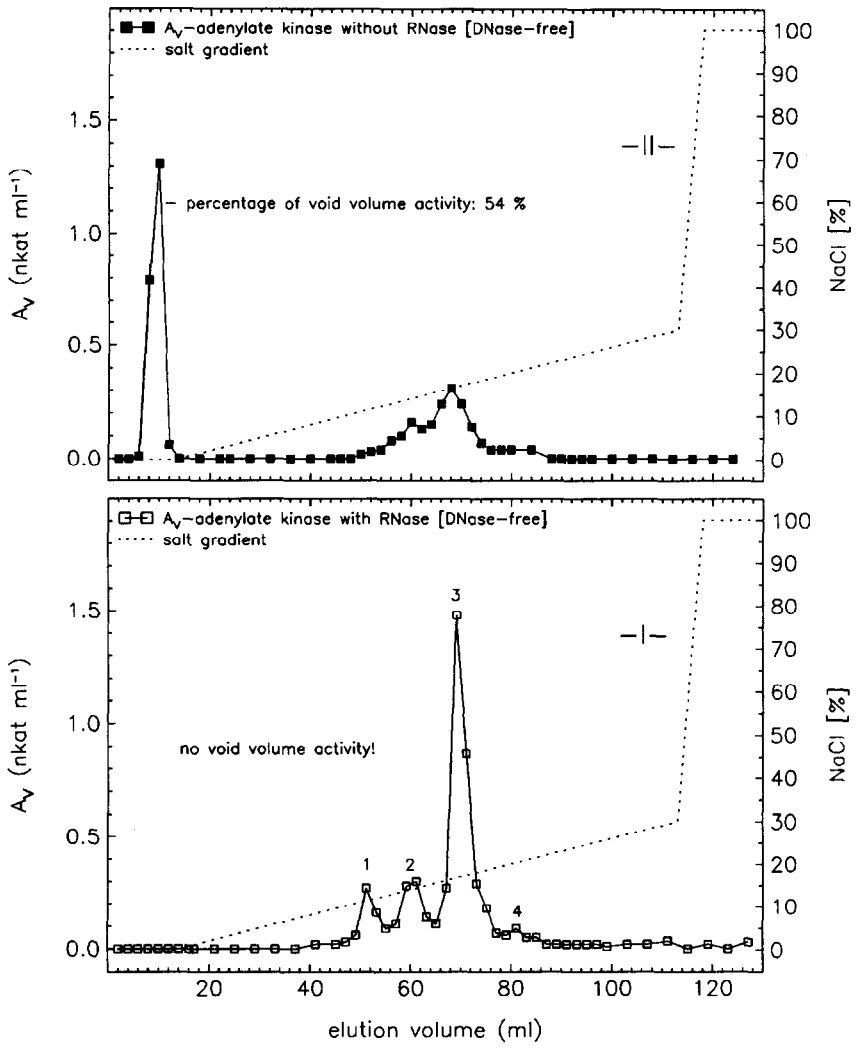

Fig. 4. Anion-exchange chromatograms of adenylate kinase activity on Mono Q. (I) With RNase (DNase-free). Protein applied, 3.2 mg; activity applied, 10 nkat; recovery, 10 nkat (100%). (II) Without RNase (DNase-free). Protein applied, 3.4 mg; activity applied, 8.4 nkat; recovery, 8 nkat (95%). Buffer: A = 40 m*M* 2-amino-2-methyl-1-propanol–HCl (pH 10.25)–14 m*M* 2-mercaptoethanol; B = 1 *M* NaCl in A. Flow-rate, 1 ml min^{-1}.

De Looze and Wagner [33–35] were able to show that chloroplast glyceraldehyde-3-phosphate dehydrogenase isoenzyme patterns could be modulated by the addition of pyridine nucleotides at the time of extraction. Moreover, aggregated ($\geq 10^6$) and disaggregated ($165 \cdot 10^3$) molecular mass forms were obtained in the presence of NAD^+ and $NADP^+$, respectively. The aggregation phenomenon could be prevented by RNase treatment. Thus, the occurrence of adenylate kinase activity in the void volume might also have its reason in aggregation of isoenzymes mediated by small molecules. Rechromatography of void volume activity showed that it is composed of all adenylate kinase activities. Gel filtration of different materials showed adenylate kinase activity eluting in a range between 25 and 35 kilodalton, thus excluding covalent aggregation of adenylate kinases (data not shown).

Another explanation for void volume activity could be that the factor responsible for void volume activity might block binding sites on Mono Q, thus leading to void volume activity. If this is the case, it is not a specific phenomenon for adenylate kinase but might concern other proteins also. This can be clarified by examination of other enzymes.

Nevertheless, the influence of RNase treatment on the binding of adenylate kinase activity to Mono Q may suggest that ribonucleoprotein interactions could be involved in the occurrence of void volume activity. As no leader sequences have been characterized for adenylate kinases, the question arises of how proteins are directed to different organelles. Therefore, post-translational modifications might be responsible for protein targeting. According to the signal hypothesis [36] for translocation of proteins across membranes, ribonucleoprotein interactions might play a role in light-regulated targeting [37].

The question of why the observed phenomenon occurred only with Mono Q and not with other chromatographic material might be explained by the pore size of these materials. Mono beads have a pore size of about 5 nm, so that larger or modified molecules are not able to enter the pores. The nature of the factors affecting void volume activity and dependence on light treatment is the aim of further investigations.

ACKNOWLEDGEMENT

We thank Dr. S. Frosch for reading the manuscript and helpful discussions.

REFERENCES

1 L. Noda, in P. D. Boyer, H. A. Lardy and K. Myrbaeck (Editors), *The Enzymes*, Vol. VIII, Academic Press, New York, 1973, p. 279.
2 W. E. Criss, G. Litwack, H. P. Morris and S. Weinhouse, *Cancer Res.*, 30 (1970) 370.
3 S. E. Brolin, in H. U. Bergmeyer (Editor), *Methods of Enzymatic Analysis*, Vol. III, Verlag Chemie, Weinheim, 1983, p. 540.
4 R. A. Fildes and H. Harris, *Nature (London)*, 209 (1966) 261.
5 S. Frosch and E. Wagner, *Can. J. Bot.*, 51 (1973) 1521.
6 S. Frosch and E. Wagner, *Can. J. Bot.*, 51 (1973) 1529.
7 U. Haertlé, *Dissertation*, Universität Freiburg i. Br., Freiburg i. Br., 1977.
8 E. Wagner, U. Haertlé, I. Kossmann and S. Frosch, *Endocytobiology*, Vol. II, Walter de Gruyter, Berlin, New York, 1983, p. 341.
9 W. E. Criss, V. Sapico and G. Litwack, *J. Biol. Chem.*, 245 (1970) 6346.
10 W. E. Criss, *J. Biol. Chem.*, 245 (1970) 6352.
11 D. E. Atkinson, *Biochemistry*, 7 (1968) 4030.
12 J. Bomsel and A. Pradet, *Biochim. Biophys. Acta*, 162 (1968) 230.
13 J. W. Stucky, *Eur. J. Biochem.*, 109 (1980) 269.
14 L. A. Kleczkowski and D. D. Randall, *Photosynthetica*, 22 (1988) 112.
15 L. A. Kleczkowski and D. D. Randall, *Plant Physiol.*, 81 (1986) 1110.
16 M. D. Hatch, *Aust. J. Plant Physiol.*, 9 (1982) 287.
17 L. G. Butler and V. Bennett, *Plant Physiol.*, 44 (1969) 1285.
18 M. D. Hatch, C. R. Slack and T. A. Bull, *Phytochemistry*, 8 (1969) 697.
19 C. R. Slack, M. D. Hatch and D. J. Goodschild, *Biochem. J.*, 114 (1969) 489.
20 K. Birkenhead, D. Walker and C. Foyer, *Planta*, 156 (1982) 171.
21 R. Hampp, M. Goller and H. Ziegler, *Plant Physiol.*, 69 (1982) 448.
22 A. E. Karu and E. N. Moudrianakis, *Arch. Biochem. Biophys.*, 129 (1969) 655.
23 S. Murakami and H. Strotmann, *Arch. Biochem. Biophys.*, 185 (1978) 30.
24 S. Frosch, E. Wagner and B. G. Cumming, *Can. J. Bot.*, 51 (1973) 1355.
25 G. E. Schulz, M. Elzinga, F. Marx and R. H. Schirmer, *Nature (London)*, 250 (1974) 120.
26 W. Sachsenheimer and G. E. Schulz, *J. Mol. Biol.*, 114 (1977) 23.
27 M. Brune, R. Schumann and F. Wittinghofer, *Nucleic Acids Res.*, 13 (1985) 7139.
28 D. R. Hoagland and D. J. Arnon, *Circ. Calif. Agric. Exp. Stn.*, (1939) 327.
29 M. M. Bradford, *Anal. Biochem.*, 72 (1976) 248.
30 O. Bárzu and S. Michelson, *FEBS Lett.* 153 (1983) 280.
31 C. M. Luz, I. König, R. H. Schirmer and R. Frank, *Biochim. Biophys. Acta*, 1038 (1990) 80.
32 V.-Q. Hoang, W. Jaklitsch and M. C. Scrutton, *FEMS Microbiol. Lett.*, 78 (1991) 133.
33 S. De Looze and E. Wagner, *Physiol. Plant.*, 57 (1983) 231.
34 S. De Looze and E. Wagner, *Physiol. Plant.*, 57 (1983) 238.
35 S. De Looze and E. Wagner, *Physiol. Plant.*, 57 (1983) 242.
36 P. Walter and G. Blobel, *J. Cell Biol.*, 91 (1981) 557.
37 M. Tevini and D.-P. Häder, *Allgemeine Photobiologie*, Georg Thieme, Stuttgart, 1985, p. 259.