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Nonreversible D-Glyceraldehyde 3-Phosphate Dehydrogenase of Plant Tissues[1]

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ABSTRACT

Preparations of TPN-linked nonreversible D-glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.9), free of TPN-linked reversible D-glyceraldehyde 3-phosphate dehydrogenase, have been obtained from green shoots, etiolated shoots, and cotyledons of pea (*Pisum sativum*), cotyledons of peanut (*Arachis hypogea*), and leaves of maize (*Zea mays*). The properties of the enzyme were similar from each of these sources: the Km values for D-glyceraldehyde 3-phosphate and TPN were about 20 μM and 3 μM, respectively. The enzyme activity was inhibited by L-glyceraldehyde 3-phosphate, D-erythrose 4-phosphate, and phosphohydroxypyruvate. Activity was found predominantly in photosynthetic and gluconeogenic tissues of higher plants. A light-induced, phytochrome-mediated increase of enzyme activity in a photosynthetic tissue (pea shoots) was demonstrated. Appearance of enzyme activity in a gluconeogenic tissue (endosperm of castor bean, *Ricinus communis*) coincided with the conversion of fat to carbohydrate during germination. In photosynthetic tissue, the enzyme is located outside the chloroplast, and at *in vivo* levels of triose-phosphates and pyridine nucleotides, the activity is probably greater than that of DPN-linked reversible D-glyceraldehyde 3-phosphate dehydrogenase. Several possible roles for the enzyme in plant carbohydrate metabolism are considered.

Plant tissues contain three enzymes for the oxidation of D-G3P[3] (12). Two of these catalyze a Pi-dependent, reversible oxidation of D-G3P to 1,3-diP-glycerate; one enzyme is DPN-specific and found in both photosynthetic and nonphotosynthetic tissues, while the other uses TPN and is most probably confined to the chloroplast. Both enzymes have been extensively studied (29). The third enzyme, which is TPN-specific, oxidizes D-G3P to glycerate-3-P in a reaction which does not require Pi and is not reversible. Evidence for this enzyme activity in green leaves was reported by Axelrod *et al.* (3), Barnett *et al.* (4), and Gibbs (11). Shortly thereafter, Arnon *et al.* (2) and Rosenberg and Arnon (31) reported the properties of a partially purified preparation of the enzyme from sugar beet leaves. Recently, Jacob and d'Auzac (17) have purified the enzyme from the latex of *Hevea brasiliensis*. In this laboratory, the partially purified enzyme from pea shoots was inhibited strongly by L-G3P (19). It is possible that the scarcity of reports on the enzyme is related to the presence of this inhibitor in commercial preparations of the substrate D-G3P.

In the present investigation, the significance of this nonreversible D-G3P dehydrogenase to plant carbohydrate metabolism has been evaluated. A preparation of pea shoot enzyme, free of reversible Pi-dependent D-G3P dehydrogenase activity, has now been obtained, and the properties have been compared with those of the same enzyme partially purified from several other plant tissues. In addition, a relatively specific assay for the determination of the enzyme activity in cell-free extracts is described. Changes in the enzyme activity during the germination of peas and castor beans and the distribution of the enzyme in representative tissues were examined.

The properties and *in vivo* localization of the three D-G3P dehydrogenases were compared, and the results indicate that, in some tissues, a major portion of triose-P oxidation may be mediated by the nonreversible enzyme.

MATERIALS AND METHODS

Materials. Pea (*Pisum sativum* L. var. Progress No. 9), maize (*Zea mays* L. var. Early Fortune), peanuts (*Arachis hypogea* L. var. Jumbo Virginia), and castor bean (*Ricinus communis* L.) were grown under natural lighting in a glasshouse at 16 to 18 C or in the dark at 22 C.

The following chemicals and enzymes were obtained from the Sigma Chemical Company: ATP, dihydroxyacetone-P, DPN, D-erythrose-4-P, D-fructose-1,6-diP, DL-G3P, D-glycerate-3-P, P-glycolate, P-hydroxypyruvate, D-ribose-5-P, TPN, TPNH, G3P dehydrogenase, 3-P-glycerate kinase, and triose-P isomerase. Chloroacetol-P was a gift of Dr. F. C. Hartman through the courtesy of Dr. Louise Anderson. All chemicals were prepared as sodium salts at the recommended pH before use.

L-G3P was prepared according to the method of Venkataraman and Racker (38). The D-G3P present in 0.65 mmole of DL-G3P was converted to glycerate-3-P by G3P dehydrogenase. The L-G3P which remained was purified from the reaction mixture by gradient elution on a Bio-Rad AG 1-X8 formate column (1.8 cm^2 × 5.5 cm) using 50 ml of water in the mixing vessel and 50 ml of 0.5 M ammonium formate-0.2 N formic acid in the reservoir. The calcium salt of L-G3P was collected (38) and converted to the sodium salt before use. The concentration of D-G3P in this solution was about one-eighth that of the L-G3P.

D-G3P was prepared according to Horecker and Smyrniotis

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[3] Abbreviation: G3P: glyceraldehyde 3-phosphate.

(16). D-Fructose-1,6-diP (0.6 mmole) was incubated with aldolase in the presence of acetaldehyde which trapped the dihydroxyacetone-P. The D-G3P was then purified from this reaction mixture as described above for the L-isomer.

Concentrations of reagents in solutions were determined by enzymic assays.

Assay of Nonreversible D-G3P Dehydrogenase Activity. The enzyme was assayed by measuring the reduction of TPN. The standard reaction mixture contained, in a final volume of 1.0 ml, 50 μmoles of glycylglycine-NaOH buffer, pH 7.7, 0.2 μmole of TPN, 5 units of triose-P isomerase, 0.025 μmole of D-G3P (generated from 0.5 μmole of dihydroxyacetone-P by triose-P isomerase), and 50 to 100 μl of the nonreversible D-G3P dehydrogenase preparation (in the case of the partially purified pea shoot preparation, this volume contained 22–44 μg of protein). The reaction was started by the addition of dihydroxyacetone-P. The increase in absorbance at 340 nm was followed in a Beckman DUR spectrophotometer connected to a Gilford 2000 recorder. In a control experiment with 0.06 μmole of D-G3P, the reaction rate was the same whether this substrate was generated from 1.2 μmoles of dihydroxyacetone-P or added directly in the presence of chloroacetol-P (see below).

For some experiments, the substrate D-G3P was added directly. Triose-P isomerase was omitted from these reaction mixtures, and the triose-P isomerase present in the enzyme preparation was inhibited by the addition of 0.1 μmole of chloroacetol-P (1, 26).

Assay of Reversible D-G3P Dehydrogenase (TPN- and DPN-linked) Activity. These enzymes were assayed in the forward direction (D-G3P → glycerate-3-P) (12) or back direction (glycerate-3-P → D-G3P) (23); the latter assay was used when it was necessary to determine the activity of the TPN-linked enzyme in the presence of the nonreversible D-G3P dehydrogenase. Reaction mixtures contained, in a final volume of 1.0 ml, 50 μmoles of glycylglycine-NaOH buffer, pH 7.7, 3.3 μmoles of GSH and, in addition, for assays in the forward direction: 5 μmoles of sodium arsenate, 0.2 μmole of TPN (or DPN), and 0.5 μmole of DL-G3P; for assays in the back direction: 4 μmoles of $MgCl_2$, 4 μmoles of ATP, 0.2 μmole of TPNH (or DPNH), 5 units of 3-P-glycerate kinase, and 1 μmole of glycerate-3-P. Reactions were started by the addition of substrate, and the change in absorbance at 340 nm was followed spectrophotometrically.

Estimation of Protein. Protein was precipitated from samples of the enzyme preparations with 15% trichloroacetic acid, washed with 80% acetone, dissolved in 0.75 N NaOH, and determined by the biuret method (24).

Determination of the Activity of Nonreversible D-G3P Dehydrogenase in Cell-free Plant Extracts. Cell-free preparations were obtained from 2 to 4 g of tissue by grinding with 10 ml of a solution containing 50 mM tris-HCl buffer, pH 8, 5 mM cysteine-HCl, and 1 mM $EDTANa_4$. The homogenate was squeezed through cheesecloth, centrifuged at 12,000*g* for 15 min, and the supernatant was collected. Enzyme activity was determined using the standard reaction mixture described above (except the TPN concentration was 0.1 mM) and measuring that portion of the TPN-reduction which was inhibited by the subsequent addition of 0.2 μmole of L-G3P (the inhibition observed in all the present experiments was 90–100%).

Partial Purification of Nonreversible D-G3P Dehydrogenase from Pea Shoots. Green pea shoots were harvested 15 to 20 days after planting. All subsequent operations were at 2 to 4 C, and all centrifugations were at 15,000*g* for 20 min. Pea shoots (170 g) were homogenized with 400 ml of a solution containing 50 mM tris-HCl buffer, pH 8, 5 mM cysteine-HCl, and 1 mM $EDTANa_4$. The homogenate was squeezed through three layers of cheesecloth, centrifuged, and the supernatant was collected as the crude enzyme. This was stirred for 50 min with 110 g of dry activated charcoal which was washed thoroughly before use. The preparation was recovered from the charcoal by pouring through cheesecloth, centrifuged, and the supernatant was fractionated by the gradual addition of solid $(NH_4)_2SO_4$. During this operation the solution was maintained at pH 8 with 5 N NH_4OH. The protein which precipitated between 55 and 75% saturation with $(NH_4)_2SO_4$ was dissolved in 6 ml of the medium used for homogenization then dialyzed for 2 hr against 1 liter of a solution containing 10 mM tris-HCl buffer, pH 8, 1 mM cysteine-HCl, and 0.2 mM $EDTANa_4$. Ethanol at −20 C was added slowly to the dialyzed preparation until the concentration was 38% (v/v). Precipitated protein was removed at the centrifuge, and the supernatant was left overnight at 2 C, during which time more protein precipitated and was removed by centrifugation. The supernatant was dialyzed as described above, concentrated to 3 ml by ultrafiltration through a Diaflo XM-50 filter (Amicon Corp., Lexington, Mass.), and passed through a column of Sephadex G-200 (3.8 cm^2 × 18 cm). Fractions of 3 ml were collected, and those containing enzyme activity were combined and added to a column of diethylaminoethyl cellulose (3.8 cm^2 × 2.3 cm). The enzyme was eluted with a linear gradient of NaCl between 0.045 M and 0.113 M in 5 mM tris-HCl buffer, pH 8. Twenty-five 7-ml fractions were collected and assayed for nonreversible D-G3P dehydrogenase, TPN-linked reversible D-G3P dehydrogenase, and fructose-1,6-diP aldolase; those fractions containing activity of nonreversible D-G3P dehydrogenase only were combined and concentrated to 9 ml, as described above.

The specific activity of this preparation was 33 μmoles/hr·mg protein, representing a 20-fold increase in relative activity over the crude enzyme. The enzyme activity in the standard reaction mixture was constant for at least 3 min. The preparation was free of aldolase, reversible D-G3P dehydrogenases (TPN- and DPN-linked), and TPNH oxidase. Phosphatases active with triose-P and glycerate-3-P were present but at activities too low to interfere with the measurements reported here. The ratio of the specific activities of triose-P isomerase to nonreversible D-G3P dehydrogenase was reduced from 250 in the crude extract to 3.5 in the final preparation.

Partial Purification of Nonreversible D-G3P Dehydrogenase from Etiolated Pea Shoots, Pea Cotyledons, Peanut Cotyledons, and Maize Leaves. The purification procedure used in these experiments was similar to that described above for green pea shoots, except the Sephadex step was omitted. Preparations were obtained from etiolated pea shoots harvested 7 days from planting, pea cotyledons from mature seeds soaked 24 hr, cotyledons from peanuts germinated 17 days in the dark, and green leaves of 11-day maize plants; in the latter preparation 5% (w/v) insoluble polyvinylpyrrolidone was included in the solution for homogenization.

None of these preparations contained TPN-linked reversible D-G3P dehydrogenase activity; the DPN-linked enzyme was assayed in the pea cotyledon and peanut cotyledon preparations only and found absent in both cases.

RESULTS

Properties of the Partially Purified Nonreversible D-G3P Dehydrogenase from Pea Shoots. TPN reduction by the pea shoot nonreversible D-G3P dehydrogenase was directly proportional to the enzyme preparation added for amounts containing up to 44 μg of protein. No activity of Pi-dependent reversible

D-G3P dehydrogenase (TPN- or DPN-linked) was detected when assayed in the back direction (see "Materials and Methods"), although activity was observed after addition of crude pea shoot extract to the same reaction mixtures. These experiments confirmed that the TPN reduction observed was due entirely to the nonreversible enzyme.

Evidence was obtained that the reaction is indeed nonreversible, as reported by Rosenberg and Arnon (31). When 1 mM glycerate-3-P and 0.07 mM TPNH were incubated with the pea shoot preparation, no TPNH was oxidized. On the other hand, all TPN added to the standard reaction mixture was rapidly reduced. Stoichiometric studies indicated that 1 mole of glycerate-3-P was formed and 1 mole of TPN reduced for each mole of triose-P oxidized. Rosenberg and Arnon (31) demonstrated previously the molar equivalence of glycerate-3-P and TPN in the reaction catalyzed by the enzyme from sugar beet leaves. Also in agreement with these earlier studies (31), we observed that the pea shoot enzyme was not active when DPN replaced TPN. D-G3P could not be replaced by D-glyceraldehyde, dihydroxyacetone-P, D-erythrose-4-P, D-ribose-5-P, or D-glucose-6-P.

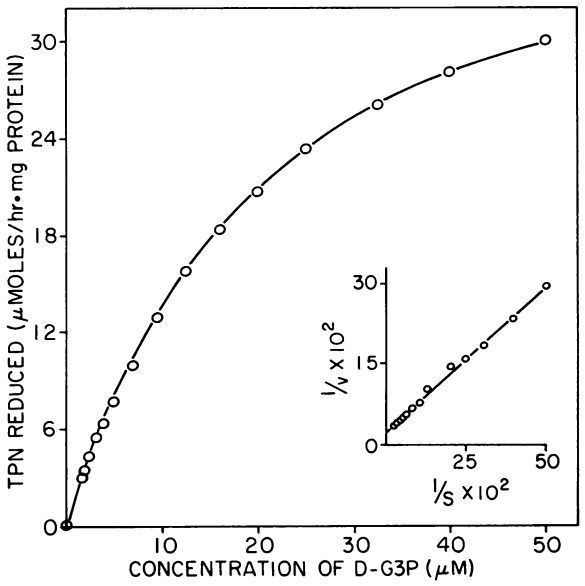

FIG. 1. Effect of D-G3P concentration on pea shoot nonreversible D-G3P dehydrogenase activity. Standard reaction mixtures were as described under "Materials and Methods," except the concentration of D-G3P was varied as shown. Inset: double-reciprocal (Lineweaver-Burk) plot of the results.

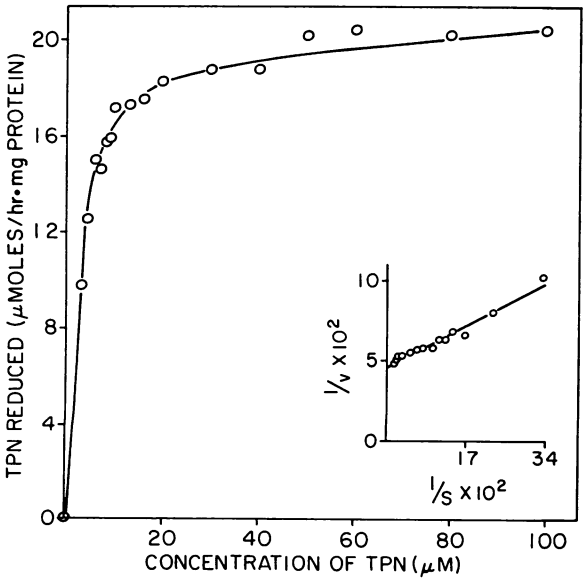

FIG. 2. Effect of TPN concentration on pea shoot nonreversible D-G3P dehydrogenase activity. Standard reaction mixtures were as described under "Materials and Methods," except the concentration of TPN was varied as shown. Inset: double-reciprocal (Lineweaver-Burk) plot of the results.

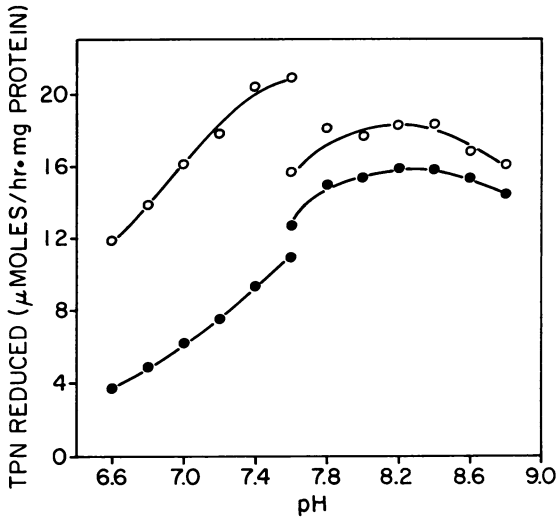

FIG. 3. Effect of pH on pea shoot nonreversible D-G3P dehydrogenase activity. Standard reaction mixtures were as described under "Materials and Methods," except the buffer was imidazole-HCl (pH 6.6–7.6) or tris-HCl (pH 7.6 to 8.8). ○: No L-G3P; ●: with 10 nmoles of L-G3P.

The *Km* of the pea shoot nonreversible D-G3P dehydrogenase for D-G3P was 22 μM (Fig. 1), very similar to the *Km* for the sugar beet leaf enzyme (31) and in contrast to the high value of 1.2 mM reported for the enzyme from *Hevea brasiliensis* latex (17). The pea shoot enzyme had a particularly high affinity for TPN as shown by the low *Km* of 3.3 μM (Fig. 2); somewhat lower affinities were reported for the enzymes from the other plant tissues (17, 31).

The effect of pH on enzyme activity, both in the absence and presence of the inhibitor L-G3P, is shown in Figure 3. Appreciable activity was observed at all pH values between 6.6 and 8.8; however, L-G3P considerably decreased activity at lower pH values. Previously, a comparatively low activity at pH 7.0 was reported for the sugar beet leaf enzyme (31); however, a mixture of the D- and L-isomers of G3P was added to the reaction mixtures in those experiments.

A number of compounds inhibited the activity of nonreversible D-G3P dehydrogenase (Fig. 4). Strong inhibition by L-G3P was reported recently from this laboratory (19). Further experiments showed D-erythrose-4-P was a more potent inhibitor; enzyme activity was reduced 50% by 5 μM D-erythrose-4-P (Fig. 4B) compared to 20 μM L-G3P required for a similar inhibition (Fig. 4A). These two compounds have been reported to reduce the activity of aldolase (22) and aspartate aminotransferase (25), and in the latter case Nisselbaum *et al.* (25) pointed out that the compounds possess both free aldehyde and phosphoryl groups which may have been required for the observed effect; higher homologues (ribose-5-P and glucose-6-P) which exist predominantly in the internal hemiacetal configuration had no effect. In the present experiments, the pea shoot nonreversible D-G3P dehydrogenase was only slightly inhibited by D-ribose-5-P (Fig. 4C) and was not significantly

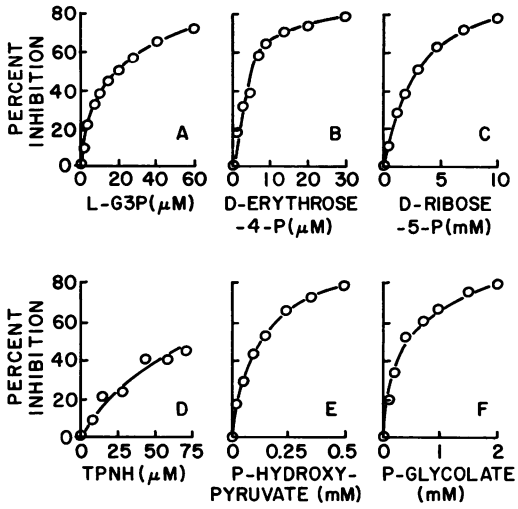

FIG. 4. Inhibition of pea shoot nonreversible D-G3P dehydrogenase activity by L-G3P (A), D-erythrose-4-P (B), D-ribose-5-P (C), TPNH (D), P-hydroxypyruvate (E), and P-glycolate (F). Reaction mixtures were as described under "Materials and Methods" for the standard assay (A to E, except in D only 0.01 μmole TPN was added) or with chloroacetol-P (F). Inhibitors were added at the concentrations shown. Control experiments showed there was no TPNH oxidation when pea shoot preparation was incubated with each inhibitor at the highest concentration used. In A to E the activity of triose-P isomerase, assayed in the direction dihydroxyacetone-P → D-G3P (28) was not affected by the inhibitors at the highest concentrations used.

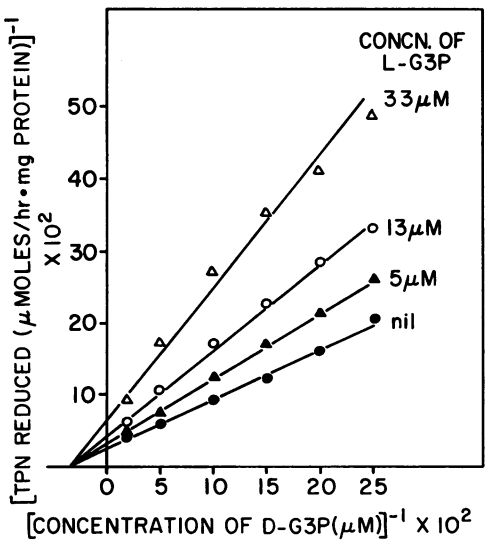

FIG. 5. Effect of D-G3P concentration on pea shoot nonreversible D-G3P dehydrogenase activity at several concentrations of L-G3P. Standard reaction mixtures were as described under "Materials and Methods," except the concentration of D-G3P was varied, and L-G3P was added as shown.

affected by 2 mM D-glucose-6-P; hence, the inhibitions shown in Figure 4, A and B, may also have been dependent on free aldehyde and phosphoryl groups.

Increased concentrations of D-G3P relieved the L-G3P inhibition (Fig. 5). When interpreted according to the nomenclature of Cleland (8), the results in Figure 5 show that the L-G3P inhibition was of the linear noncompetitive type with a *Km* of 22 μM. It is possible that the L-G3P binds to the active site in a manner analogous to the binding of the substrate D-G3P.

The products of the nonreversible D-G3P dehydrogenase reaction inhibited the enzyme activity. TPNH was a relatively strong inhibitor (Fig. 4D), but glycerate-3-P was far less effective: at 2 mM the enzyme activity was inhibited only 28%. A similar inhibition of the enzyme from *Hevea brasiliensis* latex was reported by Jacob and d'Auzac (17), but these authors could find no TPNH inhibition.

P-Hydroxypyruvate and P-glycolate also inhibited the pea shoot nonreversible D-G3P dehydrogenase activity, the concentrations required for 50% inhibition being 0.13 mM and 0.38 mM, respectively (Fig. 4, E and F). Enzyme activity was not significantly inhibited by either 0.5 mM hydroxypyruvate or 2 mM glycolate. Other compounds tested and found to have little or no influence on enzyme activity were 0.5 mM dihydroxyacetone-P, 2 mM P-enolpyruvate, 2 mM D-fructose-6-P, 2 mM D-fructose-1,6-diP, 1 mM gluconate-6-P, 0.3 mM D-sedoheptulose-7-P, and 2 mM ATP. A relatively high concentration of Pi (10 mM) inhibited the enzyme activity by 20%; a similar inhibition by Pi was observed previously (17, 31).

Some of the inhibitors of nonreversible D-G3P dehydrogenase were tested for an effect on TPN-linked, reversible D-G3P dehydrogenase. P-Hydroxypyruvate and P-glycolate inhibited both enzymes: the activity of the reversible enzyme (assayed under the conditions described in Table IV) was inhibited 44% by 0.25 mM P-hydroxypyruvate and 31% by 0.5 mM P-glycolate. However, this enzyme was less sensitive than the nonreversible enzyme to L-G3P and D-erythrose-4-P: L-G3P at 200 μM inhibited activity 26%, while 55 μM D-erythrose-4-P caused only 13% inhibition.

Comparison of Nonreversible D-G3P Dehydrogenases Partially Purified from Green Pea Shoots, Etiolated Pea Shoots, Pea Cotyledons, Peanut Cotyledons, and Maize Leaves. Although the specific activity of the nonreversible D-G3P dehydrogenase varied greatly in cell-free extracts from green and etiolated pea shoots, pea and peanut cotyledons, and maize leaves, the properties of the enzyme partially purified from each of these tissues were quite similar (Table I).

Distribution of Nonreversible D-G3P Dehydrogenase. Photosynthetic tissues from higher plants contain appreciable activities of nonreversible D-G3P dehydrogenase (Table II). However, the enzyme is not confined to the green parts of plants as previously suggested (13); activity has been reported in

Table I. *Properties of Nonreversible D-G3P Dehydrogenases Partially Purified from Several Plant Tissues*

The preparation and assay of nonreversible D-G3P dehydrogenase and estimation of protein are described under "Materials and Methods." *Km* values were obtained from experiments similar to those in Figures 1 and 2.

Source of Enzyme Preparation	Specific Activity in Cell-free Extract	*Km* Values		Inhibitors				
		D-G3P	TPN	5 μM D-Erythrose-4-P	10 μM L-G3P	100 μM P-Hydroxypyruvate	120 μM TPNH	200 μM P-Glycolate
	μmoles/hr·mg protein	*μM*		*% inhibition*				
Green pea shoot	1.68	22	3.3	48	37	45	19	35
Etiolated pea shoot	0.14	18	3.5	63	54	57	22	31
Pea cotyledon	0.02	17	2.3	32	36	43	26	33
Peanut cotyledon	1.50	25	5.0	50	50	50	14	46
Maize leaf	0.83	19	3.5	64	59	46	12	41

Table II. *Distribution of Nonreversible D-G3P Dehydrogenase*

The preparation and assay of crude extracts were as described under "Materials and Methods," except the buffer in reaction mixtures was tris-HCl, pH 7.5, and both triose-P isomerase and L-G3P were omitted. Triose-P isomerase in crude extracts was assumed sufficient to generate rapidly D-G3P from dihydroxyacetone-P (37); this assumption was confirmed for those tissues in which no activity was found (see text). The TPN reduction observed below was shown not to result from glucose-6-P dehydrogenase or TPN-linked reversible D-G3P dehydrogenase activity, since the TPN reduction was much faster with dihydroxyacetone-P as substrate than with the same concentration of either D-fructose-6-P or DL-G3P.

Enzyme Source	Specific Activity
	μmoles/hr·g fresh wt
Leaves	
Pea	13
Clover	24
Carrot	18
Spinach	10
Maize	8
Oat	10
Fern	15
Cotyledon	
Pea	1
Peanut (dark-grown)	28
Marrow (dark-grown)	38
Endosperm	
Castor bean	25
Pea shoot (dark-grown)	4

germinating castor bean endosperm (5), etiolated pea stem (34) and pine seeds (27), and in the present experiments high activities were detected in three nongreen gluconeogenic tissues, and a lower activity was found in dark-grown pea shoots (Table II). In contrast, no enzyme activity was detected in several other nongreen tissues including pea root, potato tuber, carrot root, and the shoot of the saprophytic angiosperm *Monotropa* sp., or in several unicellular photosynthetic algae and bacteria (including *Chlorella pyrenoidosa, Dunaliella tertiolecta,* and *Rhodospirillum rubrum*). No activity was found in cell-free extracts of yeast and rat muscle.

Changes in the Level of Nonreversible -G3P Dehydrogenase Activity. Work in this laboratory confirmed that L-G3P, at concentrations above 100 μM, almost completely inhibits pea shoot nonreversible D-G3P dehydrogenase (19), and that the sensitivity of the enzyme from other plant tissues to this inhibitor is similar (Table I). These observations permitted a relatively specific assay of the enzyme activity in cell-free extracts by employing the standard reaction mixture (see "Materials and Methods") and by measuring that portion of the TPN reduction inhibited by the subsequent addition of 0.2 μmole L-G3P. Glucose-6-P dehydrogenase and TPN-linked reversible D-G3P dehydrogenase, two enzymes which may also catalyze TPN reduction in a cell-free extract, were much less sensitive to inhibition by this concentration of L-G3P.

By using this assay procedure, the levels of nonreversible D-G3P dehydrogenase activity in pea shoots (green and etiolated), pea cotyledons, and castor bean endosperm were followed during germination (Figs. 6–9). No activity was detected in the pea shoot embryo during early germination, but activity appeared just prior to emergence at 5 days (Fig. 6). The activity increased rapidly in green shoots during leaf expansion and remained at this high level. A similar increase occurred in etiolated pea shoots, but the final activity attained was only about one-fifth that of the green plants. In pea cotyledons the activity remained quite low.

Light markedly increased the enzyme level in dark-grown pea shoots (Figs. 7 and 8). When etiolated shoots were transferred to natural lighting, the enzyme level increased to that of green shoots within 2 days; even 1 hr of light caused a doubling of activity during the subsequent 24 hr (Fig. 7).

This latter observation suggested that phytochrome may mediate the light-stimulated increase in enzyme activity. Evidence that this is so is shown in Figure 8; red light irradiation of etiolated pea shoots caused a 75% increase in enzyme activity, but far red light largely reversed this effect. Similar effects have been reported for other extra plastid enzymes, for example, glycolate oxidase (21).

Endosperm tissue of germinating castor bean contained

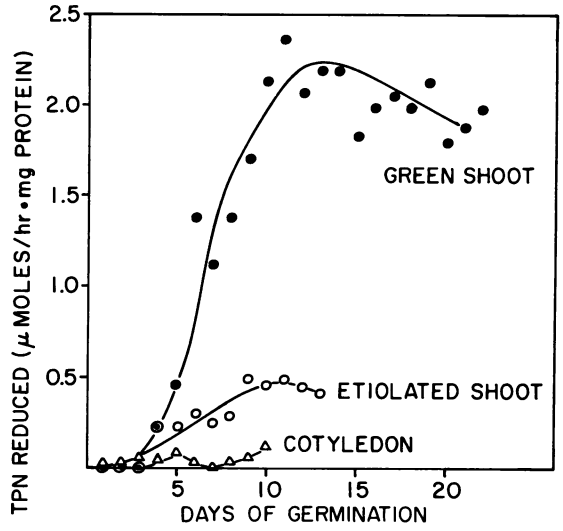

FIG. 6. Changes in nonreversible D-G3P dehydrogenase activity during germination of peas. The preparation of crude extracts, assay of enzyme activity, and determination of protein are described under "Materials and Methods." ●: Green shoots; ○: etiolated shoots; △: cotyledon.

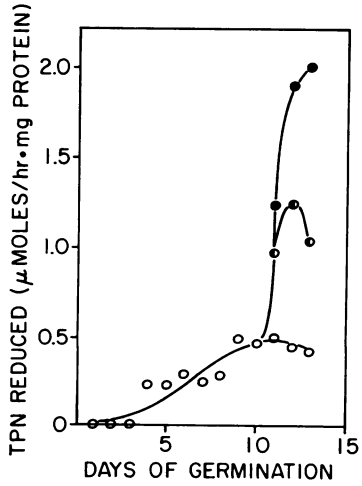

FIG. 7. Effect of light on nonreversible D-G3P dehydrogenase activity in dark-grown pea shoots. The preparation of crude extracts, assay of enzyme activity, and determination of protein are described under "Materials and Methods." ○: No light; ◐: exposed to white light for 1 hr on the 10th day; ●: transferred on the 10th day to natural light regime.

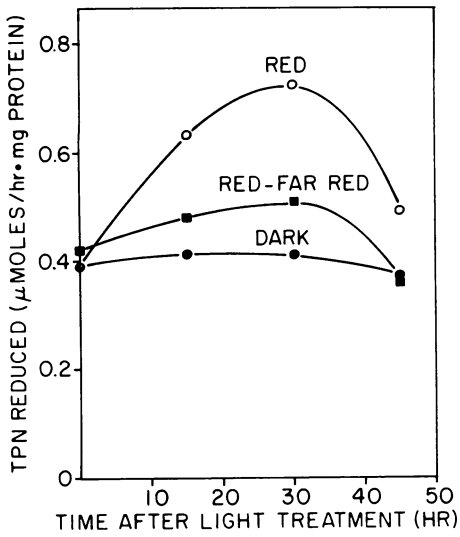

FIG. 8. Effect of red and far red light on nonreversible D-G3P dehydrogenase activity in dark-grown pea shoots. Three lots of peas, germinated 8 days in the dark, were treated separately as follows: ●: No light; ○: 15 min red light; ■: 15 min red light followed immediately by 15 min far red light. At the times indicated, crude extracts were prepared and assayed for enzyme activity and protein as described under "Materials and Methods."

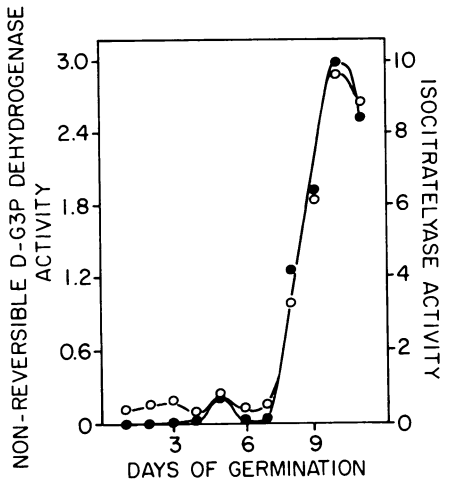

FIG. 9. Changes in the levels of nonreversible D-G3P dehydrogenase and isocitrate lyase in the endosperm of castor bean germinating at 18 C. Crude extracts were prepared and assayed for enzyme activity and protein as described under "Materials and Methods;" the activity of isocitrate lyase was determined by the method of Cooper and Beevers (9). Activities are expressed in μmoles/hr·mg protein. ○: Nonreversible D-G3P dehydrogenase; ●: isocitrate lyase.

activity of nonreversible D-G3P dehydrogenase only after active conversion of fat to carbohydrate, as evidenced by the appearance of isocitrate lyase activity (6), had begun (Fig. 9). In fact, the appearance and increase of both enzyme activities were very similar. However, glyoxysomes isolated from this endosperm on a stepped sucrose density gradient and identified by isocitrate lyase activity (9) contained no activity of nonreversible D-G3P dehydrogenase, suggesting that this enzyme is located outside the glyoxysome.

Absence of Nonreversible D-G3P Dehydrogenase in Chloroplasts. There are conflicting reports as to whether nonreversible D-G3P dehydrogenase occurs in chloroplasts (31, 34). In the present experiments, strong evidence was obtained that the enzyme is localized outside the chloroplast, since no activity could be detected in a crude preparation from isolated chloroplasts, although activity was clearly present in the supernatant fraction (Table III). This result was obtained whether the crude or partially purified preparation was assayed; in the latter case, it was possible to add a large proportion of the chloroplast protein to the assay mixture so that even low specific activities would have been detected if present. In contrast, the TPN-linked reversible D-G3P dehydrogenase, which is located in the chloroplast (23), was easily detected in the chloroplast preparation obtained here (Table III).

Table III. *Activities of TPN-Linked D-G3P Dehydrogenases (Reversible and Nonreversible) in Chloroplast and Cytoplasm of Spinach Leaves*

Spinach leaves (10 g) were homogenized for 3 sec in 40 ml of aqueous medium (modification of solution A of Jensen and Bassham [18]), and the homogenate was filtered and centrifuged 2400*g* for 90 sec. The supernatant was reserved for assay. The chloroplasts were washed briefly with the same medium, centrifuged 1900*g* for 60 sec, then broken in 20 ml of a solution containing 50 mM tris-HCl buffer, pH 8, 5 mM cysteine-HCl, and 1 mM $EDTANa_4$. Samples of each solution were partially purified by $(NH_4)_2SO_4$ fractionation and dialysed, as described for the pea shoot preparation (see "Materials and Methods"). Enzyme activities were assayed as described under "Materials and Methods;" the reversible D-G3P dehydrogenase was assayed in the back direction.

Fraction	Nonreversible D-G3P Dehydrogenase		TPN-linked Reversible D-G3P Dehydrogenase	
	Crude	Partial purification	Crude	Partial purification
	μmoles/hr·mg protein			
Chloroplasts	0	0	1.3	9.2
Supernatant	2.1	4.0	8.1	20

Comparison of the Properties and Activities of the Nonreversible and Reversible D-G3P Dehydrogenases. A preparation of TPN-linked reversible D-G3P dehydrogenase (free of the nonreversible D-G3P dehydrogenase) was obtained from pea shoots and compared with the nonreversible enzyme (Table IV). At least with respect to some properties, these two enzymes behave quite differently. The greater affinity of the nonreversible enzyme for the substrates is particularly obvious; the affinity for TPN is 40 times that of the reversible enzyme. This observation suggested that the nonreversible D-G3P dehydrogenase may be more important *in vivo* than previously believed (34). Consequently, the activities of this enzyme and the reversible D-G3P dehydrogenases (TPN- and DPN-linked) were compared in a cell-free pea shoot extract with varying substrate concentrations (Table V). Although the activities of the reversible D-G3P dehydrogenases were greater than the activity of the nonreversible enzyme at high substrate concentrations, quite the opposite situation was observed when the substrate concentrations were lower and probably closer to physiological levels (10, 14).

DISCUSSION

Very little information on the nonreversible D-G3P dehydrogenase has been reported since the discovery and initial studies of the enzyme 18 years ago (2–4, 11, 31). Recent work in this laboratory has shown that commercial preparations of G3P are unsuitable for determining the enzyme activity be-

Table IV. *Properties of TPN-Linked D-G3P-Dehydrogenases (Reversible and Nonreversible) from Pea Shoots*

The TPN-linked reversible D-G3P dehydrogenase was partially purified from a crude pea shoot extract as follows. The extract was heated to 60 C (this treatment inactivates the nonreversible enzyme [31]), cooled, centrifuged, and the supernatant was fractionated with $(NH_4)_2SO_4$; protein which precipitated between 40 and 60% saturation was dialyzed and used as the source of enzyme. Enzyme activity was assayed in the forward direction as described under "Materials and Methods," except the substitute was generated from dihydroxyacetone-P by triose-P isomerase instead of being added as DL-G3P. Activity was absolutely dependent on the presence of arsenate. The nonreversible D-G3P dehydrogenase was partially purified and assayed as described under "Materials and Methods."

Property	Non-reversible D-G3P Dehydro-genase	TPN-linked Reversible D-G3P Dehydro-genase
Km for D-G3P (μM)	22	80
Km for TPN (μM)	3.3	130
Inhibition by 0.2 mM L-G3P (%)	96	26
Inhibition by 0.1 mM chloroacetol-P (%)	3	100

Table V. *Effect of Concentration of Substrates on the Activities of Pea Shoot D-G3P Dehydrogenases*

The preparation of a crude pea shoot extract and estimation of protein were as described under "Materials and Methods." Enzyme activities were determined as described under "Materials and Methods," except the concentrtion of substrates was varied as shown; D-G3P was generated from dihydroxyacetone-P. The two TPN activities were separated as those inhibited (nonreversible enzyme) and unaffected (reversible enzyme) by the subsequent addition of 0.2 μmole of L-G3P.

Substrates	Nonreversible D-G3P Dehydrogenase	TPN-linked Reversible D-G3P Dehydrogenase	DPN-linked Reversible D-G3P Dehydrogenase
	μmoles/hr·mg protein		
200 μM TPN (or DPN) and 500 μM DL-G3P		2.4[1]	11.8[1]
200 μM TPN (or DPN) and 50 μM D-G3P	2.6	ND[2]	5.5
20 μM TPN (or DPN) and 25 μM D-G3P	1.8	ND	0.5
20 μM TPN (or DPN) and 5 μM D-G3P	0.6	ND	ND

[1] The buffer in these assays was tris-HCl, pH 8.5.

[2] Not detected.

cause of the presence of the L-isomer which is a strong inhibitor (19); this problem may not have been recognized in previous investigations. Enzyme activity may be assayed by generating the substrate enzymically from D-fructose-1,6-diP (17, 31) or dihydroxyacetone-P (19).

The properties of nonreversible D-G3P dehydrogenase, partially purified from several plant tissues, showed no significant variation from tissue to tissue, and where similar experiments have been reported for the enzyme from other tissues (17, 31), comparable results were obtained in most instances. In contrast, the distribution and activity of the enzyme varied markedly from tissue to tissue (Table II). Activity appeared to be restricted to photosynthetic and gluconeogenic tissues of higher plants, and even in these tissues the activity remained low until the capacity for photosynthesis (or gluconeogenesis) developed. The increase in leaves coincided with exposure to light and was probably phytochrome-mediated (Figs. 7 and 8), whereas in germinating, fat-storing seeds the increase was closely associated with that of isocitrate lyase (Fig. 9).

Evaluation of the role of nonreversible D-G3P dehydrogenase in green leaves has been complicated by the presence of TPN-linked reversible D-G3P dehydrogenase (31, 34). However, the latter enzyme is probably restricted to the chloroplast (23), whereas the nonreversible D-G3P dehydrogenase is most likely located outside the chloroplast ([34], Table III). In addition, while the activity of the reversible enzyme is considerably greater at higher substrate concentrations (34), the present investigation has shown that the activity of the nonreversible enzyme is greater than that of either the TPN- or DPN-linked reversible enzymes at substrate concentrations which are probably more representative of *in vivo* levels (10, 14). This observation is consistent with the greater affinity of the nonreversible enzyme for substrates; the Km values for D-G3P and pyridine nucleotide are much lower than those of the reversible enzymes ([32], Table IV). Also, since the enzyme reaction does not require ATP, ADP, or Pi, glycerate-3-P may be formed even when the ATP/ADP ratio is high. Indeed, a number of investigators have reported that a substantial portion of the TPN reduction in cell-free plant extracts supplied with D-fructose-1,6-diP was mediated by this enzyme (3, 4, 7, 11).

A role for nonreversible D-G3P dehydrogenase in green leaves may be to form TPNH in the cytoplasm from triose-P produced during photosynthesis in the chloroplast. Although whole chloroplasts are relatively impermeable to TPN and TPNH (30), the presence of nonreversible D-G3P dehydrogenase in the cytoplasm could permit an indirect transfer of photosynthetically produced TPNH from the chloroplast to the cytoplasm via a shuttle of triose-P and glycerate-3-P. An analogous scheme has been proposed by Stocking and Larson (35) for the reduction of extra plastid DPN. It is interesting that Heber and Santarius (15) found most of the cytoplasmic TPN in leaves occurred in the reduced state.

The nonreversible D-G3P dehydrogenase may also catalyze TPNH formation from glycolytically produced triose-P in the dark and thus represent a source of TPNH in the dark for photosynthetic and gluconeogenic tissues. This TPNH could be utilized for biosynthesis; Thomas and ap Rees (36) have suggested that glycolysis is required in gluconeogenic tissues for the provision of intermediates for biosynthesis, and it would seem appropriate that these tissues also contain active nonreversible D-G3P dehydrogenase which could couple TPNH formation to glycolysis. A similar situation may apply to green leaves in darkness. The activity of the enzyme may be controlled through the level of the product glycerate-3-P; this compound strongly inhibits plant phosphofructokinase (20), and it is not inconceivable that accumulation of glycerate-3-P *in vivo* would limit glycolysis thereby reducing the availability of triose-P for nonreversible D-G3P dehydrogenase.

The enzyme activity may also be regulated by some of the inhibitors shown in Table I. D-Erythrose-4-P, the most effective inhibitor, is produced from D-G3P by transketolase in the oxidative pentose phosphate pathway. When this pathway is operating, TPN reduction is coupled to the direct oxidation of glucose, and it would seem reasonable that TPN reduction by the nonreversible D-G3P dehydrogenase reaction may not be required at the same time and could be prevented by the level of D-erythrose-4-P present. The inhibition of enzyme activity

by P-hydroxypyruvate may be involved in the control of serine biosynthesis, since this compound is an intermediate in the conversion of glycerate-3-P to serine (33).

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