A DEVELOPMENTAL STUDY OF D-GLYCERIC ACID DEHYDROGENASE\textsuperscript{1,2}

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A previous publication from this laboratory (13) presented evidence for the occurrence in higher plants of a new enzyme, \textit{n}-glyceric acid dehydrogenase, which catalyzes the reversible oxidation of \textit{n}-glycerate to hydroxypruvinate in the presence of diphosphopyridine nucleotide\textsuperscript{5} according to the following equation:

\[
\begin{align*}
\text{CH}_3\text{OH} & \quad \text{CH}_4\text{OH} \\
\text{H-} & \text{C-} \text{OH} + \text{DPN}^+ \rightarrow \text{C} = \text{O} + \text{DPNH} + \text{H}^+ \\
\text{COO}^- & \quad \text{COO}^- 
\end{align*}
\]

Since the mere presence of an enzyme in a tissue does not establish a physiological role, further data are needed in order to elucidate the possible metabolic function of this enzyme. As an aid in obtaining such information, the developmental pattern of this dehydrogenase was studied. The previous investigation (13) had shown that although \textit{n}-glyceric acid dehydrogenase is widely distributed in the tissues of various higher plants, the enzyme is present in a particularly high concentration in green leaves. This localization has been substantiated by following the pattern of the increase in the enzymatic activity upon germination. Another DPN-dependent enzyme, glyoxylic acid reductase (17), was studied for comparison. Peas and wheat were selected as representative plants because of their ease of growth under laboratory conditions.

MATERIALS AND METHODS

Pea seeds (\textit{Pisum sativum} var. Alaska) or wheat seeds (\textit{Triticum aestivum} var. Pawnee) were sterilized for 15 minutes with a saturated solution of calcium hypochlorite before being soaked for 2 hours in dis-tilled water. The seeds were placed on a layer of cheesecloth supported by a wire frame that was set into enamel trays containing sufficient nutrient solution to keep the cloth moist. The nutrient solution contained 0.005 M Ca(NO\textsubscript{3})\textsubscript{2} and KNO\textsubscript{3}; 0.001 M MgSO\textsubscript{4} and KH\textsubscript{2}PO\textsubscript{4}; 2.5 \times 10^{-4} M K\textsubscript{2}HPO\textsubscript{4}; and 1.8 \times 10^{-5} M FeSO\textsubscript{4}. After being covered for the first 2 to 3 days, the trays were placed at room temperature under several white fluorescent lamps (about 300 to 500 fc of light) for 8 to 10 hours a day. These plants are called “light-grown.” Plants grown under comparable conditions except for being kept in complete darkness are referred to as “dark-grown” plants. Good growth was maintained under these conditions.

The plants were harvested at intervals and approximately 10 to 20 grams fresh weight of the portions to be analyzed were weighed, and then ground at 4°C for 1 minute in 200 ml of cold (-18°C) acetone in a Waring blender. The powder was filtered and washed with another 200 ml of cold acetone in successive small portions, and then further dried and stored at 4°C in a desiccator under vacuum with CaCl\textsubscript{2} as desiccant. This powder was extracted for 0.5 hour at 4°C in 25 to 50 ml of 0.001 M phosphate buffer (pH 7.4), filtered through glass wool, and then centrifuged at high speed to give a clear supernatant. Dry weight analyses were made on this final extract. Dialysis made no appreciable difference in the enzymatic activity and was omitted. These acetone powder preparations gave a greater total activity for glyceric acid dehydrogenase than dialyzed extracts made by grinding frozen leaves in a mortar. The ratio of the activity of glyceric acid dehydrogenase to glyoxylate acid reductase was similar in the two different preparations.

Since the equilibrium of this dehydrogenase reaction lies far in the direction of glyceric acid and DPN\textsuperscript{+}, the enzymatic activity is most easily measured by observing the oxidation of DPNH in the presence of hydroxypruvinate. Therefore, in the subsequent experiments, the oxidation of DPNH was followed in a Beckman spectrophotometer by observing the change in the optical density (log \textit{I}/\textit{I} \textsubscript{0}) at 340 m\textmu. Because DPNH is oxidized in the absence of added substrate by some of the enzyme extracts used (2), a control mixture without added substrate was always assayed in each experiment, and this rate of oxidation of DPNH was subtracted from the rate measured in a mixture with added substrate.

Suitable aliquots of the enzyme extract were added to each experimental cuvette to maintain a constant change in optical density equal to or less than 0.020 per minute over a period of 10 minutes. The final rates are expressed as the change in optical density per minute per shoot or per mg dry weight of the enzyme extract. Besides the enzyme, the experimental cuvettes contained the following components in a total volume of 3 ml: 0.2 ml of an 0.5 M phosphate buffer of pH 6.5, 5 micromoles of either hydroxypruvinate, pyruvate, or glyoxylate, 0.2 micromoles of DPNH. These reaction mixtures were read against a solution containing the complete system minus DPNH. Increasing the concentration of substrate or of DPNH did not alter the rates of enzymatic activity.

The hydroxypruvinate, kindly supplied by Dr. W. Fager, was prepared from pyruvic acid according to Sprinson and Chargaff (12). The glyoxylate bisulfite

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\textsuperscript{5} DPN\textsuperscript{+} = oxidized diphosphopyridine nucleotide; DPNH = reduced diphosphopyridine nucleotide.
addition product, a gift of Dr. I. Zelitch, was prepared from oxalic acid according to Weinhouse and Friedman (16), and was hydrolyzed with HCl before being used. The DPNH was enzymatically reduced and isolated according to Loewus et al (7).

Reproducibility of the actual spectrophotometric measurements was limited only by pipetting errors, and therefore, caused little variation in the data. However, the data obtained from this developmental study does show variation. Some of this can be attributed to uncontrolled temperature conditions during the two separate growth experiments, and some to the necessity of obtaining acetone powders in order to remove pigments obscuring the spectrophotometric assay method. The rates therefore should be considered only as minimum values for each chronological age.

RESULTS

DISTRIBUTION OF D-GlycERIC ACID DEHYDROGENASE ACTIVITY WITHIN THE Pea PLANT: Extracts from dormant pea seeds contain a measurable, but weak d-glyceric acid dehydrogenase activity (fig. 1, A). When extracts from 11-day-old pea plants were assayed for the dehydrogenase, approximately 74% of the total activity of the root shoot axis (minus cotyledons) was contributed by the leaves (leaflets, stipules and tendrils), about 20% by the stems and the remaining 6% by the roots. Similar results were obtained with 14-day-old plants. Therefore, although the enzyme is present in seeds and roots, the major portion of the activity is found in the leaves.

DEVELOPMENTAL STUDY: If an enzyme is found predominantly in leaf tissue, a developmental study should show that the increase in the enzymatic activity is correlated with the growth of the leaves. Data from such a study using pea and wheat plants are shown in figure 1, A and B. The two separate plantings are indicated by solid and open circles and each point on the graph represents a single enzymatic assay. The enzymatic activity is expressed as the change in optical density per minute. The solid lines represent the activity per shoot, while the dashed lines represent the activity per mg dry weight of the enzyme extract. Approximate wet weight data per shoot and the height of the shoots are given in table I. While there is considerable variation between the 2 separate growth experiments, the trend is similar in both cases, and the following conclusions may be drawn.

Upon germination, the increase in dehydrogenase activity is slow until the shoot begins the period of maximum growth in length associated with rapid leaf growth. The enzymatic activity in both peas and wheat increases rapidly 4 to 10 days after germination, reaching a maximum after about 10 days of growth. This increase is closely correlated with the formation of new compound leaves in the pea plant (table I). The average increase per shoot during the 4- to 10-day-old period is equivalent to a change in optical density of 1.8 per minute in peas and 0.6 in wheat. The increase in activity per mg dry weight of the enzyme extract is less dramatic, especially in the case of peas.

After the peak value at 9 to 10 days, wheat plants show a significant decrease in activity, especially when the results are expressed as the activity per shoot. This decrease is not due to the presence of any inhibitor in the preparations as no inhibition was observed when a 14-day-old wheat extract was added to the more active earlier extracts of either wheat or peas. It is still possible, however, that the enzyme is inhibited in these older plants by a substance that is no longer inhibitory in the enzyme extracts. The decrease on a dry weight basis may mean that the synthesis of the enzyme is not keeping pace with the synthesis of other protein after this peak period at 9 to 10 days. The actual loss of activity per shoot is much less apparent or perhaps not existent in pea seedlings during the 14-day period studied, but the activity per mg dry weight decreases as in wheat plants.

All of the above preparations were assayed for lactic acid dehydrogenase because this enzyme can also reduce hydroxypyruvate (9). In the root and seed preparations, DPNH was oxidized upon the addition of pyruvate as substrate, but all of these preparations were unable to reduce DPN+ in the presence

| Table I |
| --- | --- | --- | --- | --- | --- | --- |
| **Peas** | **Age in days** | **Height of shoot (cm)** | **No. cmpd. leaves** | **Gm wet wt per shoot** | **Glyceric/glyoxylic activity** | **Age in days** |
| 0 | 4.5 | 6.5 | 8 | 10 | 12 | 14 |
| Height of shoot (cm) | 1.5-2 | 3-4 | 11-15 | 14-18 | 20-22 | 24-27 |
| No. cmpd. leaves | 1 | 1.5 | 3-4 | 5 | 5 | 5 |
| Gm wet wt per shoot | 0.05 | 0.11 | 0.32 | 0.41 | 0.66 | 0.70 |
| Glyceric/glyoxylic activity | 3.5 | 6.5 | 8 | 8 | 9 | 13 | 14 |
| **Wheat** | **Age in days** | **Ave. height of shoot (cm)** | **Gm wet wt per shoot** | **Glyceric/glyoxylic activity** | **Age in days** |
| 0 | 4 | 5 | 7 | 9 | 12 | 14 |
| Ave. height of shoot (cm) | 5 | 7.5 | 14.5 | 16 | 22 | 26 |
| Gm wet wt per shoot | 0.03 | 0.05 | 0.10 | 0.12 | 0.15 | 0.19 |
| Glyceric/glyoxylic activity | 1 | 15 | 25 | 25 | 24 | 18 | 20 |
Fig. 1. D-Glyceric dehydrogenase and glyoxylic acid reductase activity in pea and wheat shoots. Graphs A and C show the enzymatic activity of D-glyceric dehydrogenase (A) and glyoxylic acid reductase (C) in peas. Graphs B and D show similar data for D-glyceric acid dehydrogenase (B) and glyoxylic acid reductase (D) in wheat. The enzymatic activity is equal to the change in optical density per minute either per shoot (circles) or per mg dry weight of the enzyme extract (triangles). Open and solid symbols represent separate growth experiments.
of lactate at pH 9, indicating the absence of a lactic dehydrogenase. The activity with pyruvate could be explained by the presence of pyruvic acid carboxylase, which would convert the pyruvate to acetaldehyde, coupled with alcohol dehydrogenase, which would then oxidize the DPNH.

Glyoxylic acid reductase activity of the same preparations follows a similar pattern of increase during the early growth of the shoot, but the actual rate of increase is not as rapid (fig. 1, C and D). For instance, the glyceric acid dehydrogenase activity of peas increases over 5 times as fast as that of glyoxylic acid reductase. The average increase per shoot from 4 to 10 days is equivalent to a change in optical density of 0.23 per minute in peas and 0.024 in wheat. This difference is partly reflected in a change in the ratio of the hydroxypyruvate to glyoxalate activity in activity was observed as shown in table II (A and B represent separate experiments). Comparable data obtained from plants placed in the light each day after germination are shown in the last column.

Although peas are not a satisfactory test material because the growth pattern is highly modified in the dark, with internodal growth predominating, similar results are obtained if one compares plants of the same size. Pea seedlings, grown for 6 days in the dark and subsequently placed in the light for 24 hours, had approximately the same activity as those left continually in the dark. In contrast to wheat plants, however, the amount of enzyme (and leaf development) was comparable to that of 4.5-day-old rather than to 7-day-old light-grown pea plants (table II).

**INTRACELLULAR LOCALIZATION:** Tolbert and Cohen (15) reported that 10% of the total activity of glycolic acid oxidase of leaves was associated with a possible chloroplast fraction. They felt that this small percent was truly a part of the particulate complement because the activity was retained in a variety of buffers at different pH values.

Preliminary studies were made to indicate the intracellular localization of d-glyceric acid dehydrogenase in leaves. Crude particulate fractions were separated from soluble enzymes in the supernatant by centrifugation of sucrose-phosphate (8) or sorbitol-borate-KCl (3) homogenates of green pea shoots at 18,000 × g for 60 minutes. The centrifuged particles were washed once with the same medium, and then both fractions were precipitated with acetone. Approximately 5 to 10% of the total activity (of the particles plus the supernatant) was associated with the washed crude particulate fraction (presumably containing chloroplasts, mitochondria, etc) while the remaining 90 to 95% appeared in the soluble fraction of the supernatant. Although the present study with peas cannot differentiate between secondary adsorption during extraction, and the valid localization of this small percent of activity with leaf particles, the majority of the enzyme is certainly soluble under the conditions employed.

**DISCUSSION**

The metabolic function of the two organic acids, glyceric and hydroxypyruvic, are not well known. Glyceric acid is of interest because of its possible relationship to the early photosynthetic intermediate, 3-phospho-d-glycerate. Free glyceric acid has also been identified among the early products of photosynthesis (1), but it is not clear whether this is merely the result of phosphatase activity. In a study of the synthesis of ascorbic acid in cress seedlings, Isherwood, Chen, and Mapson (6) observed that an increase in d-glyceric acid was generally associated with the ascorbic acid increase which occurs during the first 5 days of growth after germination. Although the relationship between these two substrates was not elucidated, d-glyceric acid did not appear to be a precursor of ascorbic acid, nor was ascorbic acid the precursor of d-glyceric acid.
Regardless of the relationship between ascorbic and glyceric acids, it is significant that D-glyceric acid has been identified as a major acid constituent in a higher plant, and that the increase in glyceric acid content might coincide with the increase of the enzyme D-glyceric dehydrogenase. Although the present developmental study was not concerned with cress seedlings, water cress shoots (Nasturtium officinale) are an active source of the enzyme, giving a total change in optical density of 0.26 per minute per mg dry weight of an extract of an acetone powder. While Isherwood and coworkers suggest that the glyceric acid in cress seedlings could arise from the activity of 3-phosphoglycerate phosphatase or glycerol dehydrogenase, the D-glycerate could be due to the action of D-glyceric acid dehydrogenase upon hydroxypyruvate. This of course, necessitates the explanation of the origin of hydroxypyruvate within the plant.

Hydroxypyruvate has been isolated from extracts of mammalian tissues after incubation with serine (11). A similar isolation has not been made from plant tissues, but the recent investigations (5, 10) of the enzyme, transketolase, indicate that hydroxypyruvate can serve as a source of active aldehyde in the formation of pentose phosphate from triose phosphate.

Possible interrelationships between enzymes and substrates which increase upon germination or which are concentrated in leaf tissue need to be further elucidated.

**SUMMARY**

D-Glyceric acid dehydrogenase is predominantly a leaf enzyme, although low activities are found in roots and seeds of wheat and peas. The main increase in enzymatic activity during the early growth of the shoot is associated with maximum leaf development. The enzyme is found in comparable amounts in dark- and light-grown plants of similar size and is predominantly a soluble enzyme. Comparisons are made with glyoxylic acid reductase, which shows a similar but smaller increase during the early growth of the shoot.

**LITERATURE CITED**