Biochimica et Biophysica Acta, 661 (1981) 199–204
Elsevier/North-Holland Biomedical Press

BBA 69364

CHARACTERIZATION AND PARTIAL PURIFICATION OF DL-GLYCEROL-1-PHOSPHATASE FROM *DUNALIELLA SALINA*

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(Received February 27th, 1981)

Key words: DL-*Glycerol-1-phosphatase; Osmoregulation; Glycerol metabolism; (Dunaliella salina)*

A specific DL-glycerol-1-phosphatase (glycerol-1-phosphate phosphohydrolase, EC 3.1.3.21) has been identified in the halotolerant alga *Duniella salina*. The enzyme is highly specific for DL-glycerol 1-phosphate, requires magnesium for activity and has a neutral pH optimum. High sensitivity toward sulfhydryl reagents suggests the existence of a sulfhydryl group in close proximity to the active site. Due to instability the enzyme was only partially purified (40-fold). Activity measurements following polyacrylamide electrophoresis showed the enzyme to have a molecular weight around 86 kdaltons. It is suggested that the enzyme plays a major role in the mechanism of osmoregulation in *Dunaliella*.

Introduction

The halotolerant algae *Dunaliella* are dependent on their internal concentration of glycerol as means to osmotically balance the salinity of their environment [1,2]. The intracellular glycerol concentration changes proportionally to the NaCl concentration of the external medium which can range from below 0.5 M to saturation. Furthermore, adjustment of the intracellular glycerol concentration occurs readily and rapidly. Clearly, *Dunaliella* must possess an easibly accessible metabolic pathway by which glycerol can be rapidly synthesized and removed.

A scheme for a glycerol cycle has been proposed [3,4], based upon identification of two enzymes which seem to be unique to *Dunaliella* and may reasonably be assumed to be involved in such a pathway: (a) Dihydroxyacetone reductase (glycerol: $NADP^+$ 2-oxidoreductase (dihydroxyacetone-forming), EC 1.1.1.156), which catalyzes the interconversion of glycerol and dihydroxyacetone via the reduction and oxidation of $NADP^+$, and has been studied by two groups [5–7]; (b) Dihydroxyacetone kinase which has recently been partially purified and characterized [8,9]. Activity of a NAD^+-dependent DL-glycerol-1-phosphate dehydrogenase (EC 1.1.1.8) has also been demonstrated in the crude extract of *Dunaliella* (Ref. 10 and Brown, personal communication). However, it has not been purified or characterized thus far.

All proposed pathways for glycerol biosynthesis require the involvement of phosphorylated three-carbon compounds as intermediates. Clearly, therefore, some means must exist in *Dunaliella* to hydrolyze the esterified phosphate to permit the observed large accumulation of free glycerol.

Antia and Watt [10] using *p*-nitrophenylphosphate as a substrate demonstrated activity of phosphatase(s) at pH 4.8, 7.5 and 10.5 in a cell-free extract of *Dunaliella tertiolecta*. However, substrate specificity was not studied. Wegmann [11] reported briefly activity of a glycerol phosphatase in an extract of *Dunaliella* but did not report on any of its characteristics.

In this study we describe the existence, the partial purification and characterization of a specific, neutral and Mg^{2+}-dependent DL-glycerol-1-phosphatase from the alga *Dunaliella salina*. The enzyme is present in other *Dunaliella* species as well as in *Asteromonas* [4], all of which osmoregulate with glycerol.

Materials and Methods

All chemicals were of reagent grade quality. Biochemicals were obtained from Sigma Chemical Company; DEAE-cellulose (DE52) from Whatman; and Sephadex G-50 (superfine), Sephadex G-150 (superfine) and electrophoresis protein standards from Pharmacia.

The purification procedure of the enzyme is summarized in Table I. *Dunaliella salina* were grown as previously described [2] in 3.5 M NaCl. About 70 l algal culture were harvested by centrifugation (approx. 2 000 × *g*) at room temperature. From this point on all operations were carried out at 0–4°C. After cooling on ice for 10 min the concentrated algae were subjected to osmotic shock by the addition of 9 vol. ice-cold 10 mM Tricine (pH 7.4). The mixture was continuously stirred for about 30 min in order to permit complete cell rupture. The resulting mixture (cell fragments) was centrifuged at 48 000 × *g* for 15 min to remove cell debris and the supernatant was collected (cell-free extract). The pellet was washed with 9 vol. of 10 mM Tricine (pH 7.5) and again centrifuged. This supernatant was added to the above collected 'cell-free extract'.

While continuously mixing $(NH_4)_2SO_4$ was slowly added to the extract to a final concentration of 3.6 M. The mixture was stirred for an additional 15 min, centrifuged at 48 000 × *g* for 15 min, the supernatant removed, and the pellet resuspended in a minimum volume (about 35 ml) of suspension buffer (10 mM Tricine (pH 7.4)/0.5 M glycerol). The $(NH_4)_2SO_4$ was removed by the method of Penefsky [12] from aliquots of both the supernatant and the resuspended pellet before testing for enzymic activity and protein content. The supernatant contained no detectable activity.

The resuspended pellet was dialyzed for 4 h against about 2.5 l suspension buffer, changing the buffer about midway. (The dialysis tubing was pretreated by boiling in 0.4 M $NaHCO_3$/10 mM EDTA and stored in 50% ethanol). The enzyme solution was diluted with suspension buffer (about 2 vol.) until the conductance was less than 2 $m\Omega^{-1}$ which is the conductance of the suspension buffer containing 30 mM NaCl (dialyzate). It was then applied to a 2.1 × 26 cm column of DEAE-cellulose (Whatman DE-52) pre-equilibrated with the suspension buffer. To avoid overloading the column, dialyzate was applied until the yellow-orange color covered no more than one-third of the column length.

The column was washed with suspension buffer containing 30 mM NaCl until no protein appeared in the eluate followed by a 1 000 ml linear gradient of 30–130 mM NaCl in the suspension buffer. The activity of DL-glycerol-1-phosphatase and the protein concentration were determined for the collected fractions. Two peaks of DL-glycerol-1-phosphatase were generally found. The activity in the small peak which eluted around 43 mM NaCl was not significantly purified. It showed, nevertheless, specificity toward DL-glycerol 1-phosphate (no activity with glycerol 2-phosphate) and required Mg^{2+} for activity (see below), but was not studied any further. The major peak of activity was eluted around 63 mM NaCl. The fractions with the highest specific activity were pooled, diluted with 3 vol. suspension buffer and concentrated by adsorption to a 1 ml DEAE-cellulose (Whatman DE-52) columns pre-equilibrated with suspension buffer. The protein was eluted in three 1-ml

TABLE I

PURIFICATION OF DL-GLYCEROL-1-PHOSPHATASE FROM *DUNALIELLA SALINA*

For details see Materials and Methods.

Fraction	Volume (ml)	Protein (mg)	Total activity (units)	Specific acitivity (units/mg protein)	Purification (-fold)	Yield (%)
Cell fragments	290	4 050	612	0.15	1	100
Cell-free extract	451	1 191	513	0.43	3	84
Dialyzate	151	1 068	574	0.54	4	94
DEAE-cellulose Peak I	248	25	7	0.28	2	1
DEAE-cellulose Peak II	976	30	183	6.1	41	30

fractions of the suspension buffer containing 0.2 M NaCl. Glycerol was immediately added to a final concentration of 2 M, and the enzyme was stored at −20°C in small aliquots which were diluted with water before use.

The activity of DL-glycerol-1-phosphatase was measured by incubating the enzyme in a reaction mixture containing in a total volume of 1.0-ml: 20 mM Tricine (pH 7.0)/5 mM $MgCl_2$/10 mM DL-glycerol 1-phosphate. At appropriate time intervals 50% $HClO_4$ (to a final concentration of 4.5%) was added to samples. The denatured protein was precipitated by a 2 min centrifugation in a microfuge and the samples were stored at 4°C. The inorganic phosphate released in each sample was measured as previously described [13]. When necessary total phosphate concentration was also determined as described [13]. The rate of the enzymic reaction was calculated from the linear slope of phosphate released vs. time. Phosphatase activity on *p*-nitrophenylphosphate was followed spectrophotometrically at 405 nm. The activities of dihydroxyacetone reductase and dihydroxyacetone kinase were measured by the methods described by Ben-Amotz and Avron [5] and Lerner and Avron [8], respectively.

A unit of phosphatase activity is defined as the amount of enzyme which releases 1 μmol inorganic phosphate/min at 25°C. Protein concentration was determined as described by Kochert [14].

Vertical acrylamide-slab gel electrophoresis was performed with the lower, separating gel (11 cm high), composed of 7.5–10% bisacrylamide solution (30% acrylamide/0.8% acrylamide) in a Tris-HCl buffer (pH 8.8), and the upper or stacking gel (3 cm high) composed of 3% bisacrylamide in a Tris-HCl buffer (pH 6.8). The running buffer was 25 mM Tris-glycine (pH 8.9). Electrophoresis was carried out with an electrophoresis apparatus from Hoffer at 4°C at 150 mV for about 270 min or until the dye front of bromphenol blue began to be eluted from the gel.

Protein staining of the gels was carried out by the method of Diezel et al. [15]. Localization of DL-glycerol-1-phosphatase was performed by incubating the gel in the reaction mixture described above with the addition of 0.1% $Pb(NO_3)_2$, which after about 0.5 h resulted in a distinct white band.

Results

Enzyme stability. In order to determine the best conditions for the stabilization of DL-glycerol-1-phosphatase, cell-free extract was incubated in the presence of various concentrations of $MgCl_2$, NaCl, DL-glycerol 1-phosphate, dithiothreitol, EDTA and glycerol over a period of 8 days at 4°C. The enzyme lost about 5% of its activity per day in the crude extract (Fig. 1). Addition of $MgCl_2$, and to a lesser degree addition of NaCl, accelerated the loss of activity. This inactivation was irreversible since removal of the salt by passing the sample though a Sephadex column did not restore activity. The natural decay and the salt-induced acceleration were retarded by high glycerol concentrations but even in the presence of 2 M glycerol activity was lost at an appreciable rate in the presence of salts.

Addition of 0.1 mM EDTA, 10 mM dithiothreitol or 10 mM DL-glycerol 1-phosphate did not significantly affect the stability of the enzyme in the crude extract (data not presented). When the partially purified enzyme was stored at −20°C in the presence of 2 M glycerol/0.2 M NaCl/10 mM Tricine (pH 7.4), 100% activity was maintained for a period of two months.

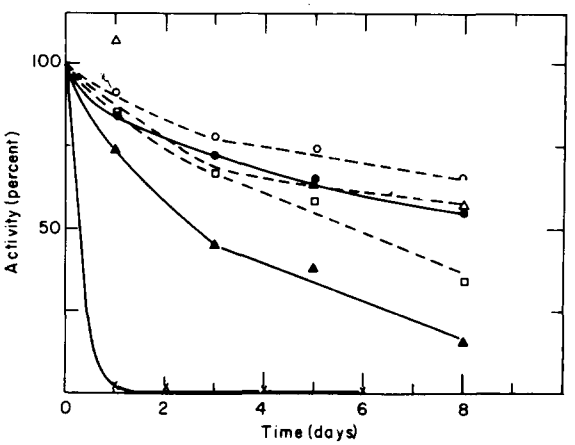

Fig. 1. The effect of salts and glycerol on enzyme stability. The following were added to a cell-free extract maintained at 4°C and containing 10 mM Tricine (pH 7.4) and approx. 0.2 M NaCl (●——●): 2 M glycerol (○ - - - - - ○); 0.2 M NaCl (▲——▲); 2 M glycerol + 0.2 M NaCl (△ - - - - - △); 0.5 M glycerol + 0.2 M NaCl (□ - - - - - □); 0.15 M $MgCl_2$ (X——X). Samples were removed at the times indicated and assayed.

Purity. As a result of the inability to fully stabilize DL-glycerol-1-phosphatase attempts at further purification were not successful. The partially purified preparation was 40-fold purer than the cell fragments (Table I). Contamination by other proteins was quite evident when the preparation was subjected to electrophoresis.

The partially purified enzyme had no detectable dihydroxyacetone reductase or dihydroxyacetone kinase activity. The reductase was present in the void volume and the wash of the DEAE-cellulose column, and the kinase was still retained on the column when the phosphatase was eluted.

Molecular weight. The partially purified enzyme preparation was subjected to electrophoresis on a 7.5–10% gradient of polyacrylamide. When the gel was assayed for DL-glycerol-1-phosphatase activity, only one band could be seen. This band corresponded to a molecular weight of about 86 kdaltons when compared to molecular weight standards.

Substrate specificity and affinity. The apparent K_m values for the partially purified DL-glycerol-1-phosphatase were calculated from Lineweaver-Burk plots at two different Mg^{2+} concentrations. It is evident from Fig. 2, that the apparent K_m for glycerol 1-phosphate is dependent on the Mg^{2+} concentration while the V is not. An increase in Mg^{2+} results in an increase in the apparent K_m toward DL-glycerol 1-phosphate. The real substrate of the enzyme is most likely a Mg^{2+}-glycerol 1-phosphate complex, with excess free Mg^{2+} being a competitive inhibitor (see below). Addition of 2 M glycerol to the reaction mixture resulted is only minor effects on the K_m and V values (data not shown).

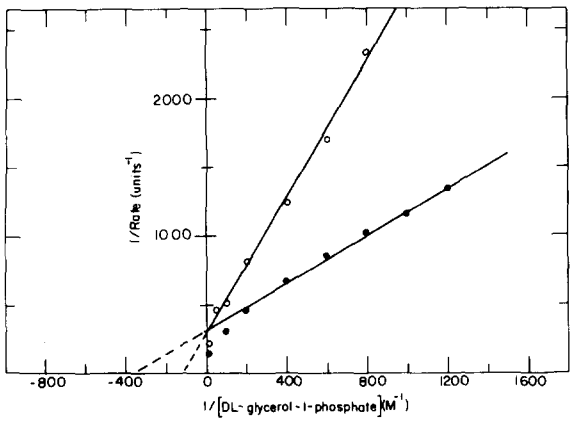

Fig. 2. The apparent K_m of DL-glycerol-1-phosphatase for DL-glycerol 1-phosphate. The reaction mixture contained: 20 mM Tricine (pH 7.0); the indicated concentration of DL-glycerol 1-phosphate; 10 mM $MgCl_2$, K_m = 2.7 mM (●———●) or 30 mM $MgCl_2$, K_m = 8.5 mM (○———○), and 0.35 μg/ml of the partially purified enzyme.

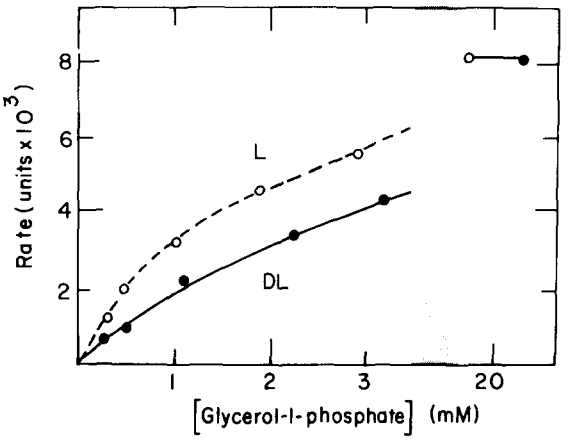

Fig. 3. Activity of DL-glycerol-1-phosphatase as a function of varying concentrations of DL-glycerol 1-phosphate or L-glycerol 1-phosphate. The reaction mixture contained: 20 mM Tricine (pH 7.0)/5 mM $MgCl_2$; the indicated concentrations of DL-glycerol 1-phosphate (●———●) or L-glycerol 1-phosphate (○ - - - - - ○), and 0.7 μg/ml partially purified enzyme. The absolute concentrations of the substrates were determined by the total phosphate content of each reaction mixture.

DL-Glycerol-1-phosphatase is highly specific toward its substrate. No activity was detected for any of a variety of plausible substrates, including glycerol 2-phosphate, DL-glyceraldehyde 3-phosphate dihydroxyacetone phosphate, D-erythrose 4-phosphate, D-glucose 6-phosphate, D-fructose 6-phosphate, D-fructose-1,6-diphosphate, phosphatidic acid, ATP, ADP and *p*-nitrophenylphosphate. Only DL-propanediol 1-phosphate was poorly hydrolyzed. Even the crude cell-free extract showed almost no phosphatase activity toward glyceraldehyde phosphate or dihydroxyacetone phosphate.

It was of interest to determine whether the phosphatase was stereospecific. Incubation of the partially purified enzyme with either DL-glycerol 1-phosphate or L-glycerol 1-phosphate resulted in total hydrolysis of the substrate. However, from the data presented in Fig. 3, it is evident that the enzyme prefers the L-form which is hydrolyzed more rapidly than the D-form at limiting substrate concentrations.

Mg^{2+} dependence and specificity. There was no

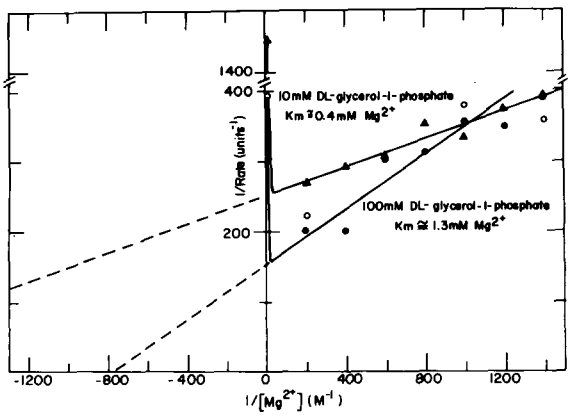

Fig. 4. The apparent K_m of DL-glycerol-1-phosphatase for Mg^{2+}. The reaction mixture contained: 20 mM Tricine (pH 7.0)/$MgCl_2$ at the indicated concentrations/100 mM DL-glycerol 1-phosphate in the absence (●———●) or presence of 2 M glycerol (○———○), or 10 mM DL-glycerol 1-phosphate (▲———▲), and 0.7 μg/ml of the partially purified enzyme.

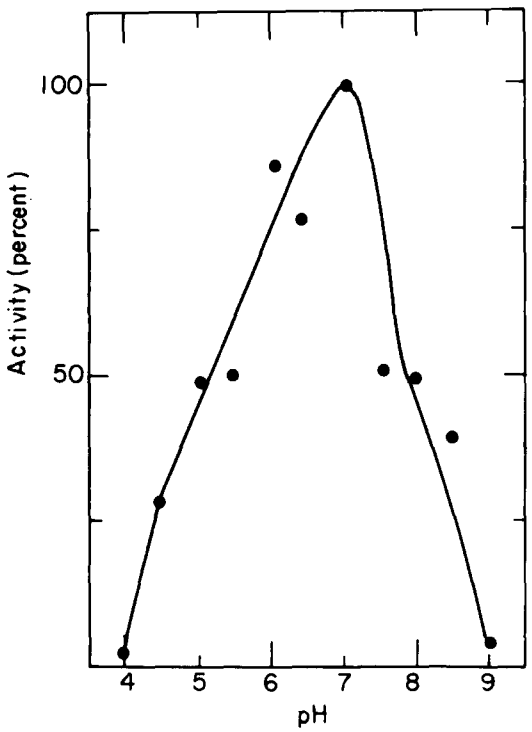

Fig. 5. pH profile of partially purified DL-glycerol-1-phosphatase. The reaction mixture contained 20 mM Tris-maleate adjusted to the pH indicated/20 mM DL-glycerol 1-phosphate/10 mM $MgCl_2$/2.4 μg/ml of the partially purified enzyme.

detectable activity of the partially purified DL-glycerol-1-phosphatase in the absence of Mg^{2+}. The apparent K_m and V for Mg^{2+} showed a small dependence on the concentration of DL-glycerol 1-phosphate which increased with increasing substrate concentration (Fig. 4). High concentrations of Mg^{2+} (above 0.1 M) strongly inhibited the enzyme indicating that excess free Mg^{2+} has an inhibitory effect. The magnitude of the inhibition was dependent on the substrate concentration. Addition of 2 M glycerol to the reaction mixture had no significant effect on either the K_m or V for Mg^{2+}.

Several divalent and monovalent cations were tested as possible substitutes for Mg^{2+}. Mn^{2+} was somewhat more effective than Mg^{2+}, but all other cations tested including Co^{2+}, Zn^{2+}, Ni^{2+}, Ca^{2+}, Ba^{2+}, Na^+ and K^+, showed slight to no activity. Ca^{2+} was inhibitory (approx. 80%) when added with equimolar concentrations of Mg^{2+}.

pH activity curve. The dependence of activity on pH is shown in Fig. 5. There is a rather sharp optimum around pH 7.0, with essentially no activity at pH 4.0 or 9.0. Thus, DL-glycerol-1-phosphatase can not be classified as either an acidic or an alkaline phosphatase.

Enzyme activation and inhibitors. The strong inhibition by low concentrations (0.1 μM) *p*-hydroxymercuribenzoate (Table II) indicates a major role for sulfhydryl groups in the enzymic reaction. The small increase in activity by EDTA may be due to the chelation of contaminating heavy metal ions which could interact with the highly sensitive sulfhydryl group. The enzyme was essentially insensitive to cyanide.

TABLE II

EFFECT OF INHIBITORS AND ACTIVATORS

Reaction mixture contained 20 mM Tricine (pH 7.0)/10 mM DL-glycerol 1-phosphate/5 mM $MgCl_2$/0.7 μg/ml protein. 100% activity refers to 4 units/mg protein.

Inhibitor or activator	Activity (percent of control)
p-Hydroxymercuribenzoate, 0.1 μM	19
KCN, 10 mM	80
$NaAsO_2$, 10 mM	28
Sodium EDTA, 0.1 mM	125
Sodium tartrate, 10 mM	100

Discussion

Activity of a DL-glycerol-1-phosphatase has been found to be present in the cell-free extract of *Dunaliella salina*. The phosphatase is completely specific for DL-glycerol 1-phosphate. This high specificity indicates that DL-glycerol-1-phosphatase is likely to be involved in the mechanism of osmoregulation in these algae.

Osmoregulation in *Dunaliella* is achieved by the synthesis and removal of glycerol [2,3,7,10]. It was previously suggested [4] that when the algae are faced with an increase in the external salt concentration dihydroxyacetone phosphate, produced photosynthetically or from stored polysaccharides (via the pentose phosphate shunt or glycolysis), is reduced to glycerol 1-phosphate by an NAD^+-dependent glycerol-1-phosphate dehydrogenase. Dephosphorylation to glycerol was postulated to occur via a glycerol-1-phosphatase which this communication establishes to exist in *Dunaliella* as a highly specific enzyme.

The studies reported here indicate that DL-glycerol-1-phosphatase acts on a Mg^{2+}-glycerol 1-phosphate compex as its real substrate. This is supported by several findings: the enzyme has no activity in the absence of Mg^{2+}; the apparent K_m for glycerol 1-phosphate depends on the Mg^{2+} concentration, and vice versa; and excess free Mg^{2+} inhibits the DL-glycerol-1-phosphatase. Since low concentrations of *p*-hydroxymercuribenzoate severely inhibit the enzyme the involvement of a sulfhydryl group at the active site is indicated. It is likely that the site of binding of the Mg^{2+} in the Mg^{2+}-glycerol 1-phosphate complex involves the sulfhydryl group [17].

A similar DL-glycerol-1-phosphatase has been described in the yeast, *Candida utilis* [18,19], which is able to utilize glycerol as its major carbon source. This enzyme is also specific for DL-glycerol 1-phosphate with some preference for the L-isomer, has a pH optimum around 6.5, and requires Mg^{2+} for activity, but is rather insensitive to *p*-hydroxymercuribenzoate and strongly inhibited by tartrate.

The properties of the DL-glycerol-1-phosphatase described here make it likely that it plays an important role in the mechanism of osmoregulation in *Dunaliella*. Since this enzyme catalyzes the essentially irreversible reaction in the pathway of glycerol synthesis [3,4], it may also serve as a possible controlling site in the pathway. Further studies along this line of thought are presently in progress.

Acknowledgements

The authors would like to gratefully acknowledge Dr. Ami Ben-Amotz for helpful discussions throughout the progress of this work and Mr. Haim Budai's help in growing the algae.

References

1 Craigie, J.S. and Mclachlan, J. (1964) Can. J. Bot. 42, 777–778
2 Ben-Amotz, A. and Avron, M. (1973) Plant Physiol. 51, 875–878
3 Ben-Amotz, A. and Avron, M. (1978) in Energetics and Structure of Halophilic Microorganisms (Caplan, S.R. and Ginzburg, M., eds.), pp. 529–541, Elsevier, Amsterdam
4 Ben-Amotz, A., Sussman, I. and Avron, M. (1981) in Experientia (Mislin, H., ed.), Birkhauser Verlag, Basal, in the press
5 Ben-Amotz, A. and Avron, M. (1973) FEBS Lett. 29, 153–155
6 Ben-Amotz, A. and Avron, M. (1974) Plant Physiol. 53, 628–631
7 Borowitzka, L.J. and Brown, A.D. (1974) Arch. Microbiol. 96, 37–52
8 Lerner, H.R. and Avron, M. (1977) Plant Physiol. 59, 15–17
9 Lerner, H.R., Sussman, I. and Avron, M. (1980) Biochim. Biophys. Acta 615, 1–9
10 Antia, N.J. and Watt, A. (1965) J. Fish. Res. Can. 22, 793–799
11 Wegmann, K. (1981) in Proc. 5th International congress on Photosynthesis (Akoyunoglu, G., ed.), Vol. II. Balaban International Sciences, Philadelphia, in the press
12 Penefsky, H.S. (1977) J. Biol. Chem. 252, 2 891–2 899
13 Ames, B.N. (1966) Methods Enzymol. 8, 115–117
14 Kochert, G. (1978) in Handbook of Phycological Methods (Hellebust, J.A. and Craigie, J.S., eds.), pp. 91–93, Cambridge University Press, Cambridge
15 Diezel, W., Kopperschlager, G. and Hofmann, E. (1972) Anal. Biochem. 48, 617–620
16 Michal, G. and Beutler, H. (1974) in Methods of Enzymatic Analysis (Bergmeyer, H.U., ed.), pp. 1 314–1 319, Academic Press, New York
17 Barron, E.S.G. (1951) Adv. Enzymol. 11, pp. 201–266
18 Tsuboi, K.K. and Hudson, P.B. (1956) Arch. Biochem. Biophys. 61, 197–210
19 Gancedo, C., Gancedo, J.M. and Sols, A. (1968) Eur. J. Biochem. 5, 165–172