RESPIRATION OF THE PEA PLANT. METABOLISM OF HEXOSE PHOSPHATE AND TRIOSE PHOSPHATE BY CELL-FREE EXTRACTS OF PEA ROOTS[1,2]

MARTIN GIBBS,[3] JOAN M. EARL AND JEAN L. RITCHIE

BIOLOGY DEPARTMENT, BROOKHAVEN NATIONAL LABORATORY, UPTON, LONG ISLAND, NEW YORK

The urge to characterize the carbon transformations in photosynthesis has rapidly resulted in much evidence for the presence of two pathways of carbohydrate metabolism, the Embden-Meyerhof-Parnas and the direct oxidation pathway in the green leaves and seeds of higher plants (2, 3, 4, 6, 8, 11, 13, 24). However, investigations concerning these pathways in other portions of the plant have been limited (6, 7, 12, 25).

The present investigation was undertaken to establish whether the primary enzymes of the two pathways are present in the roots and, if so, whether the pathways are similar to those in chlorophyll-containing tissue. The pea plant (12 to 15 days) was selected for this study.

EXPERIMENTAL MATERIALS

Glucose-6-phosphate (G-6-P) was prepared as the heptahydrate of the barium salt by the procedure of Seegmiller and Horecker (21). The crude salt was dissolved in a small amount of HCl, adjusted to pH 8.0 with saturated $Ba(OH)_2$ and left overnight at 4 to 5° C. The insoluble heptahydrate which crystallized out was washed with ethanol and acetone. Enzymatic analysis (17) showed it to be 99.3 % pure.

Fructose-6-phosphate (F-6-P) was purchased from the Schwarz Laboratories as the barium salt. It was found free of G-6-P and FDP.

A commercial preparation of the barium salt of fructose-1,6-diphosphate (FDP) was converted to the potassium salt and the contaminating inorganic phosphate removed with magnesia mixture. The product used was free of inorganic phosphate and contained 97.5 % FDP as determined enzymatically (20). This FDP was used in the aldolase experiments. In all other experiments involving FDP, the commercial preparation was used.

The barium salt of 6-phosphogluconate (6-PG) was prepared by the method of Seegmiller and Horecker (21). Enzymatic analysis indicated 82 % 6-PG and traces of G-6-P.

Phosphoglyceric acid (PGA) was purchased from the Schwarz Laboratories. Phosphorus analysis (total phosphorus-hydrolyzable phosphorus) showed it to be at least 99 % pure.

Triphosphopyridine nucleotide (TPN) was prepared by the method of LePage and Mueller (18). It contained 31 % TPN (analyzed with G-6-P dehydrogenase) and no DPN (analyzed with alcohol dehydrogenase). A sample (72 %) purchased from the Sigma Chemical Company was also used.

Diphosphopyridine nucleotide (DPN) and adenosine triphosphate (ATP) were purchased from the Schwarz Laboratories.

Lactic acid dehydrogenase, G-6-P dehydrogenase, phosphohexose isomerase and the α-glycerol phosphate dehydrogenase mixture (used to assay FDP) were kindly supplied by Dr. B. L. Horecker. Aldolase was kindly supplied by Dr. E. Racker.

METHODS

PREPARATION OF PEA ROOT EXTRACTS: *Pisum sativum* variety Alaska (Roger Brothers Seed Co., Idaho Falls, Idaho) was used throughout this investigation The seeds were soaked overnight in tap water at room temperature, then transferred to flats containing acid-washed sand or gravel. The material was watered daily with tap water. The temperature in the house was 16 to 20° C. During the winter (November through March) the solar radiation was supplemented with artificial light from 8 A.M. to 5 P.M. However, during periods of little solar radiation, some batches of plants became etiolated; these plants were not used. The plants were similar to those used in a previous investigation (11). The roots were cut off slightly below the cotyledons. They were washed in tap water, followed by a washing in distilled water. Extracts were made of this material in two ways: 1) 70 gm of roots were ground in the cold room (4° C) with an equal weight of acid-washed, cold, sharp sand and 5 ml of cold 0.5 M $KHCO_3$ and 5 ml ethylene diamine tetra-acetic acid (versene), pH 8.0 (30 mg/ml). The brei was strained through 4 thicknesses of cheesecloth and centrifuged at 18,000 × g for 20 minutes. The supernatant solution had a light tan color, its volume was 50 to 100 ml and its pH was approximately 7.6. This preparation will be designated hereafter as extract I. 2) Approximately 50 gm of roots were blended for 1 minute in a chilled Waring blendor with 400 ml of acetone cooled to − 18° C. The homogenate was filtered rapidly with suction. The dry powder was reblended with another 300 ml of acetone. When dry, the powder was screened through a plastic No. 20 sieve, then stored at − 18° C in a desiccator containing P_2O_5. Extracts were made by grinding the powder with 0.1 M tris (hydroxymethyl)-amino-methane (TRIS) buffer pH 8.0 for 30 minutes at room temperature. The mixture was centrifuged at 18,000 × g for 20 minutes. On occasion the supernatant material was passed through glass wool. This preparation is extract II.

ANALYTICAL DETERMINATIONS: Inorganic phosphate was determined by the procedure of Fiske and Subbarow (10). Inorganic phosphate liberated by

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[3] Present address: Department of Botany, University of Pennsylvania, Philadelphia 4, Pennsylvania.

hydrolysis during 20 minutes in 1 *N* KOH at room temperature was used as a measure of triose phosphorus. Iodine oxidation to differentiate the trioses was carried out according to Stumpf (23). The method of Mejbaum (19), modified by extending the heating period to 40 minutes, was used to determine pentose; D-arabinose (Pfanstiehl) was used as standard. G-6-P was determined by its ability to react with TPN in the presence of G-6-P dehydrogenase. F-6-P was determined in a similar manner with the addition of phosphohexose isomerase. FDP was determined by a method of Racker (20).

ESTIMATION OF DEHYDROGENASE ACTIVITY: Dehydrogenase activity was determined by following the rate of reduction of TPN or DPN at 340 mμ in 1-cm cells in a Beckman spectrophotometer, model DU, at room temperature. Usually the cell contained 0.2 ml extract, 10 μM of $MgCl_2$, 0.1 μM of TPN or DPN, 125 μM of glycylglycine buffer or 100 μM of TRIS buffer in a total volume of 3.0 ml. The reaction was started by the addition of 0.1 ml substrate to both cells, the blank cell lacking TPN or DPN. The inhibitor studies were carried out by incubating the test system with the poison for 5 minutes before addition of substrate.

RESULTS AND DISCUSSION

ENZYMES OF THE DIRECT OXIDATIVE PATHWAY, *G-6-P and 6-PG Dehydrogenases:* As demonstrated for most material (2, 11, 14), the direct oxidative pathway consists of two TPN-linked dehydrogenases which converts G-6-P to pentose phosphate and CO_2 via 6-PG. Spectrophotometric data indicate that the cell-free preparations of pea root also possess TPN-specific enzymes catalyzing this pathway.

Properties of G-6-P and 6-PG Dehydrogenases: The pH-activity curves for both dehydrogenases, using extracts of acetone powders, were determined in 0.04 M glycylglycine. While G-6-P dehydrogenase has an optimum pH at approximately 7.8, 6-PG dehydrogenase has its highest activity at approximately pH 7.5. The pH optimum for G-6-P dehydrogenase is similar to that found for yeast (16) and rat liver (14). The pH optimum for 6-PG dehydrogenase is close to the value of 7.6 found for purified yeast 6-PG dehydrogenase by Horecker and Smyrniotis (16), but is in contrast to the high pH optimum of 9.0 for the rat liver enzyme (14). To rule out TPN reduction by further products of 6-PG metabolism, ribose-5-phosphate (3.4 μM) was tested under the same conditions; no TPN reduction occurred.

The Effect of Inhibitors and Activators on the Dehydrogenases: Mg^{++} (3×10^{-2} M) doubles the rate of G-6-P dehydrogenase while 6-PG dehydrogenase activity is increased by one-half. High concentrations of iodoacetamide (1×10^{-2} M) inhibit both dehydrogenases slightly (20 %). However, the dehydrogenases are inhibited completely by 1×10^{-3} M *p*-chloromercuribenzoate. These data are similar to those reported for the liver dehydrogenases (14).

Stoichiometry of 6-PG Oxidation: The crude enzyme preparations used in these studies possess enzymes capable of reoxidizing DPNH but reoxidize TPNH slowly or to a limited extent. In order to accumulate the reaction products and avoid the use of stoichiometric amounts of TPN it was necessary to furnish a mechanism for the oxidation of the reduced coenzyme. This was accomplished by coupling the oxidation reaction to the reduction of pyruvate to lactate in the presence of rabbit muscle lactic acid dehydrogenase. The equations for the coupled systems are:

$$\text{6-Phosphogluconate} + \text{TPN} \longrightarrow \text{pentose phosphate} + CO_2 + \text{TPNH}$$

$$\text{Pyruvate} + \text{TPNH} \longrightarrow \text{lactate} + \text{TPN}$$

In agreement with these equations the data in table I show that for each mole of CO_2 and pentose phosphate formed, a mole of pyruvate is reduced. The reduction of pyruvate is accomplished by a stoichiometric amount of lactate, determined by the procedure of Barker and Summerson (5). The appearance of small amounts of F-6-P (0.5 μM) and traces of G-6-P (0.03 μM) indicates a further metabolism of the pentose phosphate in a manner previously described (13). Similar data have been obtained with turnip root (6) by coupling the oxidation to the reduction of glutathione.

Recently a second pathway of 6-PG oxidation (9, 26) alternate to that catalyzed by 6-PG dehydrogenase has been described. Attempts to find this second pathway in our pea root preparations have been unsuccessful.

GLYCOLYTIC ENZYMES, *The Conversion of FDP to Hexosemonophosphate (HMP):* In TRIS extracts of acetone powder, FDP is utilized and HMP is formed (fig 1). The utilization of FDP is considerably more rapid than the formation of HMP. The addition of Mg^{++} resulted both in a more rapid utilization of FDP and in a greater formation of HMP. Magnesium probably activates a FDP phosphatase. Further analysis of the HMP showed it to contain 72 % G-6-P

TABLE I

STOICHIOMETRY OF PHOSPHOGLUCONATE OXIDATION

CONC	PHOSPHOGLUCONATE μM	PYRUVATE μM	CO_2 μM	PENTOSE μM	LACTATE μM
Initial	12.0	24.0	..	4.4	...
Final	2.0	15.0	8.2	11.7	11.1
Δ	10.0	9.0	8.2	7.3	11.1

The test system contained, in addition to the initial quantities shown in the table: 100 μM glycylglycine buffer, pH 7.5, 0.31 μM of TPN, 20 μM of $MgCl_2$, 0.3 mg of lactic acid dehydrogenase, and 1.0 ml extract I, in a total volume of 2.05 ml. The gas space contained nitrogen; temperature 30° C. The reaction was begun by tipping 6-PG and TPN from one side arm and stopped by addition of 0.2 ml of 50 % TCA from a second side arm. Total CO_2 includes CO_2 liberated by the TCA. The figures in the table are corrected for endogenous reactions by running a control in which phosphogluconate was omitted.

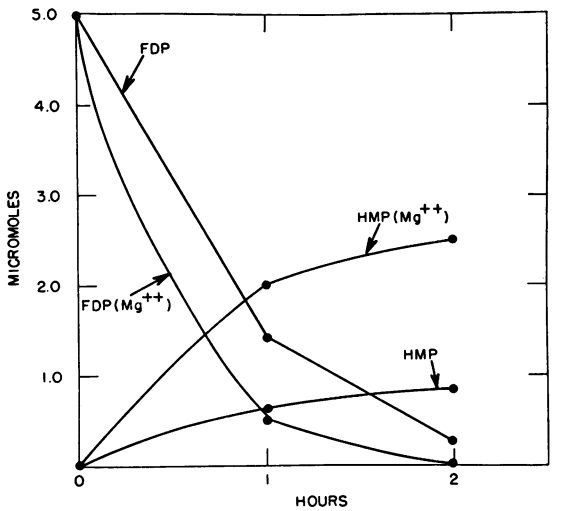

FIG. 1. Hexose monophosphate formation from fructose diphosphate. The incubation mixtures contained 5.0 μM of FDP, 1.0 ml of extract II, 20 μM of $MgCl_2$ (control contained water) in a total volume of 1.3 ml. The pH was 8.0. Incubation was at 37.5° C. At the intervals indicated, aliquots were diluted with 4 volumes of water, heated for 2 min at 100° C and centrifuged to remove the coagulated protein. Aliquots were assayed for HMP and FDP.

and 28 % F-6-P. It is evident that the expected stoichiometry of a mole of hexosemonophosphate formed per mole of FDP utilized was not obtained. This was probably caused by a further metabolism of the HMP by a series of transketolase and transaldolase reactions.

Rate of TPN Reduction by G-6-P, F-6-P and FDP: Table II presents typical rates of TPN reduction by the crude extracts when the substrate is G-6-P, F-6-P or FDP and shows the effect of Mg^{++} on the rates. It can be seen that 1) the versene treated extracts possess enzymes catalyzing reduction of TPN only with G-6-P or F-6-P as substrate, 2) the addition of Mg^{++} increases the rate of reduction with these substrates but also permits reduction by FDP. Reduction of TPN by G-6-P is catalyzed by G-6-P dehydrogenase while reduction by F-6-P and FDP is apparently caused by a conversion of these substrates to G-6-P. It is evident that the enzymes catalyzing the conversion of FDP and F-6-P to G-6-P are more affected by Mg^{++} than G-6-P dehydrogenase.

TABLE II

RATE OF TRIPHOSPHOPYRIDINE NUCLEOTIDE REDUCTION BY GLUCOSE-6-PHOSPHATE, FRUCTOSE-6-PHOSPHATE AND FRUCTOSE-1,6-DIPHOSPHATE

PHOSPHATES	Mg^{++}	NO ADDITION
G-6-P	0.019	0.008
F-6-P	0.015	0.003
FDP	0.006	0.000

The figures represent the change in optical density at 340 mμ from 0.5 to 1.0 min in a test system given in the methods section. The extracts are type I.

Aldolase: This enzyme was measured by the hydrazine fixing technique in which the triose phosphate, formed by the splitting of FDP, reacts with hydrazine to yield triose phosphate hydrazones thereby allowing the reaction to go to completion. On incubation of 50 μM of FDP with the crude extract for 30 minutes, 14.4 μM of alkali-hydrolyzable phosphate was formed indicating the presence of aldolase. On further analysis, the triose phosphate fraction was found to be 60 % glyceraldehyde-3-phosphate (G-3-P) and 40 % dihydroxyacetone phosphate. Similar data with pea root extracts have been reported by Stumpf (25).

Glyceraldehyde-3-Phosphate (G-3-P) Dehydrogenase: As seen in figure 2, the extracts contain a DPN-linked G-3-P dehydrogenase. TPN is inactive in this system. The reaction does not proceed in the absence of arsenate. Also 1×10^{-3} M iodoacetamide completely inhibits the enzyme. Aged enzyme preparations can be reactivated by cysteine. These properties suggest that the root possesses a typical G-3-P dehydrogenase as was also found in pea seed (22). The reoxidization of DPNH on addition of acetalde-

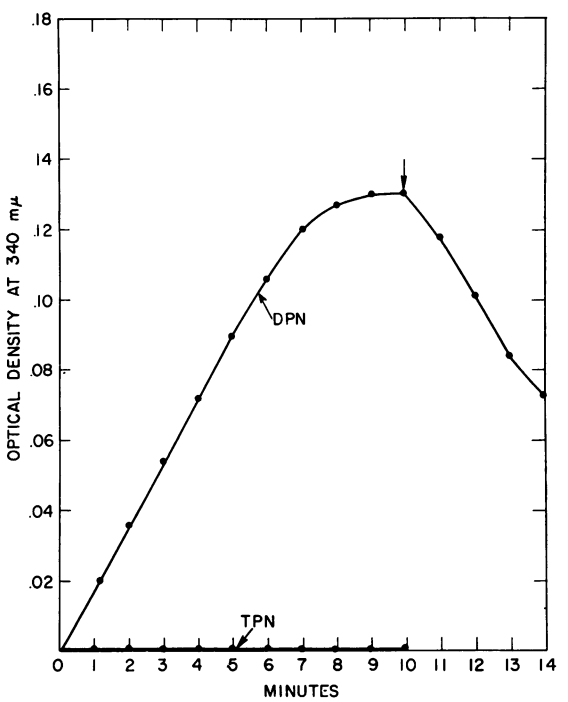

FIG. 2. Presence of triosephosphate dehydrogenase. The absorption cell contained veronal buffer, pH 8.0, 52 μM of Na arsenate, 60 μM of NaF, 12 μM of cysteine, 0.05 ml of extract I, 0.1 μM of DPN or 0.095 μM of TPN in a total volume of 3.0 ml. The reaction was begun by addition of 1.5 μM of DL-G-3-P. At arrow, 50 μM of acetaldehyde added. Readings were made against a blank containing all components except G-3-P.

hyde indicates that roots of this age still possess an alcohol dehydrogenase.

Effect of Versene on G-3-P Dehydrogenase: Inhibition of G-3-P dehydrogenase by iodoacetamide and reactivation by cysteine suggests that the enzyme depends on sulfhydryl groups for activity. Since root tissue tends to accumulate heavy metals which inhibit sulfhydryl enzymes, it was of interest to determine the effect of versene, an agent capable of removing such metals, on the activity of the enzyme during the preparation of the crude extracts. Figure 3, curve 1, indicates that the enzyme is inactive when the tissue is ground in the absence of versene but partially active when the chelating agent is present. However, as indicated in both curves, cysteine is necessary to restore full activity.

Phosphatase Activity: While animal phosphatases tend to be dependent on an alkaline pH for activity, plant phosphatases require an acid pH for activity. The data in table III agree with this general statement since inorganic phosphate was liberated from G-6-P only at an acid pH. The appearance of phosphate was inhibited by fluoride but unaffected by the addition of Mg^{++}. This phosphatase activity is not specific for G-6-P since F-6-P was also dephosphorylated. Since the conversion of FDP to F-6-P occurred at pH 8 and was sensitive to Mg^{++}, it appears that this hexosediphosphatase is not the same enzyme as that liberating phosphate from G-6-P or F-6-P. The enzyme catalyzing the breakdown of FDP to F-6-P is probably similar to that isolated from liver and designated as Gomori's phosphatase (15).

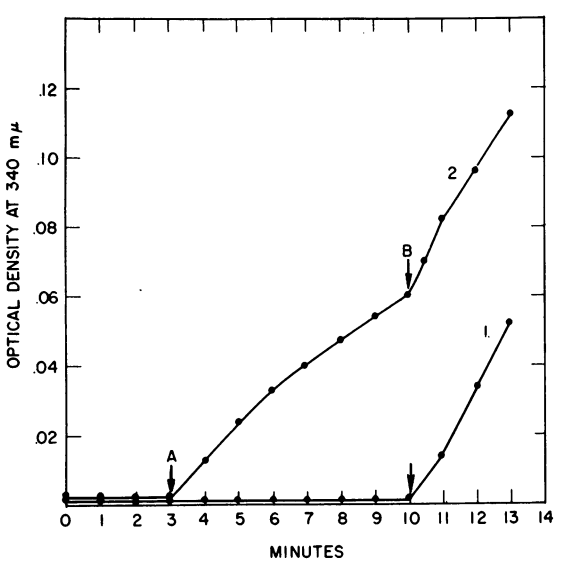

FIG. 3. Effect of versene and cysteine on triosephosphate dehydrogenase. The cuvettes contained veronal buffer, pH 7.9, 52 μM of Na arsenate, 60 μm of NaF, 0.2 μM of DPN, 0.03 ml of crystalline rabbit muscle aldolase and 0.2 ml of extract in a total volume of 3.0 ml. At arrow A, 5 μM of FDP added. At arrow B, 12 μM of cysteine added. Readings were made against a blank containing all components except DPN.

The extract used in curve 2 was prepared by grinding 9.0 gm of root tissue with 5 ml of 0.1 M $KHCO_3$ and 2 ml of versene (30 mg/ml), pH 8.0. The extract used in curve 1 was prepared without versene.

TABLE III

EFFECT OF pH ON PHOSPHATASE ACTIVITY IN PEA ROOT

MEASUREMENTS	ACID MEDIUM			ALKALINE MEDIUM		
	CONTROL	F^-	Mg^{++}	CONTROL	F^-	Mg^{++}
Final, μM PO_4	5.64	3.91	5.30	3.69	3.64	3.56
Initial, μM PO_4	3.43	3.60	3.58	3.41	3.32	3.11
Δ	2.11	0.31	1.72	0.28	0.32	0.45
pH	6.1	5.9	6.1	8.0	8.1	8.0

The test system contained 1.0 ml of extract II and 10 μM of G-6-P in a total volume of 1.2 ml. Where indicated the test system contained 20 μM of KF or 20 μM of $MgCl_2$. The mixture was adjusted to the indicated pH with 2 *N* acetic acid or 2 *N* NaOH. The incubation temperature was 30° C. At zero time and 1 hr, aliquots were diluted with 2.5 volumes of water, heated for 2 min at 100° C and centrifuged. The supernatant solution was analyzed for phosphate.

CONCLUSIONS

The present study taken together with others from this laboratory (12, 13) establishes the existence in the pea root of pathways which have been designated as direct oxidation and Embden-Meyerhof-Parnas. As in yeast, animal and other plant tissue, the dehydrogenases of the direct oxidation pathway are TPN-specific and are not inhibited by iodoacetamide. As in yeast and animal tissue, the one oxidative enzyme of the Embden-Meyerhof-Parnas scheme, triose phosphate dehydrogenase, is DPN dependent and is inhibited by iodoacetamide.

The only difference between the root and leaf with respect to breakdown of carbohydrate appears to be in the properties of triose phosphate dehydrogenase. While the root and leaf both possess a DPN-linked enzyme, the leaf tissue has, in addition, a triose phosphate oxidizing system dependent on TPN. It was noted that the absence of arsenate inhibits DPN reduction in leaf as well as root preparations. However, conclusions concerning the oxidizing enzyme(s) dependent on TPN differ. Some workers (2, 6) have reported that in crude preparations the rate of TPN reduction is unaffected by arsenate while others (1, 11) have noted an acceleration of nucleotide reduction in the presence of arsenate with partially purified preparations. These observations have given rise to the notion of a triose phosphate oxidizing enzyme unlike the classical enzyme. Recently Arnon (1) submitted evidence for such an enzyme, TPN-linked enzyme, not dependent on arsenate or phosphate plus an acceptor for activity. The enzyme(s) responsible for triose phosphate oxidation in the green leaf are under investigation in this laboratory and will be treated in a future publication.

Summary

1. Evidence is presented indicating that cell-free extracts of 12 to 15 day pea roots possess the following enzymes: glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, phosphohexose isomerase, hexosediphosphatase (fructose-1,6-diphosphate phosphatase), aldolase, triose phosphate dehydrogenase, alcohol dehydrogenase, and an acid phosphatase.

2. The glucose-6-phosphate and 6-phosphogluconate dehydrogenases are triphosphopyridine nucleotide specific and not inhibited by 1×10^{-2} M iodoacetamide.

3. The triose phosphate dehydrogenase is diphosphopyridine nucleotide dependent. The enzyme is inhibited by iodoacetamide and heavy metals and activated by cysteine. Triphosphopyridine nucleotide will not substitute for diphosphopyridine nucleotide. The absence of arsenate in the test system completely inhibits nucleotide reduction.

4. The enzyme in these extracts which converts fructose-1,6-diphosphate to fructose-6-phosphate and inorganic phosphate appears to be a specific phosphatase since it is active only at alkaline pH and is sensitive to magnesium. This is in contrast to a nonspecific phosphatase (glucose-6-phosphate and fructose-6-phosphate as substrate) which is active only at acid pH and is not affected by magnesium.

5. It is concluded that the pea root contains the direct oxidation pathway and at least the initial steps of the Embden-Meyerhof-Parnas scheme. The individual enzymes of the pea root appear to have the same characteristics as those of the green leaf with one exception; the root triose phosphate dehydrogenase is diphosphopyridine nucleotide specific.

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