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Isolation of Dihydroxyacetone Phosphate Reductase from *Dunaliella* Chloroplasts and Comparison with Isozymes from Spinach Leaves[1]

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ROBERT GEE, ARUN GOYAL, DENNIS GERBER, R. U. BYERRUM*, AND N. E. TOLBERT
Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824

ABSTRACT

A dihydroxyacetone phosphate (DHAP) reductase has been isolated in 50% yield from *Dunaliella tertiolecta* by rapid chromatography on diethylaminoethyl cellulose. The activity was located in the chloroplasts. The enzyme was cold labile, but if stored with 2 molar glycerol, most of the activity was restored at 30°C after 20 minutes. The spinach (*Spinacia oleracea* L.) reductase isoforms were not activated by heat treatment. Whereas the spinach chloroplast DHAP reductase isoform was stimulated by leaf thioredoxin, the enzyme from *Dunaliella* was stimulated by reduced *Escherichia coli* thioredoxin. The reductase from *Dunaliella* was insensitive to surfactants, whereas the higher plant reductases were completely inhibited by traces of detergents. The partially purified, cold-inactivated reductase from *Dunaliella* was reactivated and stimulated by 25 millimolar Mg^{2+} or by 250 millimolar salts, such as NaCl or KCl, which inhibited the spinach chloroplast enzyme. Phosphate at 3 to 10 millimolar severely inhibited the algal enzyme, whereas phosphate stimulated the isoform in spinach chloroplasts. Phosphate inhibition of the algal reductase was partially reversed by the addition of NaCl or $MgCl_2$ and totally by both. In the presence of 10 millimolar phosphate, 25 millimolar $MgCl_2$, and 100 millimolar NaCl, reduced thioredoxin causes a further twofold stimulation of the algal enzyme. The *Dunaliella* reductase utilized either NADH or NADPH with the same pH maximum at about 7.0. The apparent K_m (NADH) was 74 micromolar and K_m (NADPH) was 81 micromolar. Apparent V_{max} was 1100 μmoles DHAP reduced per hour per milligram chlorophyll for NADH, but due to NADH inhibition highest measured values were 350 to 400. The DHAP reductase from spinach chloroplasts exhibited little activity with NADPH above pH 7.0. Thus, the spinach chloroplast enzyme appears to use NADH *in vivo*, whereas the chloroplast enzyme from *Dunaliella* or the cytosolic isozyme from spinach may utilize either nucleotide.

Chloroplastic and cytoplasmic forms of DHAP[2] reductase have been isolated from spinach leaves (5). The chloroplast form is stimulated up to sixfold by reduced thioredoxin from spinach but less by thioredoxin from *Escherichia coli*. The leaf cytoplasmic form is stimulated about twofold by fructose 2,6-P_2 (6). These leaf isoforms cannot be detected in crude homogenates because of the presence of inhibitors including lipids, membranes, and peptides (5). Because the reaction as a glycerol-P dehydrogenase (EC 1.1.1.8) was but a trace at its pH optimum, 9.3, using unphysiologically high concentrations of glycerol-P and NAD, we have considered the enzyme to function *in vivo* as a NADH:DHAP reductase (5). The algal reductase utilizes both NADH and NADPH (15), but reexamination of the leaf reductases, indicates the chloroplast isoform seems to utilize NADH *in vivo*, while the cytosolic isoform might use either NADH or NADPH at pH 7 or lower.

When *Dunaliella tertiolecta* is salt stressed, this alga produces glycerol as a product of photosynthesis or glycolysis as an osmoticum (1, 14). A high activity of DHAP reductase is present in extracts of *Dunaliella* (2, 7, 12, 14). One purpose of this paper is to compare the properties and regulation of DHAP reductase from *Dunaliella* with those of the two isoforms in leaves. Marked difference in many respects have been found from the three sources, *Dunaliella* chloroplasts, spinach leaf chloroplasts, and leaf cytosol. In previous work the algal DHAP reductase was found to be unstable after isolation in low yield from Blue Sepharose affinity columns (12), and, therefore, it was necessary first to study the isolation and stability of the algal enzyme. Brown *et al.* (2) showed by association with marker enzymes that the enzyme was most likely of chloroplast origin, and we have confirmed this with isolated chloroplasts from *Dunaliella*.

MATERIALS AND METHODS

Plant Material. *Dunaliella tertiolecta* cells were cultured in 1 L Fernbach flasks with gentle agitation and aeration with 5% CO_2 in air under fluorescent lights (80 $\mu E \cdot m^{-2}s^{-1}$) (3). *Spinacia oleracea* L. cv Long Standing Bloomsdale was grown in the greenhouse during an 8 h light and 16 h dark day (5).

Isolation of intact photosynthetically active chloroplasts in high yield from the *Dunaliella* is described elsewhere (11). After isolation using a sorbitol/Percoll gradient, the chloroplasts were washed by resuspension in buffer followed by centrifugation. Chloroplasts were resuspended in the buffer for enzyme isolation, which was 100 mM Tris, 20 mM ascorbate, and 10 mM mercaptoethanol at pH 6.9 (5).

Chemicals. Most chemicals were from Sigma Chemical Corp. Dihydroxyacetone phosphate, obtained as the dimethylketal dimonocyclohexylamine salt, was hydrolyzed with Dowex 50 H^+ as described by Sigma. Sephacryl S-200 and Sephadex G-25 were from Pharmacea Corp., ultrapure $(NH_4)_2SO_4$ was from Swartz/Mann Inc., and DEAE-cellulose was DE-52 from Whatman Ltd. Thioredoxin from *Escherichia coli* was from Calbiochem, and a mixture of thioredoxin f and m from spinach leaves was provided by B. B. Buchanan. The reduction of thioredoxin and details of its use in DHAP reductase assays were similar to the tests with the leaf chloroplastic enzyme (5, 18).

Enzyme Isolation. The chloroplastic and cytoplasmic isozymic forms from spinach leaves were separated on Sephacryl S-200,

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[2] Abbreviation: DHAP, dihydroxyacetone phosphate.

after first making a 35 to 70% saturated $(NH_4)_2SO_4$ fractionation from a leaf homogenate (5). Leaf enzymes prepared in this manner were stored frozen in 2 M glycerol with the buffer from the chromatography column which contained 20 mM Tris and 10 mM mercaptoethanol. This concentration of glycerol would inhibit the enzyme by about 40%, but the activity has been stable for over 1 year, and the glycerol had little inhibitory effect after dilution during the assay.

The enzyme from *Dunaliella* or isolated algal chloroplasts was partially purified by the following procedure. Cells were cultured continuously with 5% CO_2 in air for 1 week, harvested by centrifugation at about 1,500*g* for 5 min, and washed once with fresh growth medium. The cells or isolated chloroplasts were resuspended in 10 mL of an isolation buffer at pH 6.9 containing 100 mM Tris, 20 mM ascorbate and 10 mM mercaptoethanol, and broken by slow release from a Yeda press after being held at 1,200 p.s.i. for 5 min. The breaking procedure was repeated, and the homogenate was centrifuged at 45,000*g* for 30 min. The supernatant was immediately placed on a 3 × 18 cm column of DEAE-cellulose and eluted with a linear 0 to 1.0 M KCl gradient in a buffer containing 25 mM Tris at pH 6.9 and 10 mM mercaptoethanol.

The isozymes from both spinach and *Dunaliella* were assayed as the reductase with 1 mM DHAP and generally 150 μM NADH or NADPH. The assay buffer was a mixture of 33.3 mM each of Mes, Hepes, and Tricine. The pH curves were run in the same buffer after adjustment to the indicated pH. The spinach cytoplasmic form was assayed at pH 7.2 and the spinach chloroplastic form was assayed at pH 6.9 at their approximate pH optima (this text). The enzyme from *Dunaliella* was assayed at pH 7.0. Specific activities are expressed as μmol NADH oxidized·h^{-1}·mg^{-1} Chl based on Chl analysis of the original homogenates from which the enzymes were isolated. One mM DTT was routinely added to the assay to stimulate the spinach chloroplastic and cytosolic isozymes. The assay for the *Dunaliella* reductase contained DTT only when indicated. Because the reductases were inhibited by concentrations of NADH over 200 μM (5), the concentration of the NADH stock solution was accurately determined from its absorption at 340 nm.

RESULTS

Preparation of DHAP Reductase from *Dunaliella*. A very active DHAP reductase was present in the homogenate of *D. tertiolecta* prepared by breaking the cells with a Yeda press and the activity did not increase upon removal of membrane and particulate fractions by high speed centrifugation. The specific activity in the crude homogenate was about 350 to 400 μmol of NADH oxidized·h^{-1}·mg^{-1} Chl. In contrast, both isoforms of DHAP reductase in homogenates from leaves were totally inhibited by lipids, membranes, and peptides components (5, 6). The total measurable activity after isolating both DHAP reductases isoforms from leaves was about 20 μmol·h^{-1}·mg^{-1} Chl. Thus, the amount of DHAP reductase activity in *Dunaliella* is about 20-fold that in leaves even though, the amount of enzyme detected in leaves is substantial.

In the crude homogenate, the DHAP reductase from *Dunaliella* was stable for at least a day at room temperature or at 4°C. However, at least 63% of the activity was lost during $(NH_4)_2SO_4$ fractionation by precipitation between 35 to 70% saturation, as used for isolation of the enzyme from leaves. The *Dunaliella* DHAP reductase in the $(NH_4)_2SO_4$ fractions could be further purified by chromatography through Sephacryl S-200, as had been done successfully with isoforms from spinach leaves (5). However, chromatography on Sephacryl S-200 was discontinued, because the reductase from *Dunaliella* was obtained in only 18% yield, and it eluted from the column in the void volume suggesting a molecular weight greater than 250,000, perhaps because of protein polymerization. Rather the crude, centrifuged homogenate was applied directly to a DEAE-cellulose column to avoid inactivation by $(NH_4)_2SO_4$ fractionation. The high specific activity made direct chromatography of the homogenate feasible. The elution profile of DHAP reductase from *Dunaliella* exhibited only one major form of the enzyme from the DEAE cellulose column (Fig. 1). The recovered activity was 55 to 60% of the original activity in the homogenate. Haus and Wegman (12) had recovered only 6% of the activity by a chromatographic procedure using a Blue Sepharose affinity column. A small second peak of DHAP reductase activity from the DEAE-cellulose column was sometimes observed near the elution position of the cytosolic form of the reductase from spinach leaves. A continuing investigation has indicated that other isoforms of DHAP reductase are present in *Dunaliella* (our unpublished data), but that the major form is always the one which chromatographs on a DEAE cellulose column as shown in Figure 1.

Stability, Heat Reactivation, Effect of DTT and Thioredoxin. Investigations of the loss of activity of the partially purified DHAP reductase from *Dunaliella* suggest that the protein readily unfolds and refolds to form the active enzyme. Addition of DTT or mercaptoethanol conferred no additional activity to the homogenate or partially purified enzyme immediately after isolation (Table I, A). Reduced thioredoxin did increase the activity. Once separated on a DEAE-cellulose column the reductase activity was very labile with most of the activity disappearing when stored overnight at 4°C or −18°C. However, sufficient stability has been achieved by storage in 2 M glycerol and subsequent heat reactivation (Table I) to keep *Dunaliella* preparations for at least a month. The isolated reductase from leaves has been stable for over 1 year in 2 M glycerol at −18°C without the need for heat reactivation. Glycerol was therefore added to the fractions as soon as they eluted from the DEAE-cellulose column. Two molar glycerol inhibited the *Dunaliella* DHAP reductase about 50%, but during the assay the glycerol concentration was reduced below 0.2 M where it was not inhibitory.

The *Dunaliella* enzyme seemed to be cold labile. The activity in the homogenate was stable overnight at room temperature but lost over 80% of its activity if frozen at −18°C. In addition when the reductase fraction from the DEAE cellulose column was pooled, glycerol added to a final concentration of 2 M, and the

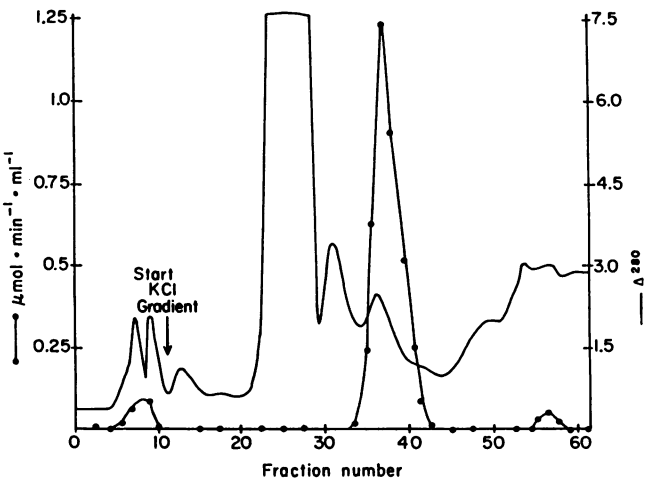

FIG. 1. DEAE-cellulose chromatography of DHAP reductase from extracts of *Dunaliella*. (——) A_{280} for protein, (●——●) NADH:DHAP reductase activity. The main peak of activity eluted similarly from algal extracts or from the soluble stroma fraction from isolated chloroplasts. The small second peak in fractions 55–57 was not present in chloroplasts. A linear KCl gradient from 0 to 1 M in 20 mM Tris buffer (pH 6.9) was used to elute the column.

Table I. *Reactivation and Stimulation of DHAP Reductase from Dunaliella by Heat and by Reduced Thioredoxin*

The enzyme had been isolated on a DEAE-cellulose column and was assayed with 150 μM NADH. When used DTT concentration was 1 mM and there was 10 μg of *E. coli* reduced thioredoxin per ml. The thioredoxin in the presence of DTT would be reduced. Specific activity was calculated on the original Chl content of the *Dunaliella* homogenate.

Pretreatment	Additions	Activity
		$\mu mol \cdot h^{-1} \cdot mg^{-1}$ Chl
A. Newly prepared, partially purified enzyme from DEAE column (no glycerol)	O	395
	DTT	380
	DTT + thioredoxin	515
B. Stored overnight at −18°C with 2 M glycerol	O	83
	DTT	281
	DTT + thioredoxin	390
C. Stored overnight at −18°C with 2 M glycerol; activated at 30°C for 15 min	O	260
	DTT	480
	DTT + thioredoxin	890
D. Stored overnight at 4°C without glycerol; activated at 30°C for 15 min	O	90
	DTT	70
	DTT + thioredoxin	188
E. Stored overnight at 4°C with 2 M glycerol	O	170
	DTT	160
	DTT + thioredoxin	386
F. Stored overnight at 4°C with 2 M glycerol; activated at 30°C for 15 min	O	280
	DTT	281
	DTT + thioredoxin	447
G. Stored overnight at room temperature with 2 M glycerol	O	423
	DTT	463

solution stored overnight at −18°C, about 75% of the activity was lost (Table I, A and B). DTT, however, restored activity of this fraction to about 75% of the original value. When the enzyme was stored overnight at −18°C in 2 M glycerol and then incubated at 30°C for 15 min, activity was about 70% of the original value (Table I, C). DTT further stimulated activity about twofold, and *E. coli* thioredoxin, reduced with DTT, stimulated activity almost fourfold. If the DTT were removed by Sephadex G-25 filtration after it had reduced the thioredoxin, the same stimulation was observed (data not shown). Storage at 4°C in the absence of glycerol (Table I, D), even when incubated afterward at 30°C for 15 min, resulted in 75% loss of activity, and only some of the activity could be restored by the addition of reduced thioredoxin (DTT and thioredoxin). However, storage at 4°C overnight in 2 M glycerol resulted in much less loss of activity (Table I, E). Heat treatment restored most of the activity in these preparations stored at 4°C with glycerol (Table I, F) and the enzyme was further activated by reduced thioredoxin. Enzyme stored overnight in 2 M glycerol at room temperature retained the full activity of the enzyme preparation from the DEAE column (Table I, G). Although this condition induced no cold inactivation, further storage at room temperature resulted in gradual irreversible loss of activity over several days. When reactivating the enzyme after storage at 4° or −18°C, the optimum incubation conditions were temperatures between 25°C to 35°C (Fig. 2A) for times of 15 to 30 min (Fig. 2B).

It appeared from these experiments that the best storage condition for *Dunaliella* DHAP reductase was in 2 M glycerol at −18°C with the Tris, mercaptoethanol, KCl buffer from the chromatography column, after which the activity could be restored by incubation at 30°C for 20 min. Such preparations of *Dunaliella* reductase have been stored up to one month at −18°C and 5 days at 4°C with retention of most of the activity after heat reactivation.

Chloroplast Location of DHAP Reductase. Intact photosynthetically active chloroplasts from several liters of *Dunaliella* cultures grown on 5% CO_2 in air were isolated in 30 to 40% yield by centrifugation in a Percoll/sorbitol gradient (11). The intact chloroplast fraction from the gradient was diluted 10-fold with isolation medium, and then concentrated by centrifugation. After resuspension in small volumes of isolation buffer the plastids were broken by slow passage through a Yeda Press after being held at 1200 p.s.i. for 5 min. DHAP reductase activity in the broken chloroplast fraction had the same measured specific activity, 350 μmol of NADH oxidized $\cdot h^{-1} \cdot mg^{-1}$ Chl, as did the original homogenate. The reductase activity from the broken chloroplasts was in the soluble stroma fraction after centrifugation, and the activity was apparently as stable as in the homogenate. When activity in the soluble fraction from the broken chloroplasts was chromatographed on DEAE-cellulose, the elution profile was identical with the elution profile of the reductase from the whole cell preparations except that no small peak was ever observed (data not shown). It is concluded that the major DHAP reductase in *Dunaliella* is in the chloroplast as has been proposed by Brown *et al.* (2).

pH Optimum and Pyridine Nucleotide Specificity. The pH optimum of the DHAP reductase reaction from *Dunaliella* was 6.9 (Fig. 3). A little glycerol phosphate dehydrogenase activity could be detected with a maximum at about pH 9.3. These activities are similar to those from spinach chloroplasts and arguments have been presented to indicate the enzyme functions *in vivo* as a reductase (5). At pH 9.3, 20 mM glycerol phosphate and 2 mM NAD were required to detect any measurable dehydrogenase reaction and even then the activity was 16-fold less than the reductase activity at its pH optimum of about 6.9. In these respects DHAP reductase from *Dunaliella* and leaves are similar.

The DHAP reductase from *Dunaliella* utilizes both NADH

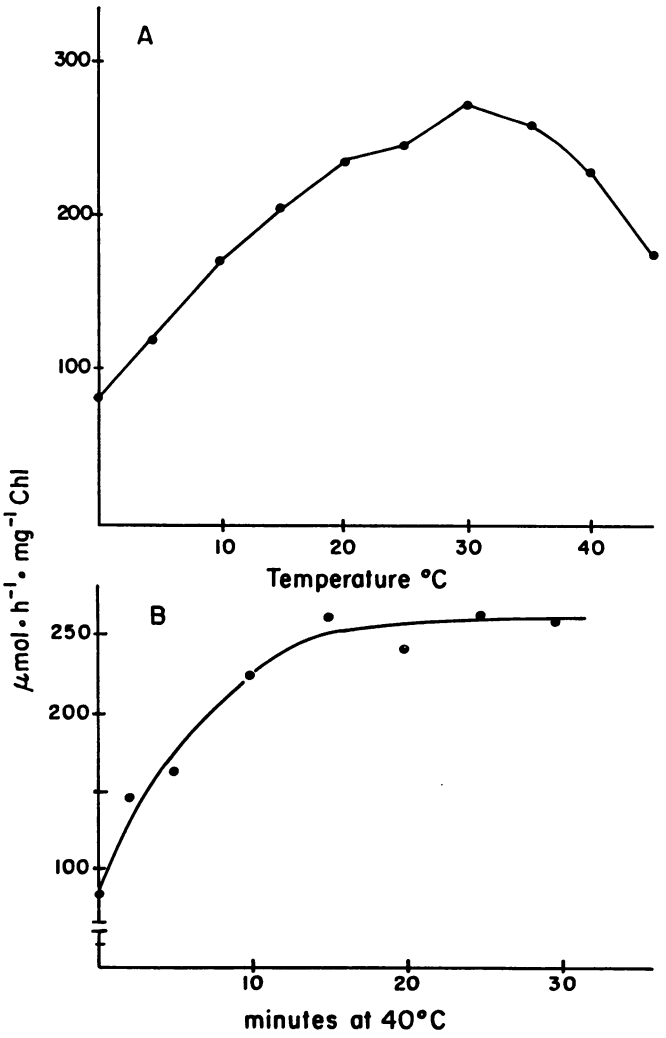

FIG. 2. Heat activation of DHAP reductase from *Dunaliella*. A, Temperature optimum for activation in 20 min; B, time for activation at 40°C.

and NADPH (Fig. 4) as previous reported (15). The pH optimum with either nucleotide was in the range of 7.0. The measured activity with NADH was about twice that with NADPH. Based on the activity in the cell extract of *Dunaliella*, the highest measured rate with 150 μM NADH was about 350 $\mu mol \cdot h^{-1} \cdot mg^{-1}$ Chl, whereas the highest rate with NADPH was 150. Apparent V_{max} values of 1100 $\mu mol \cdot h^{-1} \cdot mg^{-1}$ Chl with excess NADH or 537 with NADPH were estimated by Eadie-Hofstee plots, which extrapolates to V_{max} as if there were no inhibition by higher concentrations of reduced pyridine nucleotides. From kinetic studies (data not shown), the apparent K_m values were similar, being K_m (NADH) of 74 μM and K_m (NADPH) of 81 μM. Real K_m values were not measurable due to pyridine nucleotide inhibition at higher concentrations. Because the major *Dunaliella* DHAP reductase is located in the chloroplast where NADPH is the principle reduced pyridine nucleotide, it is likely that NADPH is the substrate for this reductase *in vivo*. Goyal *et al.* (8) found concentrations of 130 μM NADPH and 20 μM NADH in whole cells of *Dunaliella* in the light. In the chloroplast, the ratio of NADPH to NADH concentrations would probably be even greater. Thus, DHAP reductase in *Dunaliella* chloroplasts would probably use NADPH as the reductant.

The use of either NADPH or NADH by the *Dunaliella* chloroplastic enzyme (Fig. 4) made it necessary to reexamine the

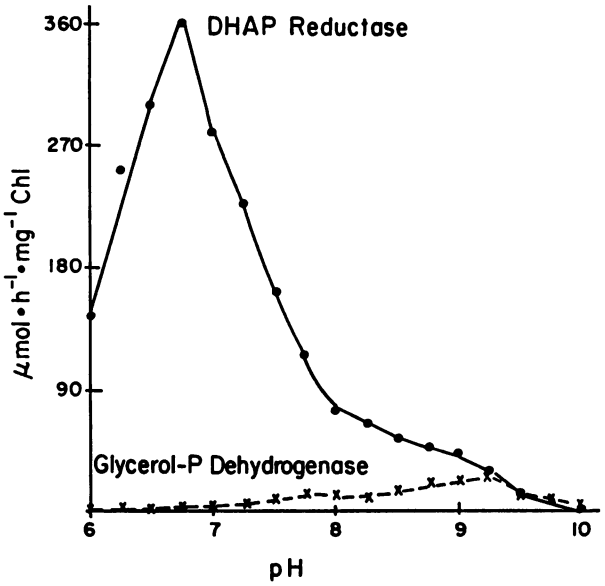

FIG. 3. The pH optima for the NADH:DHAP reductase and NAD:glycerol phosphate dehydrogenase activities from *Dunaliella*. The enzyme was from a DEAE-cellulose column (Fig. 1), and the assays were run with 150 μM NADH or NAD immediately before storage and in the absence of glycerol and without other additions for increasing reductase activity.

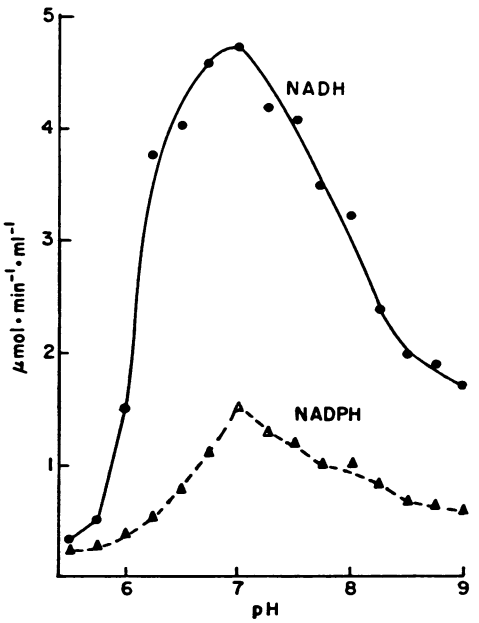

FIG. 4. Reduced pyridine nucleotide specificity for DHAP reductase from *Dunaliella*. The enzyme had been partially purified from a DEAE-cellulose column (Fig. 1) and stored overnight at 4°C in 2 M glycerol. Before the assays the preparation was heat activated at 30°C for 20 min. Assays were run with 150 μM reduced pyridine nucleotide.

reported NADH specificity of the leaf DHAP reductases (5). The DHAP reductase isoform from spinach leaf chloroplasts, after chromatographic separation from the cytosolic isoform, had the pyridine nucleotide specificity shown in Figure 5A. The pH optimum with NADH was at about 6.9 and with NADPH at about 6.0. At pH values over 6.9 there was little activity with NADPH and at a pH above 7.5, a pH to be expected in chloroplasts, there was no activity with NADPH. With NADH at pH 6.9 the apparent K_m was 41 μM and the measured V_{max} was 11 $\mu mol \cdot h^{-1} \cdot mg^{-1}$ Chl with DTT. With NADPH at a pH

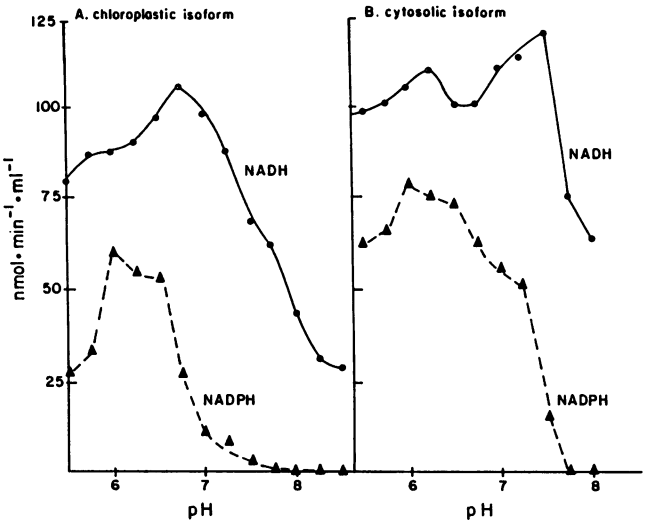

FIG. 5. Reduced pyridine nucleotide specificity for DHAP isozymes from spinach leaves. The two activities had been separated by chromatography on a Sepharacyl S-200, and stored at 4°C in 2 M glycerol. A, Chloroplastic form was isolated from mature leaves directly from the greenhouse. B, Leaf cytosolic isoform was from plants held in the dark for 3 days to increase the cytosolic and decrease the chloroplastic enzyme activity (7). Thus the apparently equal cytosolic activity relative to the chloroplast form is not typical of fresh spinach. (●——●) assayed with 150 μM NADH, (▲-----▲) assayed with 150 μM NADPH.

of 6.0 the apparent K_m was 95 μM and the measured V_{max} was 9 $\mu mol \cdot h^{-1} \cdot mg^{-1}$ Chl with DTT. Addition of thioredoxin, inhibitors, or salts has not altered these relative specificities for NADH or NADPH. Therefore, the DHAP reductase isoform in leaf chloroplasts, which have a pH of 7.5 to 8.5, would appear to require NADH. Regulation of the NADH concentration in the chloroplast might be a mechanism of regulating glycerol-P formation. In the cytosol of the leaf with a more acidic pH range of 6.5 to 7.5, either NADPH or NADH might be used, but the preferred nucleotide appears to be NADH (Fig. 5B). For the cytosolic isoform the apparent K_m (NADH) was 57 μM and K_m (NADH) was 20 μM. Activity with NADPH for both forms of DHAP reductase increased in the range of pH 6 or lower. As a general observation, other reductases that are considered to be specific *in vivo* for NADH, such as peroxisomal NADH:hydroxypyruvate reductase, have considerable activity with NADPH at a lower pH (17). The reason for a double pH optimum for the cytosolic spinach DHAP reductase with NADH (Fig. 5B) is not known, but the more acidic optimum does not occur for the chloroplastic form from either spinach or *Dunaliella*. The peak of activity at about pH 6 with NADH coincides with the NADPH pH optimum, and thus may be related to some property of pyridine nucleotide binding to the reductase.

Effect of Detergents on Activity. Severe inhibition of both forms of DHAP reductase from leaves by μg quantities of lipids has been emphasized by previous investigations (5, 6, 16). However, the presence of full activity of DHAP reductase in extracts of *Dunaliella* or its chloroplasts was indicative of no such inhibition. Addition of up to 256 μM Triton X-100 to DHAP reductase preparations from *Dunaliella* chloroplasts caused no inhibition (data not shown), whereas the enzyme from spinach leaf chloroplasts was completely inhibited by 6 μM Triton X-100 (5). Sensitivity of detergent inhibition is a pronounced difference between algae or plants reductases that may reflect differences in enzyme function. The detergent insensitive enyme in *Dunaliella* primarily produces glycerol as an osmoticum, while the lipid inhibited isoforms in leaves, which normally do not produce a large pool of glycerol, may mainly produce glycerol-P for lipid synthesis.

Salts, $MgCl_2$, and Phosphate Regulation of Dihydroxyacetone Phosphate Activity. It has previously been shown that DHAP reductase from *Dunaliella* is stimulated by salts (9) and inhibited by phosphate (13, 15). In contrast we found that DHAP reductase from spinach leaf chloroplasts was inhibited by NaCl or $MgCl_2$ and somewhat stimulated by 5 mM phosphate (6).

The activity of DHAP reductase from *Dunaliella* after isolation and storage overnight at 4°C in 2 M glycerol was restored and stimulated about threefold by addition of 100 to 150 mM NaCl or KCl with either NADH or NADPH as the reducing agent (Fig. 6A). The actual salt concentration in the assay was 100 mM higher than the molarity from the added salt because of the salt in the buffer. The molarity shown on the *x*-axis of Figure 6 reflects only the molarity of the added salt. If the assay were run with buffer diluted 10-fold, the concentration of added salt for maximum stimulation was shifted from 150 to about 250 mM (data not shown). Similar results were obtained with KBr, $CaCl_2$, and Na_2SO_4. Thus, the stimulation by 250 mM salts appears to be a general ionic effect. Salt concentrations higher than 550 mM were inhibitory. The salt stimulation increased the activity above that obtained by warming at 30°C for 15 min (Table I, F) and, therefore, the salt stimulated activity was in maximum range observed with both heat treatment plus reduced thioredoxin.

A stimulation of NADH or NADPH:DHAP reductase activity from *Dunaliella* similar to that from 150 mM NaCl (Fig. 6B) was caused by the addition of only 25 mM $MgCl_2$ or $MgSO_4$. Because $MgCl_2$ stimulation occurred at a lower concentration than with NaCl, the effect must be primarily attributed to Mg^{+2}. The Mg^{+2} stimulation of the algal DHAP reductase was consistent with a 50% inhibition of the reductase by 10 mM EDTA when NADPH was the reductant and 30% when NADH was the reductant.

Phosphate severely inhibited the NADH or the NADPH: DHAP reductase activities of the *Dunaliella* chloroplast enzyme (Fig. 7). Nearly complete inhibition occured at concentrations above 10 mM phosphate, a level of phosphate expected in the cell or chloroplast. Addition of 25 mM $MgSO_4$ or 100 mM NaCl separately only partially reversed the phosphate inhibition. On the vertical axis of the insert of Figure 7, the partially restored activity with 25 mM $MgCl_2$ and no NaCl is shown. Then addition of NaCl to the 25 mM $MgCl_2$ further reversed the phosphate inhibition to fully reactivate the enzyme.

In marked contrast to the algal reductase, the higher plant chloroplast DHAP reductase was inhibited by either NaCl or Mg^{+2} in the same concentrations used to stimulate the *Dunaliella* enzyme (Fig. 8). $MgCl_2$ 25 mM was as inhibitory as 100 to 250 mM NaCl. Similar results were obtained with NADH or NADPH.

DISCUSSION

DHAP reductases from three different sources, *Dunaliella* chloroplasts, spinach chloroplasts, and the cytoplasm of spinach leaves, have some properties in common, but there are distinct differences in their regulatory properties which seem to be associated with their physiological function and subcellular location (Table II). Two different functions are the essential role of glycerol-P in lipid synthesis in all tissues and the use of glycerol as an osmoticum by some algae. Similarly, the regulation of this enzyme from *Dunaliella* has been found to be different from the regulation of the reductases in leaves.

Two major limitations for investigation of DHAP reductase have been its lability when partially purified and the complete inhibition of the enzyme from higher plants by traces of lipids or detergents. The lability of the algal enzyme has been found to be due in part to cold inactivation. However, this inhibition can be reversed by heat, salts, or reduced *E. coli* thioredoxin. The

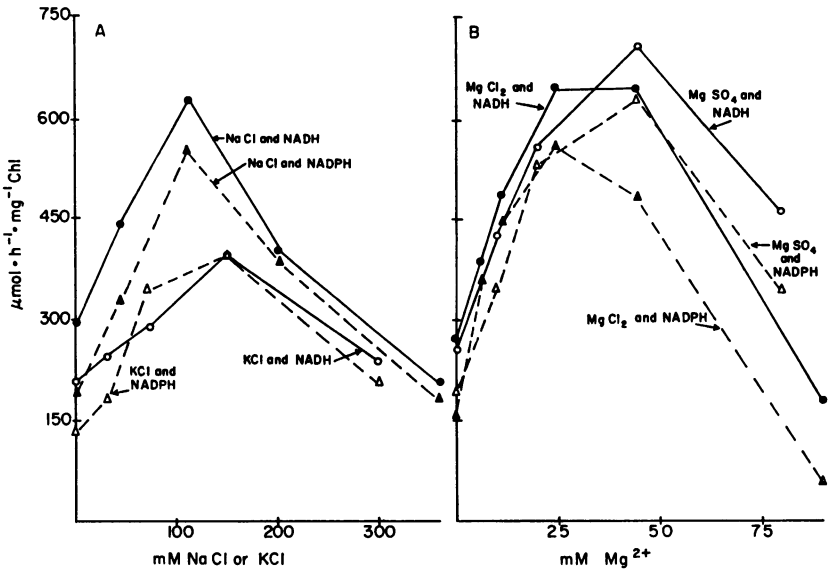

FIG. 6. Stimulation of DHAP reductase from *Dunaliella* by salts. The enzyme was from a DEAE-cellulose column and had been stored at 4°C in 2 M glycerol and the Tris, mercaptoethanol, KCl buffer from the chromatographic column. Due to a 10-fold enzyme dilution in the assay medium, the carry-over of KCl was not considered in the assay. However, the assay buffer contained 100 mM salt. The enzyme preparation was not heat activated before being assayed with 150 µM NADH or NADPH at the pH optimum for activity with each nucleotide. A, Stimulation by NaCl or KCl. (●——●) NaCl with NADH, (▲-----▲) NaCl with NADPH, (○——○) KCl with NADH, (△-----△) KCl with NADPH. B, Stimulation by $MgCl_2$. (●——●) $MgCl_2$ with NADH, (▲-----▲) $MgCl_2$ with NADPH, (○——○) $MgSO_4$ with NADH, (△-----△) $MgSO_4$ with NADPH.

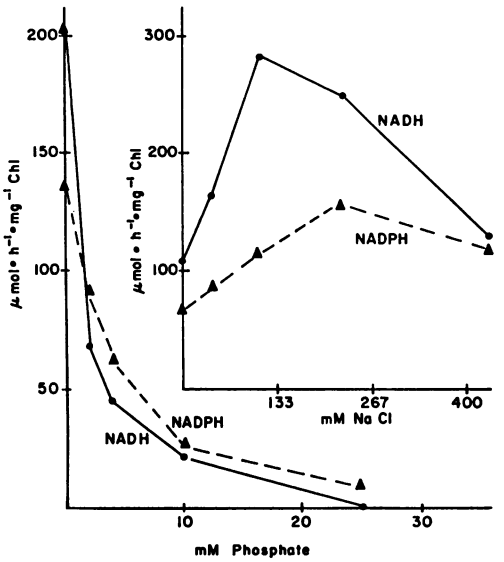

FIG. 7. Phosphate inhibition of DHAP reductase from *Dunaliella* and reversal by NaCl with $MgCl_2$. The enzyme had been isolated on a DEAE-cellulose column and stored at 4°C in 2 M glycerol. Before assaying it was heat reactivated for 20 min at 30°C. Assays were run in 333 mM each of HEPES, Tricine, and MES at pH 7.0 and with 150 µM reduced pyridine nucleotide. Data in the insert are the reversal of the phosphate inhibition by increasing NaCl concentration in the assay containing 10 mM phosphate and 25 mM $MgCl_2$. The $MgCl_2$ by itself and partially reversed the phosphate inhibition (insert with no added NaCl) and NaCl further reactivated the enzyme.

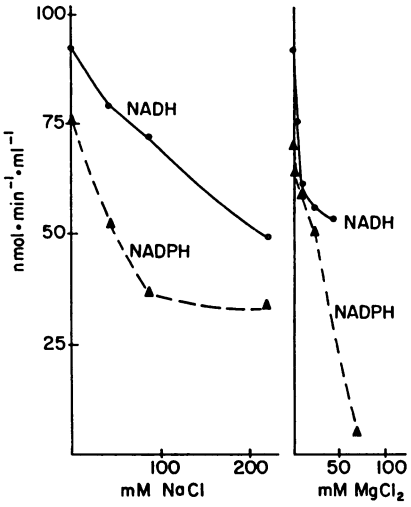

FIG. 8. Inhibition of the chloroplast form of DHAP reductase from spinach leaves by NaCl or $MgCl_2$. The enzyme had been stored at −18°C in 2 M glycerol and was assayed with 150 µM NADH at pH 7.0.

spinach leaf isozymes on the other hand were not reactivated by heat or salts, but the chloroplast enzyme was activated by spinach thioredoxin and the spinach cytosolic reductase by fructose 2,6-P_2. The chloroplastic enzyme in *Dunaliella* was inhibited slightly by fructose 2-6-P_2 (data not shown).

The DHAP reductase activity in crude *Dunaliella* cell extracts or isolated chloroplast extracts was stable during the cell extraction procedure and was readily measured in the homogenate at around 350 µmol NADH oxidized·h^{-1}·mg^{-1} Chl, which was fourfold greater than the rate of CO_2 fixation by these cells. Because of several possibilities for activation or inhibition of the enzyme the actual *in vivo* activity is hard to estimate. Activity measured in the diluted cell extract may be lower than *in vivo* activity where the enzyme could be activated by 100 mM KCl or 25 mM $MgSO_4$. On the other hand, activity in the extract could be higher than *in vivo* levels if it were inhibited 90% by 5 mM phosphate in the intact cell. Severe inhibition of the *Dunaliella* DHAP reductase by phosphate suggests that when adequate nutrients are present only lower levels of glycerol-P would be formed as needed for lipid synthesis. However, when *Dunaliella* is salt stressed, Mg^{2+} and KCl concentrations would increase in the cell (4) resulting in a reversal of the inhibition of the reductase by phosphate. The enzyme could, therefore, catalyze the reduction of large quantities of DHAP to form glycerol-P and ultimately glycerol as an osmoticum. On the other hand, DHAP reductase from spinach chloroplasts was strongly inhibited by KCl and $MgSO_4$ and somewhat stimulated by 5 mM phosphate. These opposite effects with KCl, $MgSO_4$, and phosphate on regulating DHAP reductase from algae or spinach leaves may be related to the need for glycerol as an osmoticum only in the algae. Further examination of DHAP reductase from other plants and plant tissue may suggest whether glycerol production as an osmoticum in algae, also occurs in some higher plants.

Table II. *Properties of DHAP Reductases From Dunaliella Chloroplasts, Spinach Chloroplasts, or Spinach Cytosol*

	Dunaliella	Spinach	Spinach
Subcellular location	Chloroplast	Chloroplast	Cytoplasm
pH optimum with NADH	7.0	6.9	7.2
pH optimum with NADPH	7.0	6.0	6.0
Apparent K_m (NADH)	74 μM	41 μM	57 μM
Apparent K_m (NADPH)	81 μM	95 μM	20 μM
NADH or NADPH inhibition	Over 200 μM	Over 150 μM	Over 150 μM
		$\mu mol \cdot h^{-1} \cdot mg^{-1}$ Chl	
Measured V_{max} with 150 μm NADH	350	13	4
Apparent V_{max} NADH	1100		
V_{max} after 30°C treatment and reduced thioredoxin; with 150 μM NADH	890		
Measured V_{max} with 150 μM NADPH	150		
Apparent V_{max} NADPH	537	9	
V_{max} after 30°C treatment and reduced thioredoxin; with 150 μm NADPH	532		
5 mM Pi	Inhibits 90%	Stimulates 20%	No effect
250 mM NaCl, KCl, or KBr	Stimulates inactivated enzyme	Inhibits 80%	Inhibits 80%
25 mM $MgCl_2$	Stimulates inactivated enzyme	Inhibits	Inhibits
10 mM EDTA	Inhibits	No effect	No effect
Heat activation at 30°C for 20 min	Stimulates	No effect	No effect
DTT on native enzyme	No effect	Stimulates	No effect
E. coli reduced thioredoxin	Stimulates	Little stimulation	No effect
Spinach reduced thioredoxin f and m	Slightly restores inactive enzyme	Stimulates	No effect
Fructose 2,6-P_2	Slight inhibition	No effect	Stimulates
Triton X-100	No effect at 256 μM	Inhibits 100% at 6 μM	Inhibits 100% at 6 μM

Considerable protection against irreversible inactivation of the isolated DHAP reductase with time from either higher plants or algae is accomplished by storage of the partially purified enzymes in 2 M glycerol at −18°C. After separation of the various isozymes by gel filtration on Sephacryl S-200 or by ion exchange chromatography with DEAE-cellulose, the reductase activity is lost within hours without the addition of 2 M glycerol. Glycerol stabilized the enzyme from leaves for at least 1 year when stored at −18°C. The algal enzyme lost most of its activity quickly (overnight) when stored in 2 M glycerol at −18°C. However, most of the lost activity could be restored by heat activation at 30°C for 20 min, or the addition of 100 mM NaCl or 25 mM $MgCl_2$ or 1 mM DTT. The greatest restoration of activity was achieved by the addition of reduced *E. coli* thioredoxin. After reaching a maximum activity, no further stimulation could be obtained by heat, DTT, NaCl, and $MgSO_4$. When reduced *E. coli* thioredoxin was added to the homogenate or to isolated, reactivated enzyme, additional stimulation of activity of about twofold occurred. Thus, the algal enzyme exhibited both reversible and irreversible inactivation and thioredoxin stimulation. With the isolated reductase from *Dunaliella* irreversible inactivation without added glycerol was a slow process with time at room temperature. After partial purification by either $(NH_4)_2SO_4$ precipitation or chromatography on Sephacryl S-200 or DEAE-cellulose, rapid irreversible inactivation occurred unless stored in 2 M glycerol. When stored in 2 M glycerol, inactivation in the cold was also rapid but reversible by heat or salts. The reactivation of cold inactivated enzyme and stimulation by 25 mM $MgCl_2$ or $MgSO_4$ and inhibition of the enzyme by 10 mM EDTA without added Mg^{2+} indicate a role for Mg^{2+} in the enzyme structure. Reactivation of cold partially inactive enzyme will only be understood when a detail examination of the protein structure and its active site can be undertaken. The role of reduced thioredoxin in controlling enzyme activity has been established as reducing specific disulfide bridges (18), and the results with DHAP reductase from *Dunaliella* suggest that this activation is aided by the presence of 2 M glycerol, 250 mM NaCl, 25 mM $MgCl_2$, or mild heat treatment, all of which could affect the way in which the protein is folded. The specificity of the *Dunaliella* enzyme for the procaryotic thioredoxin from *E. coli* in preference to a mixture of spinach leaf thioredoxin f and m indicates a specific role for the thioredoxin for the activation of DHAP reductase in *Dunaliella*. It would seem that the high internal cellular glycerol level, produced by *Dunaliella* as an osmoticum, might also act as a protectant for the reductase.

A distinct difference between leaf DHAP reductase isozymes and the reductase in *Dunaliella* chloroplasts has been sensitivity to lipids and detergents. Leaf reductases are totally inhibited by trace levels of lipids, whereas the reductase in *Dunaliella* is insensitive to detergents and is therefore active in the crude homogenate. We speculate that this difference may be due to the difference in function between *Dunaliella* and leaf enzyme. Glycerol-P formation initiates glycerol lipid formation which may be regulated by feed back inhibition by the lipids in the leaf.

In *Dunaliella* glycerol-P is also the precursor for the osmoticum, glycerol, and thus its DHAP reductase may be insensitive to regulation by lipids. This line of reasoning suggests that *Dunaliella* might also have another form of DHAP reductase which might be inhibited by lipids.

Phosphate concentration is another major regulatory factor that differentiates between the chloroplastic DHAP reductases from *Dunaliella* and spinach. The enzyme from the alga is inhibited about 75 to 90% by 5 to 10 mM phosphate, whereas the enzyme in spinach chloroplasts is stimulated twofold by 5 mM phosphate. One of several control processes for the DHAP reductases may be the triose phosphate shuttle which controls the level of DHAP and phosphate in the chloroplast and cytosol. DHAP reductase activity might regulate, in part, the DHAP pools and thus exert some control over the triose-P shuttle. Further investigation of DHAP reductase activities and glycerol formation in leaves may indicate a role for controlling the phosphate pools by glycerol-P formation.

The amount of DHAP reductase in *Dunaliella* chloroplasts exceeds in activity the rate of CO_2 fixation. *Dunaliella* fixes about 75 μmol $CO_2 \cdot h^{-1} \cdot mg^{-1}$ Chl (10), while, depending on the salt concentration, the activity of DHAP reductase is at least 350 μmol $\cdot h^{-1} \cdot mg^{-1}$ Chl. Certainly, therefore, this alga has the enzymatic potential for glycerol production as the main product of photosynthesis. In spinach chloroplasts the reductase level is about 13 μmol NADH oxidized $\cdot h^{-1} \cdot mg^{-1}$ Chl. However, this activity can be increased several fold by thioredoxin (5). Although glycerol-P production in plants may be more limited than in algae, the reductase activity in leaves is relatively high for a respiratory enzyme and would appear to be higher than is needed for lipid synthesis. The activity of the reductase in leaves, however, is less than the activity of enzymes directly associated with CO_2 fixation. The kinetic properties of DHAP reductase from both spinach leaves and *Dunaliella* are such that actual *in vivo* activities can not be accurately estimated because of NADH or NADPH inhibition at concentrations over 250 μM. Eadie-Hofstee plots were used to evaluate the kinetic parameters by calculating apparent V_{max} and K_m values using only the concentrations of pyridine nucleotides that do not inhibit the enzymes. These apparent values for the *Dunaliella* reductase are a V_{max} of 1100 μmol $\cdot h^{-1} \cdot mg^{-1}$ Chl and a K_m (NADH) of about 70 μm.

The pyridine nucleotide specificity of the forms of DHAP reductase from *Dunaliella* and spinach have been examined over the pH range expected in the cell. The *Dunaliella* chloroplastic reductase could use either NADH or NADPH between pH 6.5 to 8.5. However, the DHAP reductase isoforms in leaf chloroplast or cytoplasm have essentially no activity with NADPH above pH 7.5 and would, therefore, have to be dependent on the NADH pool in the chloroplasts or cytoplasm in the pH range between 7 and 8.5. At present the chloroplastic DHAP reductase from spinach leaves would appear to have no activity *in vivo* with NADPH. Previously we had reported that the leaf DHAP reductases were specific for NADH when assayed between pH 6.9 to 7.2 (5, 16). However, this concept is modified by data in the present study which indicates activity also with NADPH between pH 5.5 to 7. Whether this acidic activity with NADPH is significant in the cytosol *in vivo* is not known.

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