DEAE-Dextran Induced Increase of Membrane Permeability and Inhibition of Photosynthesis in *Dunaliella parva*

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As a prerequisite for studies of the intracellular distribution of enzymes of the glycerol cycle in *Dunaliella parva*, the effect of the polycation DEAE-dextran on the permeability of membranes for various endogenous compounds and on photosynthesis was investigated.

DEAE-dextran induces an increase of the permeability of membranes for low and high molecular weight compounds: Under the influence of DEAE-dextran low molecular compounds of the cells diffuse more rapidly along the chemical concentration gradient into the medium than macromolecules. Furthermore enzymes of the cytoplasm do occur more rapidly in the outer medium than enzymes of the chloroplasts.

Photosynthetic CO_2 fixation is inhibited already at low DEAE-dextran concentrations. This indirect inhibition is due to an unspecific efflux of compounds of low molecular weight, such as inorganic cations, metabolites or nucleotides. At high DEAE-dextran concentrations photosynthetic electron transport is directly affected at the level of the thylakoids: Electron transport is inhibited between plastoquinone and photosystem I.

The efflux characteristics of marker enzymes of DEAE-dextran treated *D. parva* cells show that under optimal experimental conditions this technique may be a suitable tool to get information about the intracellular distribution of enzymes of unknown localization in the alga.

Introduction

Diethylaminoethyl dextran (DEAE-dextran) is a synthetic polycation with a high positive charge density. The diethylaminoethyl groups of this glucose polymer bind to the negatively charged groups of phospholipids and/or proteins of biomembranes. This electrochemically binding gradually increases the native permeability of the membranes, leading in the final state to a complete lysis of the cell [1 – 3]. The reason for the increase of the membrane permeability is not exactly known. Charge neutralization may be one reason. Also changes in the secondary and tertiary structure of transmembrane proteins may be discussed as well as changes in the fluidity of membrane components by DEAE-dextran induced clustering. It is known, however, that thermal energy is required to cause irreversible changes in the membrane permeability and not just the adsorption of DEAE-dextran to the membrane.

Nevertheless, if carefully handled, the DEAE-dextran induced increase of the native permeability can be used for a differential extraction of cells: At the beginning of the incubation membranes are essentially impermeable to DEAE-dextran because of its large molecular weight (500 000 dalton) and its high charge density of 700 charges per molecule. Thus the first site of DEAE-dextran binding is limited to the plasmalemma. However, when the plasmalemma becomes permeable to DEAE-dextran after some time in its presence, then DEAE-dextran may enter the cytosol and bind to internal membranes. Thus compounds of the cytoplasm will diffuse into the extracellular medium according to their chemical concentration gradient and will do this, at least initially, more rapidly than compounds from compartments which are surrounded by additional membranes.

This principle has been used in our studies to investigate the intracellular distribution of the enzymes of the so called glycerol cycle in the halotolerant, cell-wall less unicellular green alga *Dunaliella parva*. The reason for using such an indirect approach is that with microalgae, especially with algae containing one big chloroplast per cell, the well es-

Abbreviations: BQ, *para*-benzoquinone; Chl, chlorophyll; DCMU, 3(3,4-dichlorophenyl)-1,1-dimethylurea; DCPIP, 2,6-dichlorophenyl indophenol; DEAE-dextran, diethylaminoethyl dextran; DTE, dithioerythritol; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenyl hydrazone; FeCy, $K_3[FeIII(CN_6)]$; GAP-DH, glyceraldehyde-3-phosphate dehydrogenase; G-6-P-DH, glucose-6-phosphate dehydrogenase; HEPES, 2[4-(2-hydroxyethyl)-piperazine-(1)]-ethensulfonic acid; MV, methylviologen; PGA, 3-phosphoglyceric acid; PEP, phosphoenolpyruvate, RuBP, ribulose- 1,5-bisphosphate.

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tablished methods for the aqueous or nonaqueous isolation of intact chloroplasts and other organelles are not practicable.

In this paper we describe first the basic phenomena of DEAE-dextran induced changes in the permeability of *D. parva* membranes and the consequences of these changes for photosynthetic reactions. We then describe the optimal conditions for DEAE-dextran dependent differential extraction of marker enzymes to demonstrate the principal applicability of the method for the purpose of intracellular localization of enzymes in *D. parva.* In the subsequent paper [4] we apply the DEAE-dextran technique specifically to get information about the intracellular distribution of the enzymes of the glycerol cycle in *D. parva.*

Materials and Methods

Material

Algal culture

Dunaliella parva Lerche was cultured in an inorganic culture medium under conditions as described elsewhere [10]. Algae were adapted to 0.75 M NaCl. Algae were harvested by centrifugation ($1500 \times g$, 5 min, 20 °C and resuspended, if not otherwise mentioned in a medium containing 1.5 M sorbitol and 20 mM Tris/HEPES (pH 7.6). The chlorophyll content was calculated from the absorption at 645 and 663 nm [11] of algal extracts in 80% acetone and protein determined according to the method of Lowry *et al.* [12].

Chemicals

DEAE-dextran was purchased from Pharmacia Fine Chemicals, Uppsala, (Sweden), [^{14}C]bicarbonate from Amersham & Buchler, Braunschweig (Germany) and fine chemicals and enzymes from Boehringer, Mannheim (Germany).

Methods

Photosynthetic reactions

Photosynthetic O_2 evolution and reduction of MV were measured polarographically with a Clark-type oxygen electrode under saturating, incandescent white light (30 °C, pH 7.6), whereas fixation of $^{14}CO_2$ was carried out in 10 ml glass tubes in an illuminated thermostat (pH 7.6; 30 °C). The reduction of BQ and $K_3Fe(CN_6)$ were followed spectrophotometrically from the change of the extinction at 400 nm (saturating red light, 20 °C, pH 7.6). Mg^{2+} and K^+ contents were measured by atomic absorption spectroscopy. Further details can be seen from the legends to figures and tables.

DEAE-dextan treatment

DEAE-dextran was applied in two different ways. To investigate the effect of DEAE-dextran on photosynthetic reactions and on the permeability of membranes for cations, endogenous metabolites or external substrates, DEAE-dextran was added at appropriate concentrations to the algal suspension at 30 °C and the mixture incubated at 30 °C for a further period of 5 or 10 min. The process under investigation was measured in the presence of DEAE-dextran. For differential enzyme extraction a more sophisticated treatment was applied. DEAE-dextran was added to the algal suspension under rapid mixing at 0 °C over 1 min (adsorption period, uniform adsorption of the molecules at the plasmalemma). Then mixtures were incubated at 30 °C for varying periods (time of lysis, the irreversible membrane damages occur). The raction with DEAE-dextran was stopped after the desired time of lysis by centrifugation ($5000 \times g$, 10 min, 0 °C). The pellet was discarded and supernatants stored on ice. Aliquots of the 1 ml supernantant (usually between 20 and 100 µl, for phosphatase assays 0.5 ml) were taken for the enzyme assays. Thereby a dilution of the original DEAE-dextran concentration of at least 10 fold was achieved. It was ascertained that the DEAE-dextran still present in the samples did not affect the enzyme assays *per se.*

Enzyme assays

D-glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49) and D-glyceraldehyde-3-phosphate dehydrogenase (E.C. 1.2.1.13) were measured spectrophotometrically [13]: The former reaction was measured directly, whereas the latter was coupled to the conversion of glycerate-3-phosphate to glycerate-1,3-bisphosphate by the 3-phosphoglycerate kinase (E.C. 2.7.2.3). To distinguish between the plastid and cytoplasmic form of the G-6-PDH, the reaction was carried out in the presence and absence of 50 mM DTE [9]. The G-6-PDH activity in the presence of DTE was considered to be due solely to the cytoplas-

mic form, whereas the difference between the total activity and the activity in the presence of DTE was assumed to reflect the plastid form of the enzyme.

RuBP carboxylase activity (E.C. 4.1.1.39) was measured by the method of Kaiser and Heber [14], starting with ribose-5-phosphate and ATP as substrates. PEP carboxylase (E.C. 4.1.1.31) was measured by the formation of [^{14}C]oxaloacetate from $^{14}CO_2$ and PEP [15]. The [^{14}C]oxaloacetate was stabilized by the addition of either 2,4-dinitrophenylhydrazine or malate dehydrogenase and NADH. Pyruvate kinase (E.C. 2.7.1.4) was assayed by coupling the reaction to the NADH-dependent conversion of pyruvate to lactate by the lactate dehydrogenase at pH 6.0 [13]. Fumarase (E.C. 4.2.1.2) was determined by measuring the increase in adsorption of fumarate at 240 nm (pH 7.5, 50 mM malate) [13].

Volume measurements

Cell volume was measured with a Coulter Counter (Model ZBI) connected to a channelanalyzer (Model C-1000). An orifice of 100 µm diameter was used. Packed cell volume was determined by centrifugation of cells (1500 × *g*, 20 min, 20 °C) in calibrated centrifuge tubes.

Definitions

To compare the effect of DEAE-dextran in different experiments and with different reactions we introduced the parameters T_{50} 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 × 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).

Results and Discussion

The effect of DEAE-dextran on photosynthetic reactions

Photosynthetic CO_2-fixation of *D. parva* cells is inhibited by DEAE-dextran only when the high salt medium is replaced by an equiosmolar organic medium essentially free of inorganic cations and anions (Fig. 1). The reason for this may be twofold: 1) The electrochemical competition of the cations of

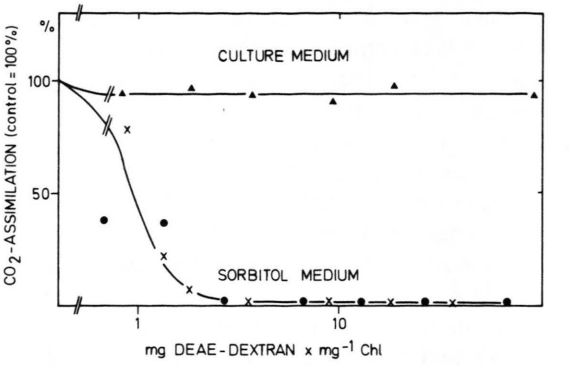

Fig. 1. The effect of DEAE-dextran on photosynthetic $^{14}CO_2$-fixation of *D. parva* cells suspended in normal culture medium containing 0.75 M NaCl (△) or in 1.5 M sorbitol medium (●, × two different experiments). Experimental conditions: 5 min preillumination in the presence of DEAE-dextran, 5 min incorporation of [^{14}C]bicarbonate (3 mM) in the presence of DEAE-dextran (30 °C, pH 7.6). Photosynthetic rates were 150 µmol CO_2 × mg^{-1} Chl × h^{-1} in the control with the salt medium, but 60 µmol CO_2 × mg^{-1} × h^{-1} in the control with the sorbitol medium.

the medium with the positively charged diethylaminoethyl groups for the negatively charged groups of the plasmalemma. 2) Neutralization of the cationic DEAE-dextran groups by the anions of the medium. In order to ensure reproducible effects of DEAE-dextran, all further experiments were carried out with algae suspended in a salt free 1.5 M sorbitol medium, buffered with TRIS/HEPES to pH 7.6. The response of photosynthetic O_2-evolution to DEAE-dextran was rapid (Fig. 2): Inhibition occured within few seconds after the addition of DEAE-dextran. It was still incomplete after 1 min, but reached a maximal effect between 2 and 5 min. This rapid effect of a rather nonpermeant compound indicates that inhibition of photosynthetic O_2-evolution must be an indirect one.

Photosystem II dependent reactions such as the reduction of BQ were highly resistant to the action of DEAE-dextran (Fig. 3), indicating that no site in the electron transport chain between the water splitting apparatus and plastoquinone was directly or indirectly affected. It is difficult to carry out a corresponding measurement for reactions requiring photosystem I only or the combined actions of both photosystems, because most of the commonly used electron donors and acceptors are poorly permeant for the plasmalemma. *E.g.* there was no reduction of MV with water as electron donor in untreated *D. parva* cells (Fig. 3A, trace 2), at least not in the tested

time range. However, with increasing DEAE-dextran concentrations a considerable, DCMU-sensitive MV-reduction was observed. This indicates that the plasmalemma becomes permeable for MV under the influence of DEAE-dextran. At very high DEAE-dextran concentrations the reaction was inhibited again, indicating an inhibition site in the electron transport chain between the water splitting apparatus and the site of MV-reduction. Together with the resistance of BQ-reduction against DEAE-dextran these results imply an inhibition site between plastoquinone and the site of MV-reduction. To elucidate this point, we measured the effect of DEAE-dextran on the MV-reduction with both water and the couple ascorbate, DCPIP as electron donors by *D. parva* thylakoids *in vitro* (Fig. 4). The photosystem I dependent electron transport from the couple ascorbate, DCPIP to MV is resistant against DEAE-dextran over a wide range of concentrations. The slight stimulation of the reaction may reflect improved access of the reactants. In contrast to this reaction, the MV-reduction with water as electron donor is readily inhibited. These results are in agreement with those of other studies [16] and suggest a DEAE-dextran inhibition site between plastoquinone and photosystem I. Such an assumption is also in agreement with the proposal that this is a rather unspecific effect of polycations: A great number of

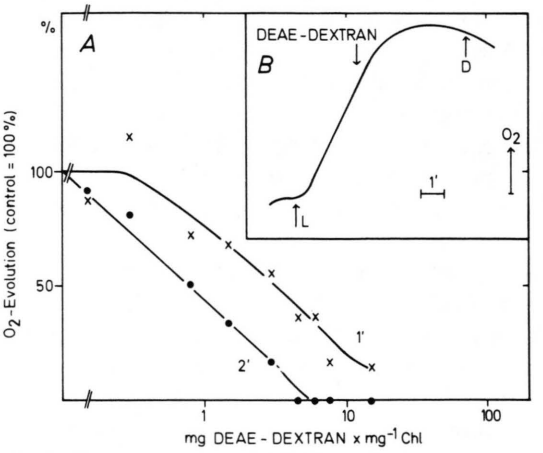

Fig. 2. Time dependent inhibition of photosynthetic O_2-evolution by DEAE-dextran in *D. parva* cells: The reaction medium contained 1.5 M sorbitol, 20 mM TRIS/HEPES (pH 7.6) and 3 mM $KHCO_3$. A: Inhibition after 1 min (×) and 2 min (●) incubation with DEAE-dextran . B: Recorder trace of the oxygen electrode demonstrating the rapid inhibition of photosynthesis by DEAE-dextran (L, light; D, dark; the arrow indicates the addition of 0.8 mg DEAE-dextran × mg^{-1} Chl).

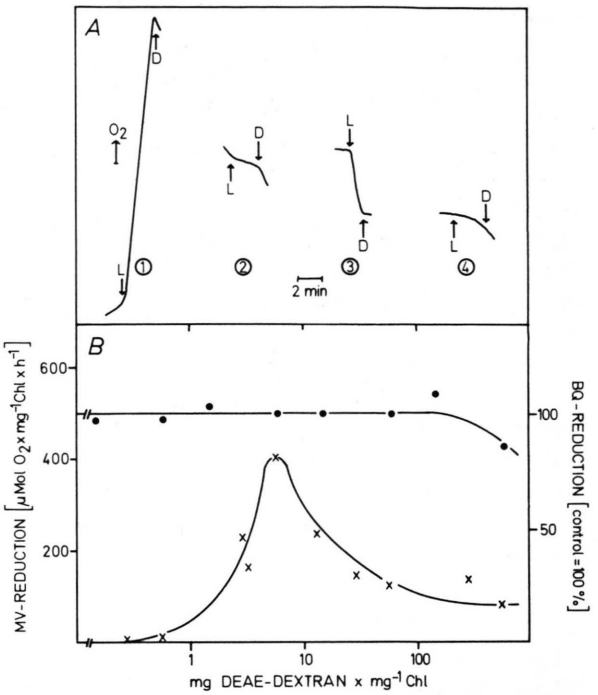

Fig. 3. The effect of DEAE-dextran on photosynthetic reduction of BQ (○) and MV (×) and *D. parva* cells (B). The reaction mixture contained 1.5 M sorbitol, 20 mM TRIS/HEPES (pH 7.6) and in addition for BQ reduction 1 mM BQ and $K_3[FeIII(CN)_6]$ and 20 mM NH_4Cl, and for MV reduction 9×10^{-6} M MV, 9×10^{-6} MFCCP and 2×10^{-4} M KCN. A: Recorder traces demonstrating photosynthetic O_2 evolution of control cells (1). Addition of FCCP, MV and KCN strongly inhibits O_2 evolution without introducing O_2-uptake (2). Only when the permeability of the plasmalemma for MV is increased by DEAE-dextran, a light-induced O_2-uptake can be observed (3), which is sensitive to 5×10^{-6} M DCMU. Preincubation with DEAE-dextran: 5 min. L: light, D: dark.

different native and artificial polycations exhibit similar effects [5–8]. The precise inhibition site is believed to be at the level of plastocyanin (8).

D_{50}-values for the various photosynthetic reactions (Table I) clearly demonstrate that the DEAE-dextran inhibition site between plastoquinone and photosystem I cannot be the reason for the inhibition of photosynthetic CO_2-fixation, because of the large differences between the D_{50}-values for inhibition of noncyclic electron transport and CO_2-fixation and the greater sensitivity of the latter reaction. Furthermore the results of the experiments with thylakoids demonstrate that the inhibition of noncyclic electron transport is due to a direct effect of DEAE-dextran, whereas it was shown above that the inhibition of CO_2-fixation must be due to an indirect effect.

Table I. D_{50} values for various photosynthetic reactions of intact cells or thylakoids from *D. parva* (average of 2–3 experiments). DEAE-dextran was always present during the assay.

Photosynthetic reaction	System	D_{50} [mg DEAE-dextran × mg^{-1} Chlorophyll]
Photosynthetic $^{14}CO_2$-fixation or O_2-evolution	intact cells	1
Electron transport from water to ferricyanide	intact cells	≫ 60
Electron transport from water to BQ	intact cells	> 100
Electron transport from water to MV	intact cells	< 20
Electron transport from water to MV	thylakoids	13
Electron transport from ascorbate, DCPIP to MV	thylakoids	> 500

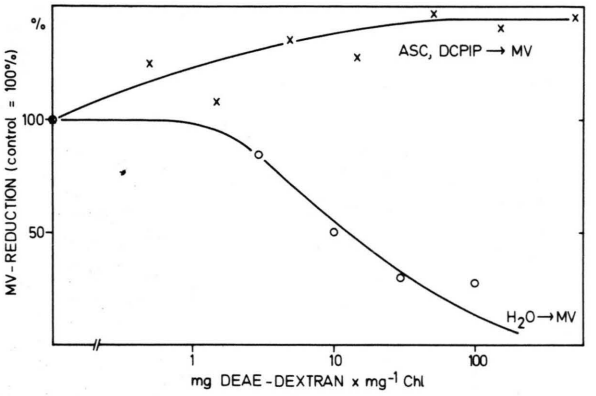

Fig. 4. The effect of DEAE-dextran on photosynthetic reduction of MV by thylakoids from *D. parva* with H_2O (○) or the ascorbate, DCPIP couple (×) as electron donors. The reaction mixture contained 4.4 mM NaCl, 4 mM $MgCl_2$, 100 mM TRIS/HEPES (pH 7.6), 9×10^{-6} M MV, 9×10^{-6} M FCCP, 2×10^{-4} M KCN and in the latter case 5×10^{-3} M ascorbate, 10^{-5} M DCPIP and 5×10^{-6} M DCMU. DEAE-dextran preincubation: 5 min (dark), then illumination in the presence of DEAE-dextran. Thylakoids were obtained from intact cells by osmotic rupture in 3 mM TRIS/HEPES (pH 7.6) (0 °C, 10 min) and centrifuging at $5000 \times g$ for 10 min.

The effect of DEAE-dextran on the permeability of membranes

The effect of DEAE-dextran on the permeability of the plasmalemma and other membranes was tested by measuring the influence of this compound on the following reactions: 1) Efflux of endogenous compounds, respectively fractions of compounds from the cell into the medium, 2) the influx of artificial compounds from the medium into the cell, 3) the packed cell volume, and 4) the behaviour of the cells as perfect osmometers.

DEAE-dextran induces in the standard sorbitol medium a significant decrease of the packed cell volume (Fig. 5). This indicates a decrease of the osmolarity of the cell sap caused by an DEAE-dextran induced increase of the permeability of the plasmalemma: At low and mediate DEAE-dextran concentrations the endogenous osmoticum glycerol (relative low molecular weight) leaks more rapidly into the medium than the external sorbitol (relative high molecular weight) can enter the cell. Only at higher DEAE-dextran concentrations both efflux of glycerol and influx of sorbitol can balance each other resulting in a more or less unchanged volume. This explanation is confirmed also by the similarity of the efflux kinetics of a ^{14}C-labelled metabolite fraction,

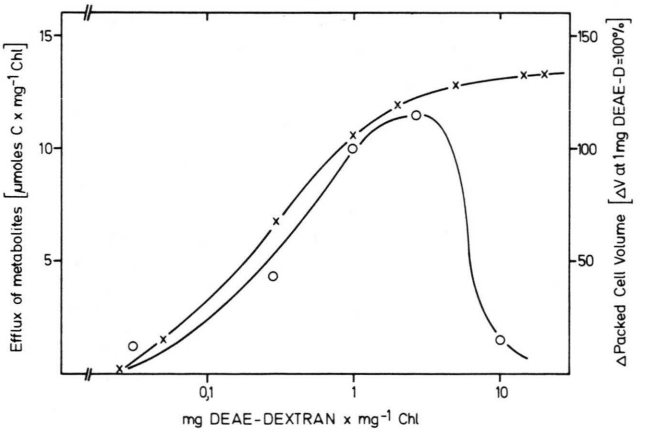

Fig. 5. The effect of DEAE-dextran concentrations on the efflux of a ^{14}C-labelled metabolite fraction (×) from *D. parva* cells (consisting mainly of glycerol) (compare Table II) and on packed cell volume (○). DEAE-dextran incubation time: 10 min at 30 °C. The average packed cell volume was 279 µl × mg^{-1} Chl, the minimal volume (= maximal Δ volume) 152 µl × mg^{-1} Chl.

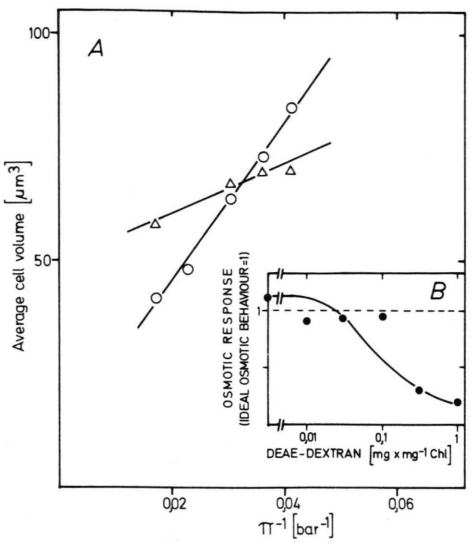

Fig. 6. Average cell volume of *D. parva* cells as function of the reciprocal osmotic potential of different NaCl-solutions (Boyle-van't Hoff plot) (A). Control (○), +1 mg DEAE × mg^{-1} chlorophyll (△). In the inset B the results of 4 experiments are summarized. The ideal osmotic behaviour (that is the slope of the control in A) was set to unity and the average deviation of DEAE-dextran treated cells calculated (compare Table II).

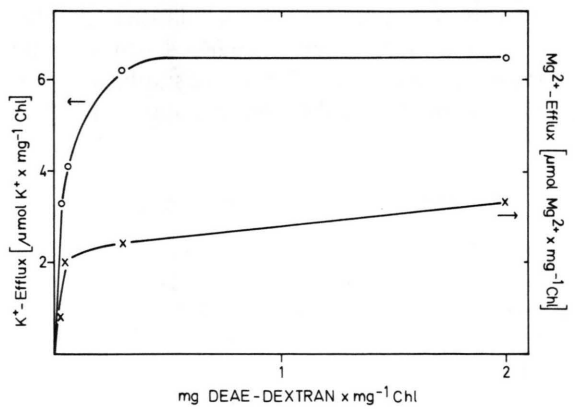

Fig. 7. The effect of DEAE-dextran concentrations on the efflux of K^+ (○) and Mg^{2+} (×) from *D. parva*. Cells were treated for 10 min with DEAE-dextran, centrifuged and the supernatant analyzed. Maximal K^+ efflux corresponds to a decrease of internal K^+ concentration of about 50 mM. The corresponding decrease in Mg^{2+} concentrations is about 25 mM.

consisting mainly of glycerol (Fig. 5). DEAE-dextran impairs also the ability of cells to react as perfect osmometers according to the Boyle-van't Hoff relationship if transfered into salt media of different osmolarity (Fig. 6). Since this reaction in contrast to the former was measured after DEAE-dextran treatment in the absence of this compound, the result demonstrates that the changes in the permeability of the plasmalemma are largely irreversible.

About half of the total K^+ content of the cells diffuses into the outer medium under the influence of DEAE-dextran, corresponding to an internal change in the concentration of about 50 mM (Fig. 7). In comparison to the monovalent K^+-efflux the efflux of the divalent Mg^{2+} is surprisingly high (Fig. 7). About 50% of the total ionic Mg^{2+} content of the cell diffuses out of the cell under the influence of DEAE-dextran, corresponding to an internal change of about 25 mM Mg^{2+}. Here it must be considered that the growth medium contains 44 mM Mg^{2+} and that in its natural habitat, the Dead Sea, *D. parva* tolerates Mg^{2+} concentrations higher than the NaCl concentrations [18].

In Fig. 8 the time dependent efflux of proteins from the cell into the medium is shown and in Fig. 9 the influx of ferricyanide from the medium into the chloroplasts (measured via the photosynthetic reduction of ferricyanide). Obviously the inhibition of CO_2-fixation must be caused by the efflux of smaller molecules (inorganic cations, metabolites, nucleotides, cofactors) rather than by the efflux of macromolecules such as proteins. This follows also from the almost tenfold higher D_{50}-value for the efflux of plastidal enzymes in comparison with the D_{50}-value for inhibition of photosynthesis (Table II).

Under the assumption that *D. parva* cells cultured in a medium with 0.75 M NaCl have a surface of about 600 $cm^2 \times mg^{-1}$ chlorophyll and that the major

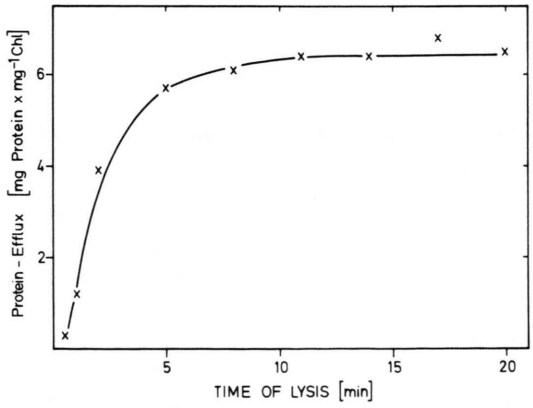

Fig. 8. The effect of DEAE-dextran (30 mg × mg^{-1} Chl) on the efflux of protein from *D. parva* cells into the medium (compare Fig. 5).

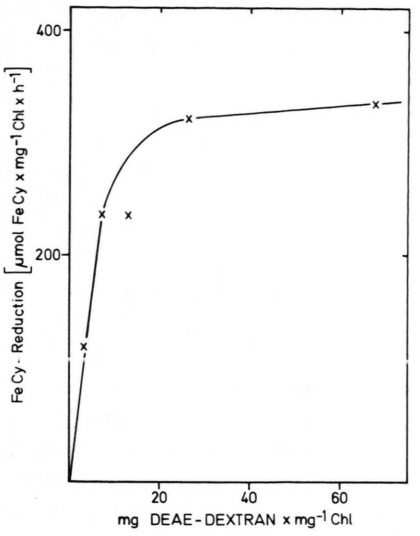

Fig. 9. The effect of DEAE-dextran (10 min at 30 °C) on photosynthetic reduction of K_3 [FeIII$(CN)_6$] in *D. parva* cells. This reaction is also an indicator for minimal influx of this compound from the medium into the chloroplast. The reaction medium contained 1.5 M sorbitol, 20 mM TRIS/HEPES (pH 7.6), 2 mM K_3[FeIII$(CN)_6$] and 20 mM NH_4Cl.

part of added DEAE-dextran molecules (high affinity) is adsorbed at the plasmalemma, it is roughly estimated that a D_{50}-value of 0.04 mg DEAE-dextran × mg chlorophyll for cation release (Table 2) corresponds to a maximal adsorption of about 8×10^{10} molecules × cm^{-2}. From the charge density of 700 positive charges per molecule it follows that maximal 56×10^{12} charges per cm^{-2} would be available to cause a 50% induction of cation release. However, it is unlikely that all charges of an adsorbed DEAE-dextran molecule bind electrochemically to negatively charged groups of the outer membrane. Thus it must be concluded that changes in the permeability of the plasmalemma are induced already by the adsorption of much less than 56×10^{12} positive charges per cm^{-2}, but probably more than 8×10^{10} charges × cm^{-2}. These values have to be compared with charge densitiy of plant membranes, *e.g.* thylakoids of about $0.5-1.6 \times 10^{13}$ charges per cm^2 [19].

Although it was not the aim of this study to obtain a preparation of intact, functional chloroplasts from *D. parva* cells by DEAE-dextran treatment, some res-

Table II. D_{50}-values for various DEAE-dextran induced effluxes and influxes in *D. parva* cells and of changes in the permeability of the plasmalemma. Efflux was measured after 10 min incubation with DEAE-dextran at 30 °C. Values for the influx of MV and ferricyanide are rough estimations from the studies of photosynthetic reduction of these compounds (compare Figs. 3 and 7). To measure the efflux of the ^{14}C-labelled metabolite fraction*, *D. parva* cells were allowed to photosynthetize in the presence of ^{14}C-labelled bicarbonate for 3 h, then centrifuged and resuspended in fresh medium. The efflux of the labelled fraction was investigated after 10 min incubation with DEAE-dextran in the dark. A major part of this fraction must be assumed to be glycerol. The osmotic response against NaCl was measured with a Coulter Counter***. After DEAE-dextran treatment cells were transferred to different NaCl-solutions and after 2 min the average cell volume measured. The ideal osmotic behaviour was set to 1. The change in packed cell volume was measured in the standard sorbitol medium. The value indicated with ** reflects average value for three different chloroplastic enzymes and was taken from Table III.

Process	D_{50} [mg DEAE-dextran × mg^{-1} chlorophyll]
Efflux of K^+ into the medium	0.04
Efflux of Mg^{2+} into the medium	0.04
Osmotic response against NaCl***	0.10
Efflux of a ^{14}C-labelled metabolite fraction into the medium*	0.30
Decrease in packed cell volume	0.40
Photosynthetic CO^2 assimilation	1.00
Influx of MV from the medium into the chloroplast of the cell	> 3.00
Influx of K_3 [Fe III $(CN)_6$] from the medium into the chloroplast of the cell	5–7
Efflux of chloroplastic enzymes from the chloroplasts into the medium**	9.00
Efflux of an endogenous soluble protein fraction from the cells into the medium	8–10

toration experiments were carried out. After inhibition of CO_2-fixation by DEAE-dextran treatment, reduction of PGA could be partially restored by adding PGA, Mg^{2+}, NADP and ATP (not shown). This demonstrates 1) that the plasmalemma and the chloroplast envelope became permeable for these compounds under the influence of DEAE-dextran, 2) that NADP reduction is still functioning in the presence of DEAE-dextran provided the efflux of internal NADP is counterbalanced by the addition of proper concentration of external NADP. Furthermore these results seem to indicate that phosphorylation is inhibited readily by DEAE-dextran and therefor ATP had to be added in order to maintain PGA reduction. Thus one important effect of DEAE-dextran treatment may be uncoupling. It should be mentioned that possible uncoupling effects of DEAE-dextran in Figs. 3, 4, and 9 were masked by the presence of uncouplers in the reaction medium. Fig. 9 demonstrates that ferricyanide which is a nonpermeant solute for both the plasmalemma and the chloroplast in their native states, becomes a permeant solute under the influence of DEAE-dextran. Thus it can be readily reduced in the light by the electron transport chain in the thylakoids. However, different from the reduction of MV *in vivo* (Fig. 3), ferricyanide reduction is not inhibited at high DEAE- dextran concentrations. This agrees with the assumption that photosynthetic electron transport is inhibited by DEAE-dextran between plastoquinone and photosystem I. In summary our permeability studies suggest that DEAE-dextran increases in an unspecific way the permeability of membranes of *D. parva* cells for a great number of different solutes. However, compounds of low molecular weight diffuse more rapidly into the medium under the influence of DEAE-dextran than compounds of high molecular weight. DEAE-dextran treatment seems to be an unsuitable method for the isolation of intact, functionable chloroplasts. However, this treatment may still be a suitable method for differential extraction of macromolecules from the compartments of the cell.

Requirement of DEAE-dextran induced enzyme efflux

The experimental conditions for an optimal efflux of enzymes were checked with G-6-P dehydrogenase as test enzyme. The efflux of this enzyme is virtually independent on time during the adsorption period at 0 °C, but significantly dependent on the time of lysis at 30 °C (Fig. 10). Optimal enzyme efflux was found at a pH of 7.6 and a temperature of 30 °C (Fig. 11). The enzyme efflux expressed on a chlorophyll basis was independent of the algal concentration during DEAE-dextran treatment, provided the ratio DEAE-dextran to the amount of algae was kept constant (not shown).

Thus the effectivness of DEAE-dextran depends strongly on the ratio of DEAE-dextran per unit membrane area. In addition it was shown that there are only minimal effects of DEAE-dextran on enzyme activities during the *in vitro* enzyme assay, at least in the range of applied concentrations. These results are in general agreement with those of other authors [2, 17]. In all further experiments standard conditions for enzyme efflux were 1 min adsorption at 0 °C, a temperature of 30 °C during lysis and a pH of 7.6 during both the adsorption and the lysis

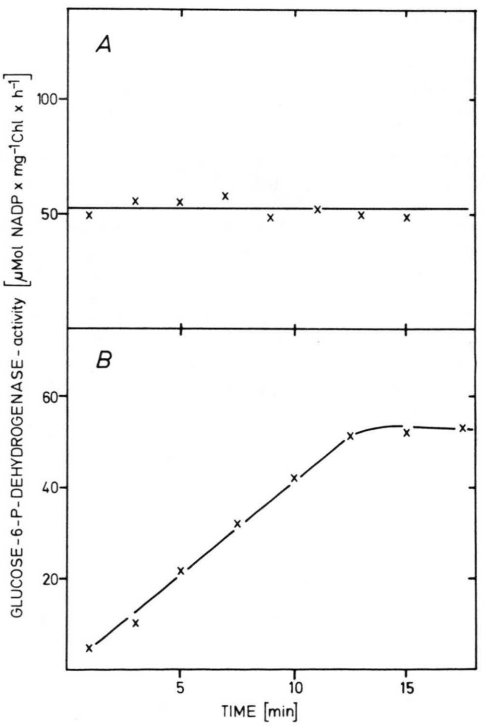

Fig. 10. The effect of DEAE-dextran (20 mg × mg^{-1} Chl) on the efflux of the G-6-P dehydrogenase. In experiment A the time of adsorption at 0 °C was varied at a constant time of lysis (10 min), whereas in B the time of lysis at 30 °C was varied at a constant time of adsorption at 0 °C (1 min). The reaction mixture during the enzyme assay contained 30 mM HEPES/NaOH (pH 7.6), 6.7 mM $MgCl_2$, 0.3 mM NADP and 1 mM G-6-P.

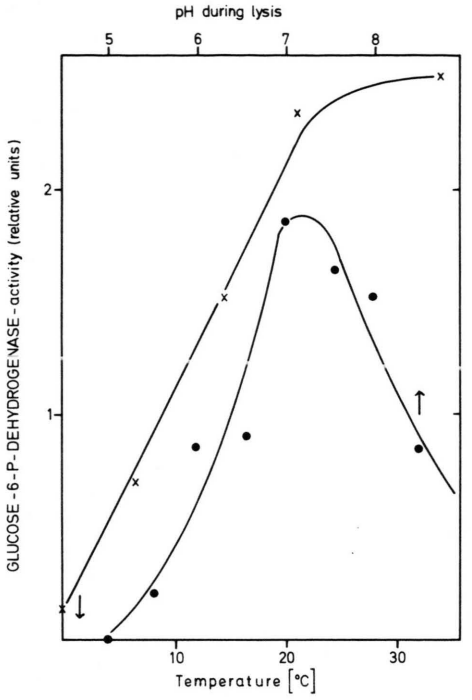

Fig. 11. The effect of temperature during lysis (●, lower abscissa) and of pH (×, upper abscissa) on DEAE-dextran induced efflux of the G-6-P dehydrogenase from *D. parva* cells. Compare Fig. 8.

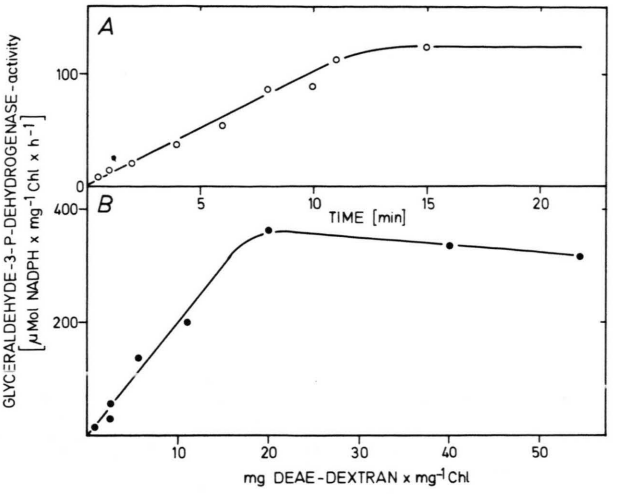

Fig. 12. The effect of time of lysis (○, A) and of DEAE-dextran concentration (●, B) on the efflux of the plastidal marker enzyme GAP_{NADP}-dehydrogenase from *D. parva* cells into the medium. DEAE-dextran concentration in A 30 mg × mg^{-1} Chl, lysis time in B 10 min. The reaction mixture contained 30 mM HEPES/NaOH (pH 7.6), 0.45 mM EDTA, 6.7 mM $MgCl_2$, 0.2 mM NADPH, 18 µg 3-phosphoglycerate kinase, 3.3 mM PGA and 3.3 mM ATP.

period. Incubation time and DEAE-dextran concentration were varied as required.

Differential extraction of marker enzymes

In order to prove the applicability of the DEAE-dextran technique for the analysis of intracellular distribution of enzymes in *D. parva*, the efflux of enzymes of known origin, so called marker enzymes were measured. The number of such enzymes is limited, especially if soluble and not membrane bound enzymes are required. Fig. 12 shows the efflux of the plastid marker enzyme GAP dehydrogenase NADP as function of the DEAE-dextran added (B) and the time of lysis (A). From such curves T_{50} and D_{50}-values were calculated. Since a certain scattering of results does occur from experiment to experiment it is of special interest of measure in one experiment the efflux of two different marker enzymes. In Fig. 13 the efflux of the plastid marker enzyme RuBP-carboxylase is compared with that of the cytoplasmic marker enzyme PEP-carboxylase. The efflux of the

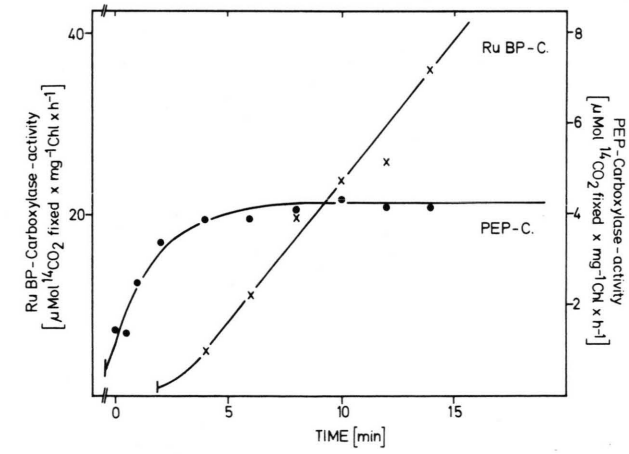

Fig. 13. The effect of DEAE-dextran (30 mg × mg^{-1} Chl) on the efflux of plastidal marker enzyme RuBP-carboxylase (×) and on the cytoplasmatic marker enzyme PEP-carboxylase (●) from *D. parva* cells into the medium. The reaction mixture for the RuBP-carboxylase assay contained 25 mM HEPES/KOH (pH 8.0), 20 mM $MgCl_2$, 15 mM $KHCO_3$ (labelled with $^{14}CO_2$), 2 mM ribose-5-phosphate, 5 mM ATP and 5 mM DTE. The PEP-carboxylase contained 20 mM HEPES/KOH (pH 7.4) 1 mM $MgCl_2$, 1 mM $KHCO_3$ (labelled with $^{14}CO_2$), 10 mM DTE and 1 mM PEP.

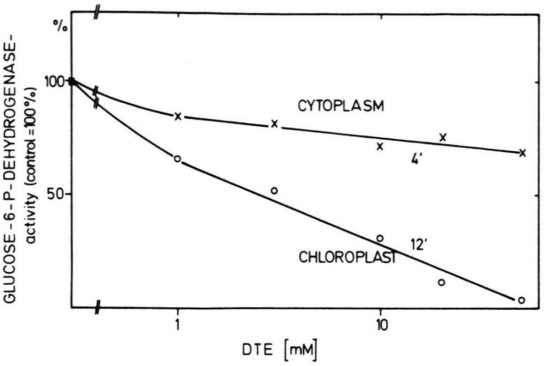

Fig. 14. The effect of DEAE-dextran (30 mg × mg^{-1} Chl) on the efflux of the G-6-P dehydrogenase from *D. parva* cells into the medium (for details see Fig. 8). The activity of the enzyme in the presence of 50 mM DTE (×) is assumed to reflect the activity of the cytoplasmatic form of the enzyme, whereas the difference of the activity in the absence and presence of 50 mM DTE is assumed to reflect the activity of the plastid form of the G-6-P dehydrogenase.

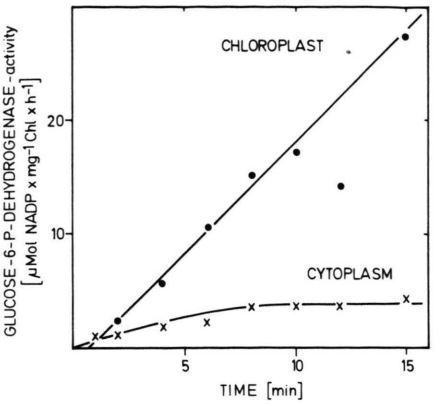

Fig. 15. The effect of DTE on the DEAE-dextran induced efflux of the G-6-P dehydrogenase from *D. parva* cells into the medium (compare Fig. 8 and 12). (×) lysis time 4 min, (○) lysis time 12 min. DEAE-dextran concentration 30 mg × mg^{-1} Chl).

latter is already saturated in this experiment when the efflux of the former is still continously increasing with time. Similar results are obtained with the G-6-P dehydrogenase (Fig. 14). This enzyme can be used as a plastid marker enzyme and as a cytoplasmic marker enzyme as well, because the former is inhibited by thiol reagents whereas the latter is not [9]: The activity of the G-6-P dehydrogenase is less inhibited by DTE in short time extracts from *D. parva* cells which should contain relatively more of the cytosolic form of the enzyme than in long time extracts which should contain more of the plastid form of the enzyme (Fig. 15).

Table III summarizes T_{50} and D_{50}-values for the tested marker enzymes. Clearly T_{50} and D_{50}-values of the tested cytoplasmic enzymes are lower than those of plastidal enzymes. We tried also to measure the efflux of fumarase from the mitochondria of *D. parva* cells, but were unsuccessful, probably because of low activity of this soluble mitochondrial enzyme (but compare 2). The efflux of enzymes from *D. parva* cells does not depend exclusively on the intracellular localization of the enzymes, but may be additionally influenced by factors such as the molecular weight, the molecular volume and the isoelectric point of the enzymes. In the subsequent paper a par-

Table III. D_{50} and T_{50} values for the DEAE-dextran induced efflux of marker enzymes from *D. parva* cells into the medium. Average of 3–7 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	Marker for	D_{50} [mg DEAE-dextran × mg^{-1} Chl]	T_{50} [min]	$\frac{\frac{D_{50\text{enzyme}}}{D_{50\text{RuBP-C}}} + \frac{T_{50\text{enzyme}}}{T_{50\text{RuBP-C}}}}{2} \times 1$
RuPB carboxylase	chloroplasts	13	8	100
GAP dehydrogenase $_{NADP}$	chloroplasts	7.13	5.5	63
Glu-6-P dehydrogenase$_{chl}$	chloroplasts	6.5	6.0	63
Average of the tested chloroplastic marker enzymes	chloroplasts	8.9	6.5	75
PEP carboxylase	cytoplasm	6.7	1.0	32
Glu-6-P dehydrogenase$_{cyt}$	cytoplasm	2.5	4.5	37
Pyruvate kinase	cytoplasm	1.4	2.2	20
Average of the tested cytoplasmic marker enzymes	cytoplasm	3.2	2.6	30

ameter is introduced, which corrects for some of these different properties of the enzymes [4]. These corrections do not dissolve the observed differences in the T_{50} and D_{50}-values.

Summarizing conclusions

In summary the results show that it should be possible by the aid of the DEAE-dextran differential extraction technique to distinguish between enzymes localized in the cytoplasm of *D. parva* cells and enzymes localized organelles which are surrounded by additional membranes [*cf.* 16, 17], *e.g.* such as the chloroplasts. However, it is difficult to distinguish *e.g.* between enzymes from chloroplasts and enzymes of mitochondria. If this is required, more sophisticated method have to be applied [17]. In a subsequent paper we apply this technique to investigate the intracellular distribution of enzymes of the glycerol cycle in *D. parva* [4]. Finally it must be mentioned that this technique can be applied only to cells lacking a cell wall, such as protoplasts [1] or naked flagellates.

Acknowledgement

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