The Intracellular Distribution of Enzymes of the Glycerol Cycle in the Unicellular Alga *Dunaliella parva*

H. Gimmler and G. Lotter

Lehrstuhl Botanik I der Universität Würzburg, Mittlerer Dallenbergweg 64, D-8700 Würzburg

Z. Naturforsch. **37 c,** 1107–1114 (1982); received June 28, 1982

Chloroplasts, Cytoplasm, DEAE-Dextran, *Dunaliella*, Glycerol Cycle

The intracellular distribution of the enzymes of the glycerol cycle in the unicellular alga *Dunaliella* parva was investigated using the technique of differential extraction by the polycation DEAE-dextran.

Results suggest a cytoplasmic localization of the DHA reductase, but a chloroplastic localization of the glycerol-3-phosphate dehydrogenase and possibly also of the glycerol-3-phosphate phosphatase.

Introduction

Glycerol is the main osmoticum of the unicellular green alga *Dunaliella* [1–4]. During the process of osmoregulation this solute has to be synthesized or degraded efficiently depending on the extent and direction of changes in the salinity of the external medium. Synthesis of glycerol starts from DHAP [5]. A glycerol-3-phosphate dehydrogenase (EC 1.1.1.8) reduces DHAP to glycerol-3-phosphate and this compound is dephosphorylated to glycerol by a specific glycerol-3-phosphate phosphatase (EC 3.1.3.21) [6]. The degradation of glycerol is catalyzed by a DHA oxidoreductase (EC 1.1.1.156) which oxidizes glycerol to DHA [7]. DHA is then phosphorylated to DHAP by a DHA kinase [8, 9]. Since DHAP is the first intermediate of glycerol synthesis, but also the end product of glycerol degradation, this pathway was called „glycerol cycle" [5]. Data about the turnover of this cycle under different osmotic conditions and its regulation are rare [10]. One cycling of DHAP requires one molecule ATP, whereas consumption and production of reduction equivalents are balanced. Such a futile cycle may have the function of a valve [11]. However, in order to prevent an excessive waste of energy it has to be highly controlled. Regulations at the levels of the two involved reversible oxidoreductases are unlikely. More probable sites for effective control of the glycerol cycle are the irreversible enzymes of the cycle that are the glycerol-3-phosphate phosphatase and the DHA kinase as well. Differential compartmentation of the enzymes of the glycerol cycle may be also involved in the regulation of this cycle. In order to identify sites and mechanism of osmoregulation we investigated in this paper the intracellular distribution of the enzymes of the glycerol cycle in *D. parva.* For microalgae no suitable, conventional methods for the isolation of intact organelles are available. This is especially valid for the cell-wall less, fragile Dunaliella with its single big cup-shaped chloroplast. Any mechanical treatment which destroys the plasmalemma is likely to damage also the chloroplast envelope (but compare reference 12). Therefore we carried out an indirect approach using the technique of differential extraction of the cells by the high molecular weight polycation DEAE-dextran [13–15]. In a previous paper [16] it was demonstrated by the use of marker enzymes that this technique allows to distinguish enzymes located in the cytoplasm from those located in chloroplasts.

Abbreviations: C, Carboxylase; Chl, chlorophyll; Cyt, cytoplasmatic; DEAE-dextran, diethylaminoethyl dextran; DH, dehydrogenase; DHA, dihydroxyacetone; DHAP, dihydroxyacetonephosphate; DTE, dithioerythritol; GAP, glyceraldehyde; Glu-6-P, glucose-6-phosphate; Gly-3-P, glycerol-3-phosphate; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; OxR, oxidoreductase; PEP, phosphoenolpyruvate; Ph, phosphatase; *p*-NPP, *para*-nitrophenylphosphate; Pyr-K, pyruvate kinase; RuBP, ribulose-1,5-bisphosphate; TRIS, trishydroxymethylaminomethane.

Reprint requests to Dr. H. Gimmler.

0341-0382/82/1100-1107 $ 01.30/0

Materials and Methods

Material

Algal culture. Dunaliella parva Lerche was cultured synchronously in an inorganic culture medium containing 0.75 M NaCl [17]. Algae were harvested by centrifugation (1500 × *g*, 10 min, 20 °C) and re-

suspended for DEAE-dextran treatment in a medium containing 1.5 M sorbitol and 20 mM TRIS/HEPES (pH 7.6).

Chemicals. DEAE-dextran was purchased from Pharmacia Chemicals, Uppsala (Sweden), [^{14}C]-$KHCO_3$ from Amersham & Buchler, Braunschweig (Germany), lactate dehydrogenase, 3-phosphoglycerate kinase and glycerol-3-phosphate dehydrogenase from Sigma München (Germany) and all other substrates and cofactors from Boehringer, Mannheim (Germany).

Methods

DEAE-dextran treatment. The differential extraction of enzymes from *D. parva* cells was carried out as described in a previous paper [16]: DEAE-dextran in the desired concentrations was added to the algal suspension under rapid mixing at 0 °C (adsorption period: uniform adsorption of the molecules at the outer plasmalemma). After 1 min samples were incubated at 30 °C for varying periods (time of lysis: The irreversible membrane damage occurs). The reaction with DEAE-dextran was stopped after the desired time of lysis by centrifugation ($5000 \times g$, 5 min, 0 °C). The supernatant was stored on ice for analysis of enzyme activity, whereas the pellet was discharged. Aliquots of the supernatant (20–500 µl, depending on enzyme activity and the sensitive of the assay) were taken for the enzyme assays. It was ascertained that the DEAE-dextran still present in these aliquots did not interfere with the enzyme assay *per se.*

To compare the effect of DEAE-dextran on the enzyme efflux in different experiments and with different reactions we introduced the parameters T_{50} and D_{50} which are defined as follows: The T_{50}-value is that time of lysis which causes half maximal reaction at a fixed concentration of DEAE-dextran ($30\ mg \times mg^{-1}$ Chl). The D_{50}-value is that DEAE-dextran concentration which causes half maximal reaction over a fixed time of lysis (10 min).

Enzyme assays

Marker enzymes. Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.13) were measured spectrophotometrically [18] following absorption changes of pyridine nucleotides at 340 nm. The former enzyme activity was determined directly whereas the latter reaction was coupled to the conversion of glycerate-3-phosphate to glycerate-1,3-biphosphate by the 3-phosphoglycerate kinase (EC 2.7.2.3). To distinguish between the plastidal and cytoplasmatic form of the Glu-6-P dehydrogenase the reaction was carried out in the presence and absence of 50 mM DTE [16, 19]. The Glu-6-P dehydrogenase activity in the presence of DTE was considered to be due solely to the cytoplasmatic form, whereas the difference between the total activity and the activity in the presence of DTE was assumed to reflect the plastidal form of the enzyme [16]. RuBP carboxylase activity (EC 4.1.1.39) was measured by the method of Kaiser and Heber [20] starting with ribose-5-phosphate and ATP as substrates. PEP carboxylase (EC 4.1.1.31) was measured by the formation of [^{14}C]oxaloacetate from $^{14}CO_2$ and PEP [21]. [^{14}C]oxaloacetate was stabilized by the addition of 2,4-dinitrophenylhydrazin or converted to malate by malate dehydrogenase and NADH. Pyruvate kinase (EC 2.7.1.4) was assayed by coupling the reaction to the NADH-dependent conversion of pyruvate to lactate by the lactate dehydrogenase [18].

Enzymes of the glycerol cycle. Glycerol-3-phosphate dehydrogenase (EC 1.1.1.8) was assayed by measuring the absorption change of NADH at 340 nm [18], final concentrations 30 mM HEPES/NaOH, pH 7.6, 0.2 mM NADH and 0.35 mM DHAP. Glycerol-3-phosphate phosphatase (EC 3.1.3.21) was assayed at pH 7.6 in the presence of 15 mM HEPES/NaOH, 5 mM glycerol-3-phosphate and 5 mM $MgCl_2$. The liberated phosphate was determined according to the method of Gerlach and Deuticke [22]. Acid phosphatase (EC 3.1.3.2) was monitored at pH 5.6 in the presence of 90 mM citrate/NaOH buffer, (respectively 30 mM HEPES/Tris for pH 7.6) and 20 mM *p*-NPP [18] by following the absorption change at 405 nm. Both phosphatase reaction were measured for 10 or 20 min and it was ensured that this was in the period of linearity. The reaction was stopped by the addition of 20% trichloroacetic acid to give a final concentration of 5%.

For the glycerol-3-phosphate assay it was necessary to add after killing equal amounts of dextran sulfat to prevent interference of the DEAE-dextran with the phosphate assay. Glycerol oxidoreductase (DHA reductase, EC 1.1.1.156) was measured according to Ben-Amotz and Avron [7] by following the absorption change of pyridine nucleotides at

340 nm at pH 7.6 in the presence of 30 mM Tris/HCl, 0.1 mM NADPH and 5 mM DHA. DHA kinase was assayed in a reaction coupled to the conversion of DHAP to glycerol-3-phosphate [8, 9]. The assay contained 100 mM tricine/HCl buffer (pH 7.6), 16 mM $MgCl_2$, 0.05 mM NADH, 2.5 mM ATP, 1 mM DHA and 1.4 units of a glycerol-3-phosphate dehydrogenase.

All enzyme reactions were measured at room temperature (20 °C). Rates were corrected for activities of untreated samples, which are the result of osmotically or mechanically ruptured cells in the suspension. These background values were usually very low, but the correction was necessary definitely for samples incubated only very short times with DEAE-dextran, respectively with very low DEAE-dextran concentrations.

Diffusion coefficients

A very rough estimation of the diffusion coefficients of the enzymes of the glycerol cycle and the tested marker enzymes was carried out in the following way: From data given by Lehninger [23] the diffusion coefficients of some globular enzymes were plotted as function of the cubic root of the molecular weight of the enzymes. The resulting straight line was used as calibration curve to get approximate values for the diffusion coefficients of the investigated enzymes. The molecular weight of the enyzmes were taken from the literature.

Results and Discussion

Time and concentration dependent DEAE-dextran induced efflux of the enzymes of the glycerol cycle of *D. parva* were determined as described under methods and in a previous paper [16]. An example is given in Fig. 1 for the glycerol-3-phosphate dehydrogenase. T_{50} and D_{50} values were intrapolated from such curves, averaged and compared with corresponding values of marker enzymes for the cytoplasm and the chloroplasts (Table I). Of special interest was the direct comparison of efflux kinetics of different enzymes in one and the same experiment. An example is given in Fig. 2: The time-dependent efflux of the glycerol oxidoreductase is much earlier saturated than the efflux of the plastidal marker enzyme GAP dehydrogenase, suggesting a cytoplasmic site of the glycerol oxidoreductase.

Some complications do occur with the glycerol-3-phosphate phosphatase, because in crude extracts

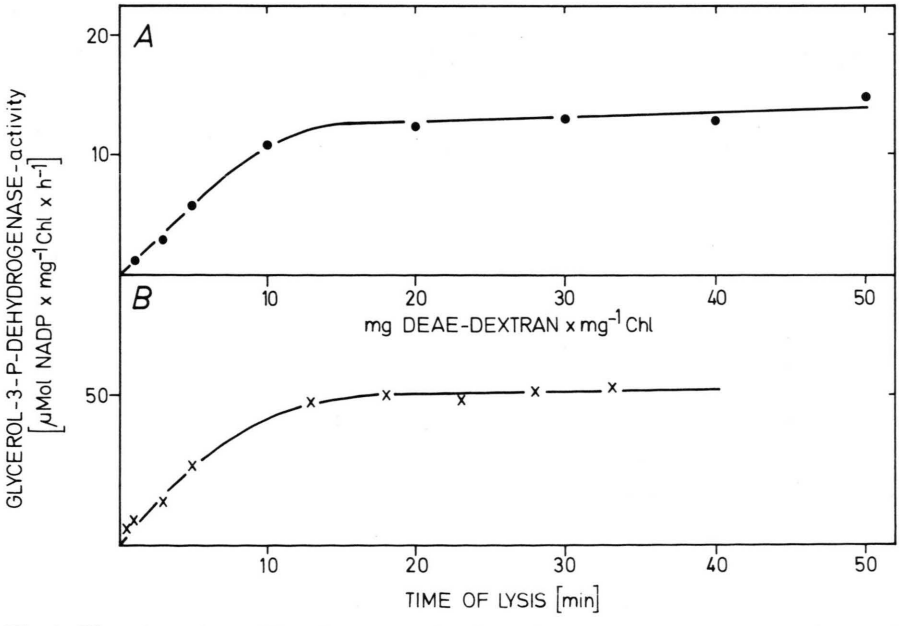

Fig. 1. Time (experiment B) and concentration (experiment A) dependent DEAE-dextran induced efflux of Gly-3-P-DH from *D. parva* cells into the medium. From such and corresponding curves T_{50}- and D_{50}-values were determined as defined under methods.

Table I. D_{50} and T_{50} values for the DEAE-dextran induced efflux of marker enzymes (data from [16]) and enzymes of the glycerol cycle and of acid phosphatase from *D. parva* cells into the medium. Average of 4–8 experiments. In the last column T_{50} and D_{50} values are normalized: Values for the RuBP carboxylase were taken as 100% and then averaged.

Enzyme	D_{50} [mg DEAE-dextran × mg^{-1} Chl]	T_{50} [min	$\frac{\left[\frac{D_{50\,\text{enzyme}}}{D_{50\,\text{RuBP-C}}}+\frac{T_{50\,\text{enzyme}}}{T_{50\,\text{RuBP-C}}}\right]}{2}\times 100$
RuBP carboxylase	13.0	8.0	100
GAP-DH (NADPH)	7.3	5.5	63
Glu-6-P-DH$_{Chl}$	6.5	6.0	63
Average of the tested chloroplastic marker enzymes	8.9	6.5	75
PEP carboxylase	6.7	1.0	32
Glu-6-P-DH$_{cyt}$	2.5	4.5	37
Pyruvate kinase	1.4	2.2	20
Average of the tested cytoplasmic marker enzymes	3.2	2.6	30
DHA oxidoreductase	1.6	1.0	13
Gly-3-P-phosphatase	–	4.2	52
Gly-3-P-DH	6.4	6.6	66
Acid phosphatase	–	10.0	125

an interference of the specific glycerol-3-phosphate phosphatase activity with the unspecific acid phosphatase activity has to be taken into account, respectively to be minimized as far as possible. The lower part of table II demonstrates that at a pH of 7.6 the activity of the unspecific phosphatase is low in comparison to the specific glycerol-3-phosphate phosphatase activity. The latter enzyme exhibits a specificity for glycerol-3-phosphate, is stimulated by Mg^{2+}, but inhibited by Ca^{2+} (upper part of Table II), whereas the acid phosphatase is not affected by divalent cations (lower part). These results agree with those of others [6] and demonstrate that even in a crude extract glycerol-3-phosphatase can be measured sufficiently exact without misleading falsifications by the effect of acid phosphatase, provided the proper experimental conditions are chosen. The second complication arises from the observation that the phosphate determination is disturbed by DEAE-dextran still present during the assays. This effect could be overcome by the addition of equimolar concentrations of the polyanion dextran sul-

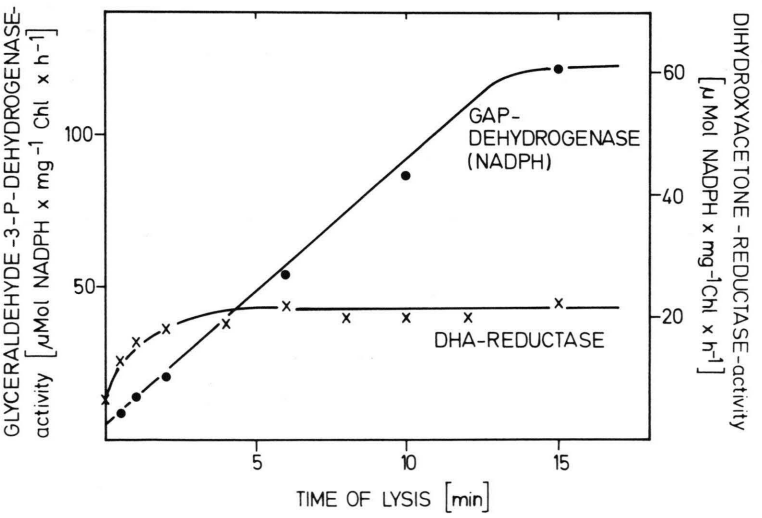

Fig. 2. Time dependent DEAE-dextran induced efflux of the GAP-DH$_{(NADPH)}$ (●, marker enzyme for the chloroplast) and of DHA oxidoreductase (×, a glycerol cycle enzyme) from *D. parva* cells into the medium.

Table II. Glycerol-3-P phosphatase and acid phosphatase activity in crude extracts from *D. parva* cells as influenced by the pH, the substrate and divalent cations. Maximal activities of both enzymes were found to be about 60 µmol phosphate liberated × mg^{-1} Chl × h^{-1}. DEAE-dextran concentration: 30 mg × mg^{-1} Chl. Values signed with *: Activities at pH 6.0 were made to 100%. The number in brackets indicate the number of experiments.

Enzyme	Substrate	pH	System	Phosphatase activity (control in the absence of divalent cations = 100%)	
				5 mM Mg^{2+}	5 mM Ca^{2+}
Glycerol-3-P phosphatase	glycerol-3-P	7.6	Supernatant of cells after 2 min treatment with DEAE-dextran	230 (3)	45 (2)
			Supernatant of cells after 15 min treatment with DEAE-dextran	249 (3)	25 (4)
	glycerol-3-P	7.6	Crude extract from osmotically shocked cells	237 (2)	28 (1)
	glycerol-2-P			29 (2)	–
	glucose-6-P			7 (2)	–
Acid phosphatase	*p*-NPP	6.0	Supernatant of cells after 2 min treatment with DEAE-dextran	90 (4)	92 (2)
		7.6		22 * (3)	–
		6.0	Supernatant of cells after 15 min treatment with DEAE-dextran	96 (4)	93 (2)
		7.6		1 * (3)	–
		6.0	Crude extracts from osmotically shocked cells	124 (2)	95 (2)

fate to the samples after killing. The efflux of glycerol-3-phosphate phosphatase into the medium is much earlier saturated than the efflux of acid phosphatase (Fig. 3). This could imply that the former enzyme is localized in the cytoplasma and the latter in a compartment, which is separated by an additional membrane from the medium, but see below.

Table I presents a direct comparison of T_{50} and D_{50}-values of the enzymes of the glycerol cycle with corresponding values of marker enzymes for the chloroplasts and the cytoplasm. The results suggest a cytoplasmic site of the DHA oxidoreductase, whereas the glycerol-3-phosphate phosphatase and the glycerol-3-phosphate dehydrogenase seem to be present either in both the cytoplasm and the chloroplasts or only in the latter. The acid phosphatase which is not involved in the glycerol cycle can be assumed to be localized in the cytosomes. The cytosome envelope is certainly rather impermeable for glycerol-3-phosphate. No suggestion can be made about the localization of the DHA kinase, because of methodical difficulties to measure sufficiently high reproducible rates of this enzyme after DEAE-dextran treatment.

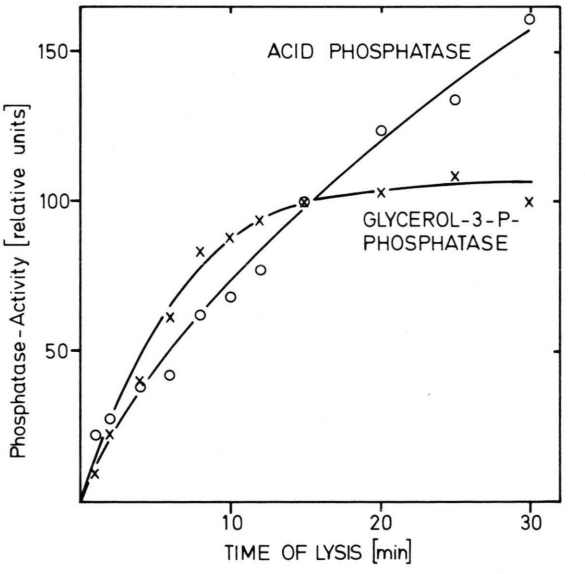

Fig. 3. Time dependent DEAE-dextran induced efflux of the Gly-3-P phosphatase (×) and the acid phosphatase (○) from *D. parva* cells into the medium. Values at 15 min were normalized to 100%. Average of 7 independent experiments. Maximal rates of both phosphatases were in the range of about 60 µmol phosphate liberated per mg Chl and h.

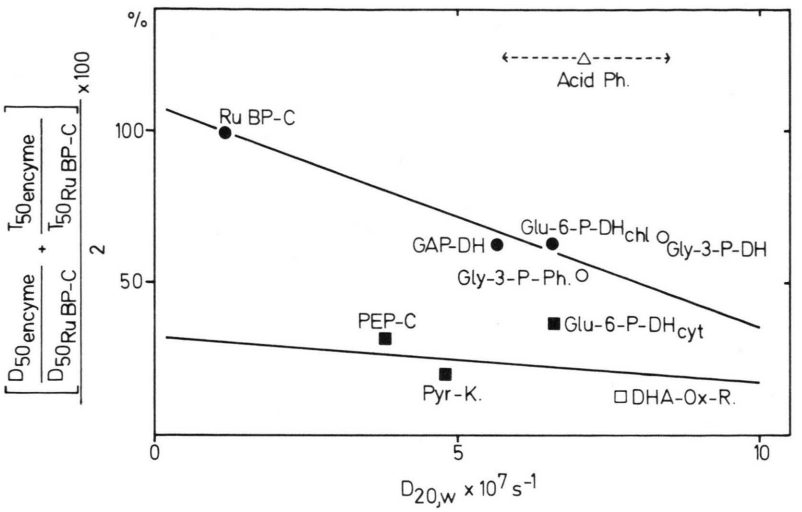

Fig. 4. Normalized efflux parameters for the DEAE-dextran induced efflux of enzymes from *D. parva* cells into the medium (compare last column of Table II) as function of diffusion coefficients ($D_{20,W}$), roughly estimated as described in Fig. 5. (●) plastidic marker enzymes, (○) suggested plastidic enzymes, (■) cytoplasmic marker enzymes, (□) suggested cytoplasmic enzyme, (△) cytosome enzyme.

Also factors others than the compartmention could influence the properties of enzyme efflux from the cells, *e.g.* the intracellular attachment of enzymes to membranes, the molecular weight, of the enzyme, respectively the molecular volume or the isoelectric point. A high molecular volume *e.g.* will certainly hamper the efflux of enzymes in comparison to enzymes with low molecular volumes. Therefore we tried a correction for at last these two parameters, by plotting the diffusion coefficient (which incorporates both parameters) as function of the efflux properties (Fig. 4). The diffusion coefficients of the enzymes of the glycerol cycle were not determined experimentally, but roughly estimated from a plot of known diffusion coefficients as function of the third root of the molecular weight of calibration proteins yielding approximately a straight calibration line (Fig. 5). If the molecular volume is the only variable in the relationship between efflux and diffusion coefficient only a single linear function should occur for all enzymes. This is obviously not the case (Fig. 4). If additional parameter determine the efflux properties, *e.g.* the compartmentation, two or more "lines" should be observed. In Fig. 4 values for the chloroplastic marker enzymes are connected to form one "line" and values for cytoplasmic marker enzymes are connected to form another "line". If treated in such a way, the value for the DHA oxireductase seems to belong to the "cytoplasmic line", whereas the values for the glycerol-3-phosphate dehydrogenase and the glycerol-3-P-phosphatase are closer to the "chloroplastic line". The value for acid phosphatase is significantly above the "chloroplastic line" for all reasonable molecular weights of this enzyme. Because of lack in information about the molecular weight the exact position cannot be determined. Nevertheless, the result may indicate a high stability of the cytosome envelope. On the other side the completely different volume of the cytosomes in comparison to the chloroplast and the cytoplasm, which have roughly equal volumes in *D. parva*, could also complicate the picture.

The view that the DHA oxidoreductase is localized in the cytoplasm and the glycerol-3-P dehydrogenase is localized in the chloroplasts of *Dunaliella* cells and partially or in total also the glycerol-3-P phosphatase agrees with recent results of Brown *et al.* [12] obtained with completely other techniques. It indicates that the chloroplast of *D. parva* is able to synthesize glycerol from precursors of photosynthesis or starch degradation, whereas the glycerol degradation to DHA (and to DHAP?) takes place in the cytoplasm (minimal hypothesis). The chloro-

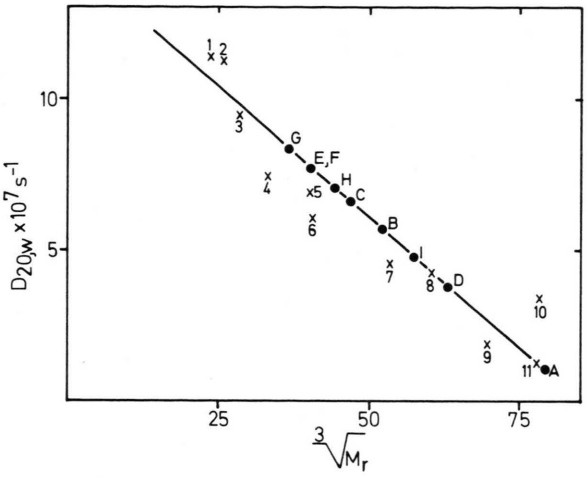

Fig. 5. Diffusion coefficients ($D_{20,w}$) of some calibration proteins (indicated by numbers) as function of the third root of the molecular weight (data from Lehninger [23]). Key: 1. cytochrome c (bovine heart); 2. myoglobin (horse heart); 3. chymotrypsin (bovine pancreas); 4. lactoglobulin (goat milk); 5. serum albumin (human); 6. hemoglobin (human); 7. aldolase; 8. catalase (horse liver); 9. urease (jack bean); 10. fibrinogen (human); 11. myosin (cod). The resulting approximate straight line was taken for rough estimation of $D_{20,w}$ of some *D. parva* enzymes of interest (as indicated by letters). Key (in brackets: source, assumed molecular weight and reference): A. RuBP carboxylase; (*Chlamydomonas*, 500000, [24]); B. GAP-DH; (*Scenedesmus*, 140000, [25]); C. Glu-6-P-DH; (*Spinacia*, 104000, [26]); D. PEP carboxylase; (*Dunaliella*, 250000, [27]; E. DHA oxidoreductase; (*Dunaliella*, 60000, [7]); F. DHA kinase; (*Dunaliella*, 65000, [8, 9]); G. Gly-3-P-DH; (*Saccharomyces*, 50000, [28]); H. Gly-3-P phosphatase; (*Dunaliella*, 86000, [6]); I. Pyruvate kinase; (*Saccharomyces*, 190000, [29]).

plast envelope is readily permeable for glycerol [30] and thus the latter can be uniformly distributed within the cell in a short time. If glycerol synthesis should take place exclusively in the chloroplasts (extended hypothesis) it is reasonable to assume that also the triggering of glycerol synthesis after hyperosmotic shock is localized in the chloroplast. However, this does not necessarily mean, that also the primary signal, which is presumably a chemical compound, is produced in the chloroplast. But its final effect is there.

If alternatively glycerol-3-P phosphatase should be present in both the cytoplasm and the chloroplast, part of the glycerol-3-P has to be transported through the chloroplast envelope. The phosphate translocator of the chloroplast envelope possesses a high capacity to exchange glycerol-3-P against phosphate [31] but the physiological significance of such a high capacity was not completely understood. In *Dunaliella* this translocator could be part of the glycerol cycle and thus be involved in the osmoregulation of this alga by exchanging plastidic glycerol-3-phosphate against cytoplasmic phosphate liberated by the action of glycerol-3-P phosphatase.

Acknowledgement

This investigation was supported by the Deutsche Forschungsgemeinschaft. The technical assistance of Mrs. G. Hartmann and of Mrs. D. Dehner is acknowledged.

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