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Enzymes of the Pentose Phosphate Pathway in the Mung-Bean Seedling

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It is well known that glucose 6-phosphate, apart from undergoing anaerobic breakdown by the glycolytic pathway, can also undergo direct oxidation to pentose phosphate which can be converted back into hexose monophosphate by a series of non-oxidative reactions. This series of reactions is now generally known as the pentose phosphate pathway. Considerable evidence has been documented in favour of the general occurrence of this pathway in a wide variety of biological tissues including plants (Axelrod & Beevers, 1956). Some of the key enzymes were purified from plant sources (Horecker, Smyrniotis & Klenow, 1953; Axelrod & Jang, 1954; Hurwitz, Weissbach, Horecker & Smyrniotis, 1956; Tabachnick, Srere, Cooper & Racker, 1958). Evidence in favour of the occurrence of this pathway *in vivo* mainly stems from the use of radioactive tracers and from the experiments with the inhibitors of the glycolytic pathway. The difficulties inherent in the use of isotopic techniques for this purpose are well recognized and the results obtained are often inconclusive (Utter, 1958). The isolation of the enzymes catalysing the reactions of the pentose phosphate pathway is another way of approach. Though considerable arguments have been made against this (Axelrod & Beevers, 1956), all other observations in favour of the operation of the pathway *in vivo* can be justified, provided that all the enzymes catalysing the reactions are found to occur in a single tissue.

In the present investigation germinating seedlings of mung bean (*Phaseolus radiatus*) have been chosen as the plant material to show the presence of all the enzymes involved in the direct oxidative

pathway. Phosphoglucose dehydrogenase, the first enzyme in the sequence, has been partially purified.

MATERIALS AND METHODS

Seedlings of *Phaseolus radiatus* L. var. N.P. 28 were grown under standard conditions. The seeds were first sterilized by immersion in aq. 0.8% formaldehyde solution for 1 hr. and then thoroughly washed with sterile water and allowed to soak in the water under sterile conditions for 1 day. The seed coats were then removed with forceps, and the seeds were spread on a moist filter paper in a sterile Petri dish and kept at $25 \pm 1^\circ$ in darkness. Seedlings were used for the experiments usually after 48 hr. of germination unless otherwise stated.

Dried baker's yeast used for the preparation of the phosphoglucose dehydrogenase was a gift from Dr Y. S. Lewis of the Central Food Technological Research Institute, Mysore, India.

Triphosphopyridine nucleotide (TPN), glucose 6-phosphate (G 6-P), diphosphopyridine nucleotide (DPN), reduced DPN (DPNH), 6-phosphogluconate (6-PG), fructose 6-phosphate (F 6-P), fructose diphosphate (FDP), ribose 5-phosphate (R 5-P), glutathione (GSH) and thiamine pyrophosphate were commercial products. Sedoheptulose 7-phosphate was a gift from Dr B. L. Horecker of the National Institutes of Health, Bethesda, Maryland, U.S.A. Xylulose 5-phosphate was prepared from R 5-P by the method of Hurwitz & Horecker (1956) with phosphoribose isomerase, which also contained phosphoribulose epimerase and a preparation of phosphoribulokinase.

Buffers. Phosphate buffers (0.1 M) of various pH values were prepared by adding the requisite volume of 0.2 M- NaH_2PO_4 (A) to the requisite volume of 0.2 M- Na_2HPO_4 (B) and diluting the mixture to 200 ml.; pH 7.0, 39 ml. of A + 61 ml. of B; pH 7.2, 28 ml. of A + 72 ml. of B; pH 7.5, 16 ml. of A + 84 ml. of B.

Table 1. *Purification of phosphoglucose dehydrogenase from mung-bean seedling*

Details of the procedure are given in the text.

Steps	Activity		Protein (mg./ml.)	Specific activity (unit/mg. of protein)
	Units/ml.	Total units		
Crude extract	0.7	273	12.9	0.06
Ammonium sulphate I	8.0	160	26.9	0.30
Gel eluate and ammonium sulphate II	12.0	24	19.1	0.66

Veronal buffer (0.1M), pH 7.5, was prepared by adding 30 ml. of 0.2M-HCl to 50 ml. of 0.2M-veronal and diluting the mixture to 200 ml.

2-Amino-2-hydroxymethylpropane-1:3-diol (tris) buffers, pH 7.0, 7.6 and 9.0, were prepared by adding the respective volumes of 47.0, 38.4 and 5.0 ml. of 0.2M-HCl to 50 ml. of 0.2M-tris and diluting each mixture to 200 ml.

The enzymes which were used in this investigation were isolated and purified in this Laboratory by methods as follows: phosphoglucose dehydrogenase was isolated and purified from baker's yeast following the method of Kornberg (1950). Phosphoglucose isomerase was isolated and purified from rabbit muscle after Slein (1950); the preparation was carried through the second ammonium sulphate fractionation and dialysed. The final diffusate was used for the assay. Aldolase was isolated and purified from rabbit muscle after the method of Taylor, Green & Cori (1948); the enzyme was used without recrystallization. Glycerophosphate dehydrogenase was isolated from rabbit muscle by the method of Racker (1947); the ammonium sulphate fraction which was precipitated between 50 and 70% saturation, was stored as a thick paste until needed. This fraction was contaminated with aldolase and triose phosphate isomerase, the presence of both of which was essential for a number of assays. Phosphoribulokinase was purified from *Spinacia oleracea* after the method of Hurwitz *et al.* (1956). The enzyme phosphoribose isomerase was isolated and purified from the acetone-dried powder of *Spinacia oleracea* (Chakravorty, 1958). This enzyme was carried through a number of steps, namely ammonium sulphate fractionation, heating at 60°, dialysis and a second ammonium sulphate fractionation. Sixtyfold purification was thus achieved.

Protein was determined by the Folin-Ciocalteu method (Lowry, Rosebrough, Farr & Randall, 1951). Phosphate measurements were made according to the method of Fiske & Subbarow (1925). Fructose was determined by the resorcinol method of Roe (1934). Assay for the pentulose was carried out by the cysteine carbazole colour reaction as described by Dische & Borenfreund (1951).

first for 1 min. and then for 0.5 min. after allowing the warmed homogenate to cool. The homogenate was centrifuged at 30 000 *g* for 15 min. Volume of the supernatant was 390 ml. (crude extract). Ammonium sulphate (113 g.) was added and the precipitate was discarded. A further 49 g. of ammonium sulphate was added and the precipitate, collected by centrifuging, was dissolved in 15 ml. of water (ammonium sulphate I). The ammonium sulphate fraction (20 ml.) was treated with 100 ml. of freshly prepared calcium phosphate gel (12.4 mg./ml.). The gel collected by centrifuging was eluted twice, first with 100 ml. then with 50 ml. of 0.1M-phosphate buffer, pH 7.5. Ammonium sulphate (34 g.) was added to 150 ml. of the eluate, the precipitate discarded and the precipitate formed by the addition of a further 18 g. of ammonium sulphate was collected by centrifuging and dissolved in 1.5 ml. of cold water. Final volume was 2 ml. (gel eluate and ammonium sulphate II). The activities at the different stages of purification are presented in Table 1. The enzyme has thus been purified 11-fold.

Preparation of the extract for the detection of other enzymes

Seedlings, after germination for 48 hr., were blended with 2 vol. of 0.1M-phosphate buffer, pH 7.0, in a Waring Blender for a total period of 1 min. (twice, each for 0.5 min.). The supernatant, collected by centrifuging, was used for the assay of the enzymes discussed below.

RESULTS

Phosphoglucose dehydrogenase and phosphogluconate dehydrogenase

These two enzymes mediate the first two oxidative steps in the pentose phosphate pathway. The presence of the two enzymes was detected by measuring the TPN reduction at 340 *mμ* with G 6-P and 6-PG as respective substrates:

Purification of phosphoglucose dehydrogenase

The following operations were done at 0° unless otherwise stated. The mung-bean seedling (100 g.) after germination for 48 hr. was homogenized with 400 ml. of phosphate buffer (0.1M), pH 7.2, in a Waring Blender at top speed,

The assay mixture contained .25 *μ*moles of veronal buffer, pH 7.5, 4 *μ*moles of G 6-P or 6-PG, 0.15 *μ*mole of TPN and suitable amount of enzyme in a total volume of 3 ml. in a Beckman cuvette of 1 cm. light path. A control cuvette containing all

Table 2. *Phosphoglucose dehydrogenase and phosphogluconate dehydrogenase activities in the mung-bean seedling after different periods of germination*

Age of seedling (hr.)	Protein (mg./ml. of crude extract)	Phosphoglucose dehydrogenase activity		Phosphogluconate dehydrogenase activity	
		Unit/ml. of crude extract	Specific activity (unit/mg. of protein)	Unit/ml. of crude extract	Specific activity (unit/mg. of protein)
24	9.9	0.50	0.05	0.50	0.05
48	7.7	0.50	0.06	0.40	0.05
72	4.8	0.27	0.05	0.25	0.05
96	3.2	0.08	0.02	0.11	0.03

the above components except the substrate (G 6-P or 6-PG) was simultaneously run. The increase in extinction at 340 m μ was followed after the addition of the enzyme. Extinction readings were corrected for the control. The increase in extinction with G 6-P as substrate measures both phosphoglucose dehydrogenase and phosphogluconate dehydrogenase activities. The difference in the rates of increase of the extinctions with G 6-P and 6-PG as substrates denotes the phosphogluconate dehydrogenase activity. One unit of enzyme is defined as that amount which causes an initial change in extinction of 1.0 per min. under the above-mentioned conditions (Kornberg, 1950).

Variation of activities of dehydrogenases with age of seedling

Some preliminary experiments were done to determine the activities of the dehydrogenases in the seedling after different periods of germination. At requisite intervals 5 g. batches of seedlings were treated as described in the Methods section for the preparation of the crude extract. This was assayed for phosphoglucose and phosphogluconate dehydrogenase activities. The results presented in Table 2 show that the activities decline considerably after germination for 72 hr. Seedlings after germination for 48 hr. were therefore invariably used for the preparation of phosphoglucose dehydrogenase.

Properties of phosphoglucose dehydrogenase

The partially purified preparation showed little phosphogluconate dehydrogenase activity and was active only with TPN and showed no activity with DPN (Table 3). The enzyme shows no Mg²⁺ ion requirement in veronal (Table 3), which was always used in the assay system. There was practically no inhibition of the enzyme activity by phosphate up to a concentration of 0.1 M, but in concentrations of 0.2 and 0.9 M about 28 and 97% inhibition was observed. The enzyme showed maximum activity at pH 7.5 and little activity below pH 6.0 and above pH 9.0. The properties of the dehydrogenase are thus similar to those of the phosphoglucose dehydrogenase from yeast (Kornberg, 1950).

Table 3. *Effect of Mg²⁺ ion, diphosphopyridine nucleotide and phosphate on phosphoglucose dehydrogenase from mung-bean seedling*

The assay system is described in the text except that veronal buffer was omitted when phosphate was tested.

Additions	Concentration (M)	Activity	
		Units/ml. of crude extract	Percentage of control
None	—	6.0	100
Mg ²⁺	0.66 $\times 10^{-2}$	5.8	97
Mg ²⁺	6.60 $\times 10^{-2}$	6.0	100
DPN*	5.0 $\times 10^{-5}$	0.0	0
DPN	5.0 $\times 10^{-5}$	5.9	99
Phosphate	0.1	5.8	97
Phosphate	0.2	4.3	72
Phosphate	0.9	0.2	3

* TPN was omitted from the assay system in this case.

Table 4. *Phosphoribose isomerase activity in extracts of mung-bean seedling*

For test conditions see text.

Protein added (mg.)	Increase in $E_{540\text{ m}\mu}$ (corrected for the blank)	Activity (unit/mg. of protein)
0.50	0.195	0.39
1.00	0.408	0.41

Phosphoribose isomerase

The occurrence of phosphoribose isomerase (PRI) in the extract was demonstrated by the formation of ribulose 5-phosphate (Ru 5-P) from R 5-P by the standard cysteine carbazole colour reaction of the pentulose (Hurwitz *et al.* 1956).

The incubation mixture contained 30 μ moles of tris buffer, pH 7.0, 1 μ mole of cysteine and 2 μ moles of R 5-P, in a total volume of 0.8 ml. The incubation was carried out at 30° for 10 min. and the reaction was stopped by the addition of 2.5 ml. of aq. 70% H₂SO₄. Immediately 0.1 ml. of 1.2% solution of cysteine hydrochloride (freshly prepared) was added, thoroughly mixed and then

0.1 ml. of ethanolic 0.1% carbazole solution was added. Absorption readings at 540 $m\mu$ were taken after 10 min. The results of the assay are presented in Table 4. One unit is defined as that which produces an absorption of 1.0 under the assay conditions.

Phosphoribulose epimerase (3-epimerase)

The presence of 3-epimerase in the germinating mung bean was demonstrated by the assay method as described by Hurwitz & Horecker (1956)

Xylulose 5-phosphate

The measurement was based on the relative stability of R 5-P to alkali. The incubation was carried out for 5 min. at 35° in a total volume of 0.2 ml. containing 10 μ moles of tris buffer, pH 7.5, 14 μ g. of phosphoribose isomerase, suitable amount of enzyme and 0.13 μ mole of xylulose 5-phosphate. The reaction was stopped by adding 0.2 ml. of 2M-KOH and the mixture was kept at 35° for 20 min. Then the orcinol-reactive material was measured by the method of Mejbaum (1939) with 40 min. heating period. A blank was run without addition of enzyme and suitable corrections were accordingly made. The results are presented in Table 5.

Transketolase

Transketolase activity of the extract was shown by measuring the formation of glyceraldehyde 3-phosphate from R 5-P and xylulose 5-phosphate as described by de La Haba, Leder & Racker (1954). Xylulose 5-phosphate was generated from R 5-P by the addition of phosphoribose isomerase (PRI) containing 3-epimerase. Glyceraldehyde 3-phosphate formed was measured by DPNH oxidation subsequent to the conversion of the former into α -glycerophosphate by the combined action of triose phosphate isomerase and α -glycerophosphate dehydrogenase.

PRI

3-epimerase

Transketolase

Triose phosphate isomerase

Glycerophosphate dehydrogenase

Table 5. *Phosphoribulose epimerase activity in extracts of mung-bean seedling*

Details of the assay system are given in the text.

Protein added (μ g.)	$E_{670 \text{ m}\mu}$ (corrected for the blank)	Alkali-stable pentose phosphate formed (μ mole)
90	0.495	0.032
180	0.915	0.059

Table 6. *Transketolase activity in extracts of mung-bean seedling*

Details of the test system are given in the text.

Protein added (mg.)	$E_{340 \text{ m}\mu}/3 \text{ min.}$	Triose phosphate formed/3 min. (μ mole)
None	0.005	0.001
0.25	0.130	0.031
0.50	0.250	0.060

The assay system contained, in a total volume of 1.5 ml., 25 μ moles of tris buffer, pH 7.6, 0.5 μ mole of thiamine pyrophosphate, 1 μ mole of MgCl_2 , 2 μ moles of R 5-P and 70 μ g. of phosphoribose isomerase. Incubation was at room temperature for 6 min., and then 0.1 mg. of α -glycerophosphate dehydrogenase (contaminated with triose phosphate isomerase) and a suitable amount of DPNH, to give an absorption reading of 0.5–0.6 at 340 $m\mu$, were added. Before adding the extract containing transketolase to start the reaction the extinction at 340 $m\mu$ was observed for a while to check whether there was any oxidation of DPNH owing to the contamination of phosphoribose isomerase or α -glycerophosphate dehydrogenase or both with transketolase activity. They were, however, found to be free of transketolase. The results obtained with the seedling extract are presented in Table 6.

Transaldolase

The transaldolase activity in the extract was detected by measuring the rate of formation of F 6-P from sedoheptulose 7-phosphate and glycer-

Table 7. *Transaldolase activity in extracts of mung-bean seedling*

For test conditions see text.

Protein added (mg.)	Substance omitted	$\Delta E_{340\text{ m}\mu}/4.5\text{ min.}$	$E_{340\text{ m}\mu}$ (corrected for the blank)	TPN reduced (μmole)
None	—	0.017	—	—
0.65	—	0.070	0.053	0.013
1.30	—	0.131	0.114	0.028
0.65	Sedoheptulose 7-phosphate	0.042	0.025	0.006

Table 8. *Phosphoglucose isomerase activity in extracts of mung-bean seedling*

For test conditions see text.

Protein added ($\mu\text{g.}$)	F 6-P formed ($\mu\text{mole}/10\text{ min.}$)	Activity (units/mg. of protein)
26	0.12	4.6
52	0.20	3.8

aldehyde 3-phosphate (Horecker & Smyrniotis, 1953). Glyceraldehyde 3-phosphate was generated from FDP by muscle aldolase. F 6-P was assayed by the reduction of TPN in the presence of phosphoglucose isomerase (PGI) and phosphoglucose dehydrogenase.

(a) FDP $\xrightleftharpoons{\text{Aldolase}}$ glyceraldehyde 3-phosphate

(b) Sedoheptulose 7-phosphate + glyceraldehyde 3-phosphate $\xrightleftharpoons{\text{Transaldolase}}$ F 6-P + erythrose 4-phosphate

(c) F 6-P $\xrightleftharpoons{\text{PGI}}$ G 6-P

(d) G 6-P + TPN⁺ $\xrightleftharpoons{\text{Phosphoglucose dehydrogenase}}$ 6-PG + TPNH + H⁺

The assay system contained, in a final volume of 15 ml., 50 μmoles of tris buffer, pH 7.0, 0.15 μmole of TPN, 0.5 μmole of FDP, 0.4 μmole of sedoheptulose 7-phosphate, 14 $\mu\text{g.}$ of aldolase, 7.4 $\mu\text{g.}$ of phosphoglucose isomerase and 7 $\mu\text{g.}$ of phosphoglucose dehydrogenase. The assay system was incubated without the seedling extract at room temperature for 5 min., in a Beckman 1 cm. cuvette, and then the extract to be tested was added. A control incubation was run excluding the crude extract as the system showed slight TPN reduction even without addition of the extract. The absorption readings were corrected for the control. Another control incubation was run, with sedoheptulose 7-phosphate omitted, to show that the F 6-P was not formed solely by the action of phosphatase, if any, on FDP. The results are presented in Table 7.

Phosphoglucose isomerase

Phosphoglucose isomerase (PGI) was determined by measuring the formation of F 6-P from G 6-P.

F 6-P was assayed by the resorcinol method of Roe (1934).

The incubation mixture contained 47 μmoles of tris buffer, pH 9.0, 1 μmole of G 6-P and the extract to be tested, in a final volume of 1 ml. The incubation was carried out at 30° for 10 min. The reaction was stopped by adding 3.5 ml. of 10M-HCl. Then 0.5 ml. of resorcinol reagent was added, mixed well and heated for 10 min. in a bath, at 80°, followed by cooling in water for 5 min. The absorbancy readings were taken at 510 m μ . Suitable blank and a standard F 6-P solution were run simultaneously. The results are presented in Table 8.

One unit of enzyme is defined as that which produces 1 μmole of F 6-P under the standard assay conditions.

DISCUSSION

The evidence presented above shows the presence of all the enzymes of the pentose phosphate pathway in extracts of germinating mung-bean seedling. Though the presence of these enzymes does not necessarily prove that the pentose phosphate pathway as a whole is operative *in vivo*, it is not unreasonable to suggest that this pathway is of significance in the germinating seedling. The activity of both phosphoglucose dehydrogenase and phosphogluconate dehydrogenase, which catalyse the initial steps of the pentose phosphate pathway, remain more or less constant up to the age of 48 hr., but after this there is a fall. Germination for longer periods than 96 hr. results in the virtual disappearance of both the dehydrogenases from the extract. Though so far there is no evidence in

favour of energy production by the pentose phosphate pathway, the occurrence of this oxidative cycle in the early life of the seedling emphasizes its close association with the high metabolic activity at this stage.

The activities of the different enzymes in the extract, as judged from the number of units per ml. and from the activity in relation to the protein content, were low in comparison with reported activities of the enzymes in extracts from animal and bacterial systems. This is, however, to be expected because of the presence of considerable amounts of non-enzymic protein (storage protein) in the seeds and in the seedling at the early stages of germination.

Phosphoglucose dehydrogenase which has been partially purified has the pH optimum and other properties similar to those of the yeast enzyme. Unlike bacterial enzyme it is specific for TPN and inactive with DPN.

SUMMARY

1. The occurrence in extracts of germinating mung-bean seedling of the following enzymes of the oxidative pentose phosphate pathway has been shown: phosphoglucose dehydrogenase, phosphogluconate dehydrogenase, phosphoribose isomerase, phosphoribulose epimerase, transketolase, transaldolase and phosphoglucose isomerase.

2. During the initial period of germination (up to 48 hr.) the amounts and specific activities of the dehydrogenases increase with time. After 72 hr., however, both start decreasing and after 96 hr. the enzymes disappear from the extracts.

3. The enzyme phosphoglucose dehydrogenase has been partially purified and its properties have been found to be similar to those of the yeast enzyme. The enzyme is triphosphopyridine nucleotide-specific, and has been successfully used in the assay of glucose 6-phosphate.

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The Metabolism of Exogenous ¹⁴C-Labelled 5-Hydroxytryptamine in Rats

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In spite of intensive study of 5-hydroxytryptamine from both biochemical and biological aspects knowledge of its metabolism is still incomplete. The fate and distribution of 5-hydroxytryptamine in rats was studied by Erspamer (1955, 1956), but more specific information about an overall metabolism is still lacking. 5-Hydroxyindolylacetic acid was established as a metabolite by Udenfriend, Titus & Weissbach (1955) and Erspamer (1955). Weissbach, Redfield & Udenfriend (1957) found 5-hydroxyindolylacetaldehyde in homogenates of

guinea-pig liver. Curzon (1957) found 5-hydroxyindolylacetic acid *O*-sulphate in urine of argentaffinoma subjects and in urine of rats after administration of 5-hydroxytryptophan, 5-hydroxytryptamine and 5-hydroxyindolylacetic acid. Chadwick & Wilkinson (1958) established 5-hydroxytryptamine *O*-sulphate as a metabolite of 5-hydroxytryptamine in incubated rat-liver homogenates. Weissbach, Redfield & Udenfriend (1958) identified 5-hydroxytryptamine *O*-glucuronide as a normal metabolite of 5-hydroxytryptamine in the urine of