PHOSPHOGLUCONIC DEHYDROGENASE IN HIGHER PLANTS

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A dehydrogenase catalyzing the oxidation of glucose-6-phosphate by triphosphopyridine nucleotide (TPN) has been shown to be widely distributed in higher plants (**2, 5**). This finding suggested that plants might form pentose by a TPN-dependent, two-step oxidation sequence similar to that already described in other organisms (**6, 8, 17**) and shown in equations 1 and 2.

(1) glucose-6-phosphate + TPN^+ + H_2O $\longrightarrow$ 6-phosphogluconate + TPNH + 2 H^+

(2) 6-phosphogluconate + TPN^+ $\longrightarrow$ pentose phosphate + CO_2 + TPNH

This paper presents evidence that enzyme systems catalyzing reaction (**2**) are widespread in higher plants. After this work was started, evidence for the presence of such enzymes in peas and spinach was presented by AXELROD and BANDURSKI (**3**).

Materials and methods

The barium salt of 6-phosphogluconate was prepared according to the method of SEEGMILLER and HORECKER (**18**). Solutions were prepared for use by dissolving the material in HCl, removing the barium with potassium sulphate, and adjusting the pH to 7.4. Various samples of barium phosphogluconate contained 1.7 to 2.0 μM of organic phosphorus per mg. and 0.01 to 0.03 μM of inorganic phosphorus per mg. The fructose content, according to the method of ROE (**16**), was not more than 0.03 μM per mg., and the glucose-6-phosphate content, according to assay with glucose-6-phosphate dehydrogenase (**11**) was 0.08 to 0.13 μM per mg. of the barium salt. The phosphogluconate content was calculated from the organic phosphorus content, after correction for the impurities. Ribose-5-phosphate was prepared by acid hydrolysis of adenylic acid or adenosine triphosphate according to the procedure described by LEPAGE and UMBREIT (**12**) and ALBAUM and UMBREIT (**1**). A preparation of TPN^+ of 70% purity was generously supplied by Dr. M. A. Mitz of Armour Laboratories. Oxidized glutathione (GSSG) was purchased from Schwartz or was prepared by oxidizing GSH with H_2O_2 as previously described (**5**). Tris (trishydroxymethyl amino-

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methane) was purchased from Commercial Solvents Corporation and recrystallized from an acetone-water mixture. Pentose was determined according to the method of MEJBAUM (**13**). The change in optical density was measured at 660 mμ, after heating in a boiling water bath for 25 minutes. Standards were made from D-ribose.

Except for the case of wheat germ and turnip root, the enzyme preparations were made by grinding the plant, with sand if necessary, but without the addition of water. Leaves were ground in the frozen state; fruits and roots were peeled and ground in a meat grinder. The coarse solids were strained off through cheesecloth and the smaller particulate matter was removed by centrifugation in the cold at 22,000 × g. All extracts were dialyzed against 0.025 M phosphate buffer (pH 7.4) for 24 hours at 3° C. Turnip root was peeled and ground in a meat grinder. The pulp was frozen with an equal volume of water, thawed, and squeezed through cheesecloth. Centrifugation and dialysis were then carried out as before. Dialyzed water extracts of wheat germ prepared as previously described (**5**) gave a high value in the orcinol test for pentose which was a disadvantage when accurate determinations of pentose formation were desired. A fractionation procedure was devised which gave a protein preparation which was rich in enzymes, but contained relatively little nucleic acid. This wheat germ protein fraction will be referred to as the WP preparation.

Wheat germ was extracted for 30 minutes at room temperature with four volumes of distilled water. The extract was strained through cheesecloth and centrifuged at 20,000 × g. for 20 minutes at 4° C. All subsequent operations were carried out in a cold room at this temperature. The supernatant was treated with $MnCl_2$ to remove nucleic acid, by a modification of the method described by KAUFMAN *et al.* (**10**). For each liter of extract, 20.4 ml. of 1.0 M $MnCl_2$ were added slowly with stirring. The solution, which had a final pH of about 5.5, was allowed to stand overnight. The heavy precipiate which formed was centrifuged off at 3,000 × g. The supernatant was stored at – 15° C for 48 hours, thawed rapidly and centrifuged again at 20,000 × g. for 20 minutes to remove additional precipitate which had appeared. The pH was adjusted to 7 with concentrated NH_4OH, and 250 gm. of solid $(NH_4)_2SO_4$ were added slowly for each liter of solution. Mechanical stirring was used during these operations, and the pH was kept at 7 by further addition of NH_4OH. After 24 hours, the bulky precipitate was centrifuged down and discarded. To each liter of supernatant, 75 gm. of $(NH_4)_2SO_4$ were then added with stirring. The precipitate was centrifuged down and dissolved in a minimal volume of 0.025 M phosphate buffer, pH 7.4, and dialyzed with stirring against the same buffer to remove the $(NH_4)_2SO_4$. The final volume was about 260 ml. of protein solution for every kilogram of wheat germ originally employed. The original extract contained about 30 mg. dry weight per milliliter after dialysis. Measurement of the ratio of the optical densities at 280 and 260 mμ indicated that about 13% of this material was nucleic acid (**19**). The amount of nucleic acid removed by the $MnCl_2$ treatment varied in different preparations, but

it was always possible by repetition of the procedure to bring the nucleic acid content down to about 1% or less. The final dry weight of this WP preparation was about 40 mg./ml.

Results

Demonstration of phosphogluconic dehydrogenase

All of the plant preparations contained the enzyme glutathione reductase (**2, 5**), which catalyzes the reaction shown in equation 3.

(3) $$GSSG + TPNH + H^+ \longrightarrow 2\,GSH + TPN^+$$

This system may be coupled with any reaction in which TPN is reduced, such as the reactions shown in equations 1 and 2. The presence of phosphogluconic dehydrogenase, therefore, could be easily detected by demonstrating that GSSG was reduced when phosphogluconate, GSSG, and a catalytic amount of TPN were incubated with the plant preparation to be tested. Table I contains representative results obtained with enzyme preparations

TABLE I

REDUCTION OF GLUTATHIONE BY 6-PHOSPHOGLUCONATE.

Source	Amount of extract	Time	Phosphogluconate added	GSSG reduced	
				Complete system	Phosphogluconate omitted
	ml.	*min.*	*μmoles*	*μmoles*	
Wheat germ	0.5	45	3.7	2.7	0.2
Cantaloupe fruit	1.6	30	10.0	3.1	0.2
Parsley leaf	1.0	30	10.0	7.6	0.1
Spinach leaf	1.0	30	5.0	3.2	0.3
Parsnip root	1.0	30	5.0	1.1	0.0
Cucumber fruit	1.6	45	10.0	7.3	0.1
Turnip root	1.6	60	10.0	2.9	0.3

made from seven different sources. Enzyme and phosphogluconate were incubated at 30° C in 3.7×10^{-2} M tris buffer of pH 7.4 with 9 μM GSSG and 30 γ TPN; total volume, 2.7 ml. The reaction was stopped by the addition of 0.3 ml. 20% HPO_3, the precipitate was centrifuged down, and an aliquot of the supernatant was titrated with iodate as previously described (**2, 5**). The enzyme preparation of wheat germ was the dialyzed water extract. The enzyme preparation of cucumber fruit consisted of the dialyzed juice concentrated to one third of the original volume by drying in the frozen state. Controls were always run in which TPN, GSSG, and phosphogluconate were omitted separately. The first two controls were uniformly low and therefore have not been included. The third control without substrate is given because it sometimes reaches an appreciable value.

No negative results were obtained in these experiments. Considerable quantitative variations were found, however, even with different preparations made from the same source. The figures must not be regarded as a quantitative measure of the amount of phosphogluconic dehydrogenase present in the plant, except in the sense that they set a minimal value (**2**).

Phosphogluconic dehydrogenase was also demonstrated spectrophotometrically by measurement of the increase in light absorption at 340 mμ when enzyme and substrate were incubated with TPN. The results confirmed the conclusions reached on the basis of the titration procedure. The specificity of the enzyme for pyridine nucleotide was likewise tested spectrophotometrically. No evidence for any reduction of diphosphopyridine nucleotide by phosphogluconate was ever found, even with enzyme mixtures that caused an almost immediate reduction of 0.2 μM of TPN.

Pentose and CO_2 production from phosphogluconate

In another series of experiments, analyses were made for pentose and CO_2 formed during the reduction of GSSG by phosphogluconate. Representative results of these experiments are shown in table II.

TABLE II

COMPARISON OF GLUTATHIONE REDUCTION WITH FORMATION OF PENTOSE AND CO_2.

Source	Amount of extract	Time	GSSG reduced	CO_2 formed	Pentose formed
	ml.	*min.*	*μmoles*	*μmoles*	*μmoles*
Wheat germ (WP)	0.3	45	8.6	6.0	5.6
Cantaloupe fruit	1.0	60	1.8	1.7	1.3
Parsley leaf	0.7	10	6.0	3.9	2.1
	0.7	20	6.9	5.5	1.6
	0.7	40	8.7	6.0	1.6
Spinach leaf	0.7	15	1.7	1.7	1.4
	0.7	45	2.7	1.9	2.2
	0.7	90	3.8	2.1	1.7
Cucumber fruit	1.6	15	4.0	2.3	3.8
	1.6	30	7.0	6.4	6.7
	1.6	60	9.1	7.4	4.8
Turnip root	1.6	20	1.8	0.6	0.4
	1.6	40	2.8	1.5	1.4
	1.6	60	3.2	1.6	1.6

The reactions were carried out in double arm Warburg vessels in an atmosphere of N_2, with yellow phosphorus in the center well. Reaction mixtures were identical with those described for table I, except that 10 μM of phosphogluconate were used in all cases. After incubation, 0.3 ml. of 20% HPO_3 was tipped in, and the CO_2 formed during the reaction was calculated by the standard procedure, with corrections for CO_2 initially present. The contents of the vessels were centrifuged, and aliquots of the supernatants were used for pentose and GSH determinations. The results recorded in table II have been corrected for the small amounts of GSSG reduction, and pentose and CO_2 formation which occurred in the absence of added phosphogluconate. With most of the sources studied, the course of the reaction was followed with time, and a number of these experiments have been included.

In all instances where a given enzyme preparation catalyzed a reduction

of GSSG by phosphogluconate, CO_2 and pentose were found to be reaction products, but the amounts of CO_2 and pentose formed were always smaller than the amount of GSSG reduced. The above discrepancies might be accounted for both by the formation of pentoses with orcinol spectra different from that of ribose, and by the further metabolism of the pentose. The orcinol spectra of the reaction products was, therefore, examined. The parsley leaf preparation and the WP fraction from wheat germ were selected for these studies, since preliminary experiments indicated that they give quite different results. With parsley, an absorption maximum at 660 mμ was predominant at 10 minutes, but in succeeding time intervals, the spectrum shifted to give a peak at about 580 mμ. The absorption maximum at 660

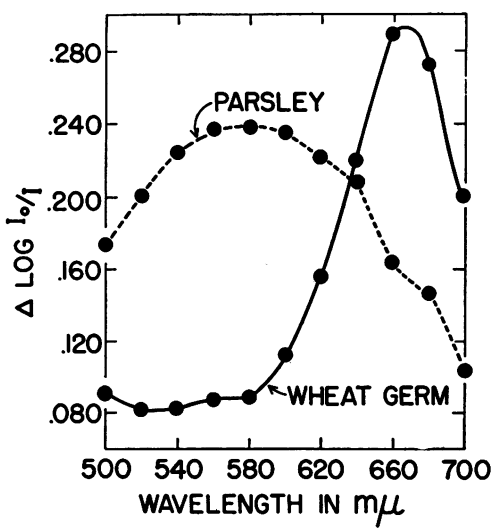

FIG. 1. The absorption curve shown for parsley was determined on a filtrate from a mixture set up as in table II for a 40-minute incubation period. The one for wheat germ was obtained from a mixture comparable to that in table II except that 0.5 ml. enzyme was used and the incubation time was 30 minutes. Absorption spectra were obtained from mixtures incubated with and without phosphogluconate. The curves represent the differences between these spectra.

mμ is characteristic of pentoses, and the seven-carbon sugar, sedoheptulose, has been reported to give a maximum at 600 mμ (**9**), whereas hexoses give absorption at shorter wave lengths. The orcinol spectra obtained with parsley indicated, therefore, that a pentose product formed initially was further metabolized, possibly in part to sedoheptulose. With wheat germ, on the other hand, there was a steady rise of the band at 660 mμ with no decline at later time intervals. The pentose which was formed was apparently not further metabolized. Figure 1 shows the characteristic difference in the orcinol spectra finally observed with the enzymes from the two sources.

Positive results were obtained when the qualitative color test specific for the seven-carbon ketose (**4, 14**) was applied to the incubation mixture with parsley leaf enzyme, but not with wheat germ. The spectral changes

of the orcinol test and the results of the heptulose color test suggested that the parsley leaf preparation contained an enzyme system metabolizing pentose in a manner similar to that described by HORECKER and SMYRNIOTIS (9). Such a system appeared to be absent in the wheat germ preparation. The action of the enzymes on ribose-5-phosphate was further investigated in order to obtain confirmation of these conclusions.

RIBOSE-5-PHOSPHATE BREAKDOWN

No action of the wheat germ WP preparation on ribose-5-phosphate could be detected. When ribose-5-phosphate was incubated with the parsley leaf enzyme, however, pentose disappeared, as shown by the decrease in the orcinol absorption band at 660 mμ. Furthermore, a reduction of GSSG occurred when TPN and GSSG were incubated with ribose-5-phosphate in the presence of the enzyme. For example, when 5 μM of ribose-5-phosphate were incubated with one ml. of enzyme for 30 minutes, 3.5 μM of pentose disappeared and 1.6 μM of GSSG were reduced. The oxidoreduction was not required for pentose breakdown, since pentose disappearance was almost as great in the absence of GSSG and TPN as in their presence. The decrease in the 660 mμ absorption band was accompanied by an increase in absorption at about 580 mμ. At the same time the qualitative heptulose color test became positive. These results confirmed the experiments done with phosphogluconate, and showed further that TPN could be reduced by pentose phosphate or by some product derived from it. A parsley leaf preparation in which glucose-6-phosphate dehydrogenase had been completely inactivated was still able to cause a reduction of GSSG in the presence of ribose-5-phosphate and TPN. This observation ruled out the possibility that the reduction of GSSG observed with ribose-5-phosphate might be due to an indirect formation of glucose-6-phosphate from pentose phosphate (15). SABLE (17) found that yeast preparations formed triose phosphate from ribose phosphate; and HORECKER and SMYRNIOTIS (9) found that when sedoheptulose phosphate was formed from pentose phosphate with a liver enzyme preparation, triose phosphate also appeared. The possibility that the reduction of GSSG in the TPN-ribose-5-phosphate system might be due to a TPN-triose-phosphate dehydrogenase was therefore entertained. Such an enzyme has been found by GIBBS (7) in pea leaves. However, the coupled reduction of GSSG by ribose-5-phosphate in the presence of the parsley leaf enzyme was completely uninfluenced by arsenate. A similar coupled reduction of GSSG independent of arsenate occurs with fructose disphosphate in the presence of the enzyme of parsley leaf and will be further described in a subsequent paper.

Discussion

The wide distribution of phosphogluconic dehydrogenase and glucose-6-phosphate dehydrogenase in higher plants suggests that this oxidative degradation of hexose occupies a significant position in plant carbohydrate metabolism. The problem of the further metabolism of pentose in these sources has been sketchily investigated in the experiments described in this

paper, and the presence of enzyme systems metabolizing pentose was proved only for the case of the parsley leaf. The data in table II show, however, that with both spinach leaf and cucumber fruit, an initial rise in pentose formation from phosphogluconate was followed by a decrease on further incubation. This may be regarded as indirect evidence that these tissues likewise metabolize pentose. AXELROD and BANDURSKI (private communication) have demonstrated pentose disappearance and sedoheptulose formation with spinach leaf enzyme.

The discrepancies in the stoichiometry shown in the results of table II could be partially explained by further metabolism of pentose. Such an explanation, however, could not account for the data obtained with wheat germ. The failure to demonstrate pentose breakdown in the case of the wheat germ preparation may have been due to inactivation of the enzymes during the procedure employed to lower the pentose content of extracts from this source.

Summary

A phosphogluconic dehydrogenase dependent on TPN has been demonstrated in wheat germ, cantaloupe and cucumber fruits, parsley and spinach leaves, and parsnip and turnip roots. Since all plant sources tested contained glutathione reductase, the oxidation of phosphogluconate was coupled with the reduction of GSSG, and pentose orcinol-reacting material and CO_2 were shown to be formed as products of such reactions. The stoichiometry indicated the occurrence of other reactions in addition to the oxidation of phosphogluconate to pentose and CO_2. A preparation from parsley leaves caused a disappearance of ribose-5-phosphate, with an appearance of the qualitative test for seven-carbon ketoses, and an unidentified TPN dependent oxidative step.

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LITERATURE CITED

1. ALBAUM, H. G. and UMBREIT, W. W. Differentiation between ribose-3-phosphate and ribose-5-phosphate by means of the orcinol-pentose reaction. Jour. Biol. Chem. **167**: 369–376. 1947.
2. ANDERSON, D. G., STAFFORD, H. A., CONN, E. E., and VENNESLAND, B. The distribution in higher plants of triphosphopyridine nucleotide-linked enzyme systems capable of reducing glutathione. Plant Physiol. **27**: 675–684. 1952.
3. AXELROD, B. and BANDURSKI, A. S. Oxidative metabolism of hexose phosphates by higher plants. Fed. Proc. **11**: 182. 1952.

4. BEVENUE, A. and WILLIAMS, K. T. Further evidence indicating the specificity of the orcinol spray reagent for keto heptoses on paper chromatograms. Arch. Biochem. Biophys. **34**: 225–227. 1951.
5. CONN, E. E. and VENNESLAND, B. Glutathione reductase of wheat germ. Jour. Biol. Chem. **192**: 17–28. 1951.
6. DICKENS, F. and GLOCK, G. E. Direct oxidation of glucose-6-phosphate, 6-phosphogluconate and pentose-5-phosphate by enzyme of animal origin. Biochem. Jour. **50**: 81–95. 1951.
7. GIBBS, M. Triosephosphate dehydrogenase and glucose-6-phosphate dehydrogenase in the pea plant. Nature **170**: 164–165. 1952.
8. HORECKER, B. L., RACKER, E., COHEN, S. S., LIPMANN, F., LAMPEN, J. O., SCHLENK, F., and DISCHE, Z. The metabolism of pentose and triose phosphates. In: A Symposium on Phosphorus Metabolism, W. D. McElroy and B. Glass, Editors. Johns Hopkins Press, Baltimore, Maryland. 1951.
9. HORECKER, B. L. and SMYRNIOTIS, P. The enzymatic formation of sedoheptulose phosphate from pentose phosphate. Jour. Amer. Chem. Soc. **74**: 2123. 1952.
10. KAUFMAN, S., KORKES, S., and DEL CAMPILLO, A. Biosynthesis of dicarboxylic acids by carbon dioxide fixation. Further studies of the "malic" enzyme of *Lactobacillus arabinosus*. Jour. Biol. Chem. **192**: 301–312. 1951.
11. KORNBERG, A. Enzymatic synthesis of triphosphopyridine nucleotide. Jour. Biol. Chem. **182**: 805–813. 1950.
12. LEPAGE, G. A. and UMBREIT, W. W. The occurrence of adenosine-3-phosphate in autotrophic bacteria. Jour. Biol. Chem. **148**: 255–260. 1943.
13. MEJBAUM, W. Über die Bestimmung kleiner Pentosemengen, insbesondere in Derivaten der Adenylsäure. Zeit. physiol. Chem. **258**: 117–120. 1937.
14. NORDAL, A. and KLEVSTRAND, R. Studies of the constituents of Crassulacean plants. I. Paper chromatographic investigations of the free sugars of some Sedum, Sempervirens, and Crassula species. Acta Chem. Scand. **5**: 85–88. 1951.
15. OCHOA, S. and STERN, J. R. Carbohydrate metabolism. Ann. Rev. of Biochem. **21**: 547–602. 1952.
16. ROE, J. A colorimetric method for the determination of fructose in blood and urine. Jour. Biol. Chem. **107**: 15–22. 1934.
17. SABLE, H. Z. Pentose metabolism in extracts of yeast and mammalian tissues. Biochim. Biophys. Acta **8**: 687–697. 1952.
18. SEEGMILLER, G. E. and HORECKER, B. L. The synthesis of glucose-6-phosphate and 6-phosphogluconate. Jour. Biol. Chem. **192**: 175–180. 1951.
19. WARBURG, O. and CHRISTIAN, W. Isolieung und Kristallisation des Garungsferments Enolase. Biochem. Z. **310**: 384–421. 1942.