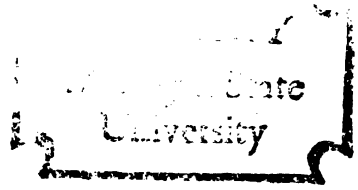
ENZYMES FOR OXIDATION OF ALPHA-HYDROXYACIDS IN ROOTS OF GREEN PLANTS

Thesis for the Degree of M. S.
MICHIGAN STATE UNIVERSITY
MAN PING KO
1975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ABSTRACT

ENZYMES FOR OXIDATION OF ALPHA-HYDROXYACIDS
IN ROOTS OF GREEN PLANTS

By

Man Ping Ko

Homogenates of young tissues of various higher plants were assayed for glycolate oxidase, a peroxisomal enzyme in green leaves of higher plants, and for glycolate dehydrogenase, a functionally analogous enzyme characteristic of certain green algae. In all the leaves examined, a glycolate dehydrogenase of the type found in green algae was not detectable although excess glycolate oxidase was always present. Glycolate dehydrogenase was also not detectable in the roots examined and glycolate oxidase was present at low level in the roots of only certain species.

Glycolate oxidase activity in the roots of watercress was found to be not different from that occurring in the leaf except in substrate specificity. The enzyme activity in the roots was particulate, capable of reducing the dye 2,6 dichlorophenol-indophenol and molecular oxygen in the presence of L(+)-lactate, glycolate and D(-)-lactate, in order of decreasing rate. The reduction was insensitive to cyanide or other inhibitors of the respiratory electron transport.

NAD-Dependent L(+)-lactate dehydrogenase was not found in the leaves, but it occurred widely in the roots. Hydroxypyruvate reductase

which was present at high level of activity in the leaves was also present in the roots. Both the lactate dehydrogenase and hydroxypyruvate reductase from roots of pea seedlings were partially purified by ammonium sulfate fractionation, DEAE cellulose ion exchange chromatography and Sephadex G-200 gel filtration chromatography. Some of their properties were examined and compared to those of the same enzymes from leaves or animals.

The root lactate dehydrogenase was nearly specific for L(+)-lactate and NAD, but the D(-)-isomer was also reduced at a very slow rate. In the reverse direction this enzyme reduced not only pyruvate but also hydroxypyruvate and glyoxylate in the presence of NADH. Optimal activity occurred at pH 9.1 in the direction of pyruvate formation and at pH 7.0 in the reverse direction. It resembled closely heart L(+)-lactate dehydrogenase in its molecular weight, electrophoretic mobility and its response to heat treatment, inhibition by excess substrate and sulfhydryl group inhibitors.

Hydroxypyruvate reductase or D(-)-glycerate dehydrogenase from the roots was specific for NADH and hydroxypyruvate and glyoxylate with a rather broad pH optimum which peaked at pH 5.9. In the reverse direction, it oxidized DL-glycerate in the presence of NAD, with optimal activity at pH 9.6. Its molecular weight, inhibition by excess hydroxypyruvate and sulfhydryl inhibitors resembled closely glycerate dehydrogenase from beef liver or leaves. Hydroxypyruvate reductase from roots was not particulate, but was present in the cytosol as it is in the liver tissue.

The possible physiological roles of these enzymes of alpha-hydroxy acid oxidation in the roots were discussed.

ENZYMES FOR OXIDATION OF ALPHA-HYDROXYACIDS IN ROOTS OF GREEN PLANTS

By

Man Ping Ko

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Biochemistry

1975

ACKNOWLEDGEMENTS

I want to express my sincere thanks to Professor N. E. Tolbert for his guidance, encouragement and support during the course of this work.

I would like to thank also Drs. D. P. Delmer and J. L. Fairley for their serving as members in my guidance committee as well as for their helpful suggestions on this manuscript.

I thank also the postdoctoral fellows, fellow graduate students and technicians in Dr. Tolbert's laboratory for the many stimulating and helpful discussions in pursuing my research.

The financial assistance of the National Science Foundation and the Department of Biochemistry, Michigan State University, is also appreciated.

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LIST OF ABBREVIATIONS

DCPIP	2,6 Dichlorophenol-indophenol, Sodium Salt
EDTA	Ethylenediamine Tetra-acetic Acid, Disodium Salt
FMN	Flavin Mononucleotide (Riboflavin Phosphate), Sodium Salt
NAD(P)	β-Nicotinamide Adenine Dinucleotide (Phosphate), Disodium Salt
NAD(P)H	β-Nicotinamide Adenine Dinucleotide (Phosphate), Reduced Form, Disodium Salt
PCMB	p-Chloromercuribenzoic Acid, Sodium Salt
Tricine	N-tris-(Hydroxymethyl)-methyl Glycine

INTRODUCTION

Interest in the metabolism of the alpha-hydroxy acids, such as glycolic acid and lactic acid, in both plants and animals is ages old and a great number of reports have appeared concerning these problems. Whereas many reports have been concerned with glycolate metabolism in photosynthetic tissues of plants, and with lactate metabolism in animals, very little attention has been devoted to the study of either acid in the nonphotosynthetic tissues of the green plants and in particular to the roots. Since virtually nothing is known about enzymes which oxidize alpha-hydroxyacids in the roots, the goal of the research was to survey for them and then to elucidate some of their properties. These exploratory experiments have dealt only with a comparison between properties of the enzymes from roots with those found in leaves or in animals.

Three enzymes have been studied: glycolate oxidase, lactate dehydrogenase and hydroxypyruvate reductase or glycerate dehydrogenase. A fourth enzyme, α-glycerophosphate dehydrogenase, has also been investigated in roots and in the leaves, although it is not an alpha-hydroxyacid oxidizing enzyme. As part of my research program, this latter enzyme was characterized from leaves of soybean and spinach and these results are being published as a separate manuscript (12).

LITERATURE REVIEW

Enzymes of Glycolate Oxidation in Photosynthetic Tissues of Plants, Algae and in Animals and Other Nonphotosynthetic Organisms

Glycolate oxidase has been extensively studied in green leaves and, recently, glycolate dehydrogenase has been shown to be a similar but quite distinct enzyme in the algae. Glycolate oxidase in the leaves (103) and glycolate dehydrogenase in the algae (76,105) have been extensively reviewed, along with other enzymes related to glycolate metabolism. Both enzymes seem important because of their role in photorespiration, in which half of the carbon fixed in photosynthesis is lost during glycolate biosynthesis and metabolism (104).

Glycolate oxidase, the first enzyme of the glycolate pathway in the peroxisomes of the leaves, was first described by Claggett, Tolbert and Burris (14) and is present in all green leaf tissues. The enzyme is a flavoprotein (49,126), with a molecular weight of 270,000 (29). It is not coupled to oxidative phosphorylation (124). The activity of the enzyme in etiolated leaves is markedly increased upon exposure of the tissue to the light (59,107), but its development does not parallel chloroplast development (27,107). The enzyme also oxidizes at a slower rate other short chain L(+)-alpha-hydroxymonocarboxylic acids to the corresponding keto acids (29), and it is therefore an alpha-hydroxyacids oxidase.

In contrast to glycolate oxidase, the enzyme that oxidizes glycolate in most unicellular green algae does not couple to oxygen as electron acceptor (15,81). In fact, the algal enzyme is not an oxidase but a dehydrogenase which can use DCPIP as electron acceptor but the natural electron acceptor is unknown (81). Glycolate dehydrogenase has been found to be of general occurrence in algae (76,105). It was in an unidentified fraction, but there are reports recently that it is in the mitochondrial (16,94) and the microbody (33) fractions. The failure to utilize oxygen as the electron acceptor during glycolate oxidation and the difference in intracellular location are not the only differences between the algal enzyme and the oxidase in higher plants. Glycolate oxidase will oxidize lactate as an alternative substrate at 1/3 the rate of glycolate but it has absolute stereospecificity for L(+)-lactate (14,126). The enzyme is insensitive to cyanide or sulfhydryl inhibitors and it is activated by the addition of FMN (29). In contrast, the algal glycolate dehydrogenase oxidizes D(-)-lactate as an alternative substrate; it utilizes L(+)-lactate only a little or not at all; it is inhibited by cyanide and sulfhydryl binding reagents; and it is not affected by FMN (35,81).

There may also be another enzyme for glycolate metabolism in marine diatoms (84), which might be distinct from either the leaf oxidase or the algal dehydrogenase. The diatom enzyme oxidizes L(+)-lactate faster than glycolate and D(-)-lactate. It is in the mitochondria (85) and its oxidation of L(+)-lactate or glycolate is coupled to oxygen uptake, and it is therefore an oxidase. The coupling to oxygen is, however, inhibited by cyanide and other electron transport

inhibitors. An enzyme of similar nature may also exist in roots of rice seedlings and in developing rice seeds (108). The enzyme in rice roots also oxidizes L(+)-lactate faster than glycolate and D(-)-lactate and the oxidation is coupled to oxygen, but its sensitivity to electron transport inhibitors has not been tested.

Enzymes of glycolate oxidation also exist in nonphotosynthetic tissues. Enzymes for catalyzing the oxidation of glycolate are present in fungi (2,28,97), bacteria (56,68), and mammalian liver and kidney (60,74,114). Glycolate oxidase from mammalian liver and kidney is similar to that in the leaf peroxisomes in intracellular localization, prosthetic group and catalytic properties (60,74). In the nonphotosynthetic tissues of the plant, i.e., the roots, glycolic acid oxidase originally was reported absent or found in very low levels of activity (52,106), and it was presumed not to exist. However, glycolic acid and glyoxylic acids were found in the roots and glyoxylic acid was suggested to be the hydrolysis product from allantoin and allantoic acids (58), which constituted more than half of the total nitrogen in some roots (78,79). Mothes and Wagner have extensively reviewed the work done on the root glycolate oxidase (80), and concluded that the activity of the enzyme was a function of state of development. Thus etiolated leaf and roots, when allowed to turn green in light had the active enzyme, while roots kept in the dark did not have the enzyme. The formation of the enzyme in the roots in light was not due to substrate activation or induction because adaptive formation was not observed when glycolic acid was given to the roots even if light was present, when light was not applied too long. They gave examples of roots not capable of greening and therefore devoid of the enzyme, but

they also cited examples of roots that turned very green in the light but still had no enzyme activity. It is therefore suggested that not only the stage of development but also the intrinsic genetic capacity to produce the enzyme was involved. Mitsui *et al.* (77) reported the existence of glycolate oxidase in roots of paddy field rice plants, upland rice plants and barn millet: the enzyme activity was low at the beginning of germination, but gradually increased after germination and then decreased again. This has been confirmed by Tolbert *et al.* (108), who also studied the enzyme in greater detail and suggested that the enzyme was a lactate oxidase rather than a glycolate oxidase on the basis that it oxidized L(+)-lactate faster than glycolate. Glycolate oxidase has also been reported in the microbody fractions of the roots of wheat (19), carrots, castor bean, potato tubers and castor bean endosperm (44). The enzyme from the castor bean endosperm has been studied and found to be somewhat different from the one in the leaves, being inhibited by cyanide (98). The property of glycolate oxidase in other nonphotosynthetic tissue has not been examined except for the rice roots. Since Tolbert *et al.* (108) found that the properties of the oxidase in rice roots were different from that in the leaf, the enzyme from the roots of some other plants was examined in this thesis.

Enzyme of Lactate Oxidation in Higher Plants

In sharp contrast to the extensive work on NAD-dependent L(+)-lactate dehydrogenase in animals, reports about the purified enzyme from plant sources have been almost nil. This is because alcohol is presumed to be the traditional end product of anaerobic respiration

in plants, while lactate is the product of anaerobic respiration in animal tissues. Thus alcohol dehydrogenase has been found to be widely distributed in plants and has been isolated, purified and studied (18,23,66,83). However, it has been known for a long time that both alcohol dehydrogenase and lactate dehydrogenase played a significant role in the anaerobic period of germination in which pyruvate was metabolized either to ethanol (11,64,70) or to lactate (4,6,17,93). Leblova *et al.* (65) found that lactate dehydrogenase was present in the seeds; its activity decreased during the first 12 hours of germination and then rose during the later stage of germination; the change in enzyme activity and lactate concentration was reciprocal. Others have found that detached rhododendron leaves and buckwheat seedlings (7,25) accumulated lactic acid during anoxia and that it rapidly disappeared upon returning the tissue to air. In attempts to explain these phenomena, lactate dehydrogenase has been isolated and partially purified. The enzyme was isolated by King from soybean cotyledon (50), and was found to be similar to the lactate dehydrogenase of muscle in mammals in many of its properties. Davies *et al.* (21) purified the enzyme from potato tuber and found it to be regulated by ATP at low pH but not at high pH. Isoenzymes of lactate dehydrogenase in meristem tissues of roots have also been reported (37), but this was not confirmed (50).

Evidence that lactate dehydrogenase is widely distributed in plants seems numerous. It was detected in a particulate fraction from rhizomes of *Equesitum* (3), in extracts from potato tubers, squash fruit and turnip roots (95). The nature of these activities has not been studied. NAD-Dependent D(-)-lactate dehydrogenase, which has

been found in bacteria (22,99), in slide mold (30), in fungi (31,87), in algae (36,117), and in certain invertebrates (67,92), has not been reported in higher plants, nor has the NAD-independent D(-)-lactate dehydrogenase which was found in algae (33,69,117), bacterial membrane (34,46,48) and even in mammals (10,112,113).

Lactate dehydrogenase from roots has not been isolated or studied, although it has been reported to be active in the meristem zone of the root tips (51).

Hydroxypyruvate Reductase in Plants and in Animals

Stafford *et al.* (95) were the first to report on this enzyme from parsley leaves by an assay involving the reduction of hydroxypyruvate to glycerate, as a NAD-linked D-glycerate dehydrogenase. At about the same time, Zelitch reported on a NADH glyoxylic reductase in spinach leaves (122) which he later crystallized from tobacco leaves (123). Later work of Holzer and Holldorf (42) and Landahn (61) indicated that NAD glycerate dehydrogenase and NADH glyoxylate reductase were the same enzyme. Further work on the enzyme by Tolbert and his associates showed that in leaves it was located in the peroxisome (111), where it functioned chiefly as hydroxypyruvate reductase in the glycolate pathway to provide one of the sources of glycerate for gluconeogenesis and synthesis of sucrose (40,45,88). A similar enzyme has been reported in livers of animals (120) and in bacteria (13,53,62). Roseblum *et al.* (89) and Sugimoto *et al.* (96) have purified the enzyme from beef liver, and Kohn and his associates (54,55) carried out extensive studies on the properties of hydroxypyruvate reductase from spinach leaves and indicated that these enzymes were very similar.

The enzymes from different sources, although differing in molecular weights, are composed of two equal subunits, and use either hydroxypyruvate or glyoxylate as substrate but have a greater activity with hydroxypyruvate. In addition, the activities of these enzymes are similarly modified by anions and are sensitive to sulfhydryl binding reagents. The enzymes from beef liver and beef spinal cord are also similar and inhibited by nucleotides (ATP) and other glycolytic intermediates (91).

In animals as well as in plants, hydroxypyruvate reductase is involved in serine metabolism (8,86,115). In the leaves, this occurs whenever there is a low level of glycine formation from glycolate (40). Hydroxypyruvate reductase is found in the cytosol of liver (74), but it exists in the peroxisomes of the leaves (111) and in a microbody-like (16) or mitochondria-like (94) fraction of the algae. The enzyme has not been studied in the nonphotosynthetic tissues of the plant, although it was reported to be in the roots (95). Its subcellular location in the roots has not been reported.

MATERIALS AND METHODS

Plant Materials

Seeds of sorghum (*Sorghum bicolor* L. var. Texas 610), corn (*Zea mays* L.), peas (*Pisum sativum* L. var. Adelman), broad bean (*Vicia faba* L.) were sterilized with 0.5% sodium hypochlorite solution for 20 minutes, rinsed with distilled water several times and then soaked in distilled water for approximately 8 hours; they were germinated in moist sterile vermiculite in wooden flats at 30° and grown in the greenhouse for 7-10 days.

Seeds of wheat (*Triticum vulgare* L. var. Eva), barley (*Horedium valgare* L. var. Liberty), and oat (*Avena sativa* cv. Victory) were sterilized in a similar manner and then germinated in the dark in moist filter papers and grown in the greenhouse in half strength Hagland's solution (41) for 10 days at 30°. Watercress (*Nasturtium officinale* R. Br.) from the local market was grown in full strength Hoagland's solution in the greenhouse at 30° for 8-10 days. During these periods, the Hoagland's solution was changed once every two days and the roots were washed with tap water twice. With this treatment, algal or microbial growth was kept at minimum and no significant growth of any microorganism was observed during these short growth periods.

Chemicals

DEAE-Cellulose (Whatman DE-52, microgranular) was obtained from Whatman and Sephadex G-200 from Pharmacia. TEMED (N,N,N',N' tetra methylenediamine), MBA (N,N'-methylenebisacrylamide) and acrylamide were from Canalco. Glycolic acid and sucrose were the products of Mann Research Laboratory. All enzymes and chemicals were from Sigma Chemical Company, J. T. Baker Chemical Company, or Mallinckrodt Chemicals. Chemicals used were of reagent or analytical grade and were used without further purification.

Preparation of Cell Free Extracts of Plant Tissues

The plant materials were rinsed several times with distilled water, blotted dry and separated into leaves and roots with razor blades or scissors. Each type of tissue was weighed to the nearest gram, minced in cold grinding medium and homogenized. The grinding medium and homogenizing procedures varied according to the type of plant material, but generally, two volumes of grinding buffer was used for each gram of tissue and the grinding medium used was 0.05 M potassium phosphate buffer at pH 7.5 with 2% (w/v) polyvinylpoly-pyrrolidone added during the grinding. The plant tissues were ground in a chilled mortar with a pestle for several minutes with or without sand. The preparations were passed through six layers of cheesecloth and centrifuged at 500 x g for 10 minutes. The supernatants were assayed for enzymes or as a starting point for subsequent enzyme isolation. All work was carried out at 0-5°.

Preparation of Particulate Fractions

For localization of enzymes in particulate fractions, either method A or method B was used. In method A, the plant tissue was minced with scissors in 1-2 volumes (w/v) of grinding medium which consisted of 0.4 M sucrose and 20 mM glycylglycine. The slurry was then ground gently with a mortar and pestle and passed through 4 layers of cheesecloth. After centrifuging at 270 x g for 10 min to remove whole cells and debris, the homogenate was decanted and centrifuged for 25 min at 37,000 x g. This produced a supernatant and a pellet, which was gently resuspended in grinding medium. The pellet, which contained the microbodies and mitochondria among other organelles, was the crude particulate fraction for enzyme assays. In all cases, catalase and cytochrome c oxidase was assayed to check for the breakage of the particles.

Method B was used only for the differential centrifugation of pea root homogenates. The tissue was homogenized in a chilled mortar and pestle with one volume by weight of grinding medium (0.5 M sucrose and 0.02 M glycylglycine at pH 7.5). The resulting slurry was squeezed through 8 layers of cheesecloth, and the pH (approximately 7) was readjusted to 7.5 with potassium hydroxide. The sap was then centrifuged at 270 x g for 20 min, and the resulting pellet was resuspended in the grinding medium. The sap was further centrifuged at 6000 x g for 20 minutes. This pellet was also resuspended in grinding buffer. A "mitochondrial" fraction was prepared by centrifugation of the sap at 37,000 x g for 20 min and resuspension of the pellet in the grinding medium. The supernatant fluid after the last centrifugation was designated the "supernatant" fraction. In each case, catalase and

cytochrome c oxidase were assayed for the localization of the particular organelles in each fraction.

Partial Purification of L(+) Lactate Dehydrogenase and Hydroxypyruvate Reductase from Pea Roots

Peas (*Pisum sativum* L. var. Adelman) were soaked for 4 to 5 hours in distilled water and germinated in moist sterile vermiculite for 7 days in the dark at 30°. The pea seedlings were then washed ten times with tap water to free the roots from vermiculite and then rinsed twice with distilled water. The roots were separated from the plants with scissors and then cut into small pieces with a sharp razor blade. The minced roots were ground in a Waring Blendor at top speed for 60 seconds with equal parts (w/v) of a buffer containing 0.05 M potassium phosphate at pH 7.5, 1 mM mercaptoethanol and 2% polyvinylpolypyrrolidone. The brei was passed through 6 layers of cheesecloth and centrifuged at 37,000 x g for 20 min. The supernatant was referred to as the "crude extract." L(+)-lactate dehydrogenase and hydroxypyruvate reductase were partially purified from this crude extract by the following steps. The results of each stage of purification procedures are summarized in Table VI for lactate dehydrogenase and in Table VII for hydroxypyruvate reductase (glycerate dehydrogenase).

(a) Ammonium sulfate fractionation

Cold saturated $(NH_4)_2SO_4$ solution at pH 7.0 was added to the crude extract to make the final solution 30% saturated with ammonium sulfate. The proteins in the solution were allowed to precipitate for 30 minutes in the cold room (4°). The precipitate was removed by centrifugation at 10,500 x g for 30 minutes and discarded. The

supernatant was made 60% saturated with respect to ammonium sulfate by adding cold saturated ammonium sulfate. The protein, precipitated between 30-60% ammonium sulfate solution, contained more than 95% of the total activities of both L(+)-lactate dehydrogenase and hydroxypyruvate reductase. The precipitate was collected by centrifugation and resuspended in a small volume of 50 mM potassium phosphate buffer at pH 7.0 and 1 mM mercaptoethanol. The solution was desalted in a Sephadex G-25 column (2.5 cm x 35 cm) before being applied to a DEAE-cellulose column.

(b) DEAE-cellulose treatment

Approximately 450 mg of the desalted protein was added to a 2.5 cm x 30 cm DEAE-cellulose (Whatman, DE-52) column previously equilibrated with several bed volumes of 5 mM potassium phosphate at pH 7.0 and 1 mM mercaptoethanol. The column was then washed with 1 bed volume of the equilibrating buffer. The proteins were then eluted at a flow rate of 0.5 ml per minute with 3 bed volumes of a linear gradient of 0 to 0.5 M potassium chloride in 5 mM potassium phosphate at pH 7.0 and 1 mM mercaptoethanol. Six-milliliter fractions were collected with a Gilson automatic fraction collector. The chloride content of the fractions was determined with a Barnstead Purity meter.

By this procedure, the L(+)-lactate dehydrogenase and hydroxypyruvate reductase were eluted separately from the column (Figure 2), with the hydroxypyruvate reductase eluting first. The fractions which showed an increase in specific activity over the previous stage were combined. The pooled proteins were concentrated by adding saturated ammonium sulfate solution until 70% saturated. These precipitated

proteins could be stored in the pellet form in the freezer at -4° for several weeks without loss of enzyme activity.

(c) Gel filtration chromatography in Sephadex G-200

The concentrated protein with lactate dehydrogenase activity from the previous step was dissolved in 4 ml of 50 mM potassium phosphate at pH 7.0 and 1 mM mercaptoethanol and applied to a Sephadex G-200 column (1.6 cm x 90 cm) previously equilibrated with the same buffer. Proteins were eluted with 300 ml (2 bed volumes) of the same buffer at a flow rate of 4 ml per hour. Fractions of 3.3 ml were collected and those with lactate dehydrogenase activities were pooled and concentrated by 67% $(NH_4)_2SO_4$ precipitation. Hydroxypyruvate reductase was partially purified in the same manner except that the buffer used was at pH 6.5. Both proteins could be kept as ammonium sulfate suspension or as a frozen pellet at -20° for a long period. The lactate dehydrogenase seemed to be more labile in solution than hydroxypyruvate reductase. In potassium phosphate buffer at pH 7.0 with 10% glycerol, lactate dehydrogenase lost 85% of its activity after 2 days at 0-4°. Hydroxypyruvate reductase, on the other hand, could be kept in 10% glycerol in 50 mM phosphate buffer with 1 mM mercaptoethanol over a period of one week at 0-4° without significant loss in activity.

Sucrose Density Gradient Centrifugation

The isolation method for microbodies followed that described by Huang and Beevers (44) with little modification. The grinding medium contained 0.4 M sucrose, 1 mM EDTA, 10 mM KCl (potassium chloride), 1 mM $MgCl_2$ (magnesium chloride), 10 mM dithiothreitol, 0.15 M Tricine

buffer adjusted to pH 7.5 and 0.5% by weight of polyvinylpyrrolidone added during the grinding. The pea roots were ground gently in medium with mortar and pestle after being first chopped to small pieces with razor blades. The homogenate was passed through 8 layers of cheese-cloth and centrifuged at 270 x g for 10 min. Ten milliliters of the supernatant was layered on a 40-ml linear gradient composed of 30-60% sucrose over a cushion of 5 ml 60% sucrose. All sucrose solutions were expressed as percentage by weight and made up in 0.1 M Tricine buffer at pH 7.5. After centrifugation for 4 hours at 21,000 rpm in a Beckman L2-65B ultracentrifuge with Spinco rotor SW 25.2, the gradients were collected in 1.5-ml fractions using a density gradient fractionator. All steps were carried out at 4°.

The fractions were assayed for catalase, cytochrome c oxidase and hydroxypyruvate reductase. The density of the fraction was determined in the Bausch and Lomb refractometer.

Biochemical Assays

All assays were run at 25° and initial rates were recorded. The spectrophotometric assays were done on a Gilford 2400S recording spectrophotometer. In all cases, the enzyme assays were tested to check if activity was dependent on protein concentration over the range used. Oxygen uptake was measured with a Rank Brothers oxygen electrode. Protein was determined by the method of Lowry *et al.* (71) using bovine serum albumin as standard. All enzyme activities were expressed as nmoles (substrates converted) per minute.

NAD-Lactate Dehydrogenase

This enzyme was assayed by following changes in absorbance at 340 nm (26). For the determination of pyruvate reduction, the 1-ml reaction mixture contained 0.1 M potassium phosphate buffer at pH 7.0, 0.01% Triton X-100, 0.12 mM NADH, appropriate amount of enzyme extract and 10 mM potassium pyruvate. For measurement of the reverse reaction, that involved the oxidation of either D- or L-lactate, the 1-ml mixture contained 0.1 M NaOH-glycine buffer at pH 9.2, 0.01% Triton X-100, 3 mM NAD, enzyme and 20 mM D- or L-lactate (lithium salt). In both cases the endogenous rate was monitored for 2 to 3 minutes and the reaction was initiated by the addition of substrates. Since pyruvate reduction lies far to the right, it was used routinely for detection of lactate dehydrogenase.

NAD D-Glycerate Dehydrogenase or Hydroxypyruvate Reductase

This enzyme was assayed the same as the lactate dehydrogenase described above except that the substrate was replaced with equimolar of Li-hydroxypyruvate or DL-glycerate (calcium salt) and the pH of the buffers were changed to 6.2 or 9.2, respectively.

Glycolic Acid Oxidase or Dehydrogenase

This enzyme was assayed by following the anaerobic reduction of 2,6 dichlorophenol-indolephenol (DCPIP) at 600 nm (109).

The reaction mixture of 2.5 ml contained 0.08 M sodium pyrophosphate at pH 8.5, 0.12 mM DCPIP, 0.01% Triton X-100, 0.1 mM FMN and either 8 mM glycolic acid (neutralized with NaOH) or 20 mM D- or L-lactate (lithium salt). The reaction was initiated by addition of

substrate after reading the endogenous rate for 5 minutes. To test for sensitivity of the enzyme to O_2 or cyanide, assays were run aerobically or in the presence of 2 mM potassium cyanide, respectively. Changes in O.D. were converted to nmoles of dye using the extinction coefficient of 21.9 cm^2/μmoles for DCPIP (1).

The oxidase was also assayed by measuring the disappearance of O_2 with a Rank oxygen electrode at 10 mvolts. The reaction mixture of 2.5 ml contained the complete mixture of the DCPIP assay minus the dye (DCPIP). Oxygen concentration was calculated from its solubility from air into water. All solutions for the assay were air saturated except the enzyme. At 25°, the reaction mixture held 685 nmoles oxygen (38). Percentage changes in oxygen concentration recorded in 5 minutes were converted to nmoles oxygen/min.

Catalase

This enzyme was used as the peroxisome marker enzyme and was assayed spectrophotometrically at 25° by following the disappearance of H_2O_2 at 240 nm (72). One to one hundred microliters of extract was added to a 3-ml mixture containing 1.25 x 10^{-2} M H_2O_2 and 1/15 M sodium phosphate at pH 7.0. The disappearance of H_2O_2 was calculated by using the extinction coefficient of 0.036 cm^2/μmole.

Cytochrome c Oxidase

Cytochrome c oxidase, the mitochondrial marker, was assayed by following the oxidation of reduced cytochrome c at 550 nm (110). The enzyme was placed in the bottom of the 1-ml quartz cuvette and 20 μl of 1% digitonin was added. After mixing and a 1 min incubation period, 0.9 ml of 0.05 M potassium phosphate buffer at pH 7.0 was added. The

reaction was then initiated by the addition of 100 µl of solution containing 10 mg/ml cytochrome c reduced with dithionite. A sample without the enzyme was used in order to measure the rate of nonenzymatic oxidation of reduced cytochrome c. The extinction coefficient of 21.0 cm^2/µmole for cytochrome c was used to calculate the amount of cytochrome c oxidized (73).

Estimation of Molecular Weight by Gel Filtration Chromatography

Gel permeation chromatography was used to estimate the molecular weights of lactate dehydrogenase and hydroxypyruvate reductase from roots. A column of Sephadex G-200 (1.6 cm x 90 cm) was equilibrated with 50 mM potassium phosphate at pH 7.0. The column was calibrated with the following proteins as molecular weight standards: cytochrome c (M.W. 12,400), ovalbumin (M.W. 43,000), bovine serum albumin (M.W. 68,000), yeast alcohol dehydrogenase (M.W. 150,000), and catalase (M.W. 240,000). Two runs were done to calibrate the column. In one run, mixture of 20 mg of each cytochrome c, ovalbumin and alcohol dehydrogenase in a total volume of 4 ml was applied to the column and eluted with the equilibrating buffer by the upward flow technique. Fractions of 3.3 ml were collected and the positions of the proteins were determined by reading O.D. 280 and enzyme activity. In a second run, a mixture of myoglobin, serum albumin and catalase was applied and the procedure was repeated. The same procedures were repeated with the root lactate dehydrogenase or hydroxypyruvate reductase. The logarithms of the molecular weights of the protein standards were plotted against the K_{av}. The excluded volume of the column was determined by using blue dextran (M.W. 2,000,000).

Electrophoretic Studies

The system was modified after Davies (20) and Williams and Reisfeld (119) and the solutions were as follows:

L_1: 36.3 gm Tris (tris(hydroxymethyl)aminomethane), 48 ml 1 N HCl, 0.46 ml TEMED (N,N,N',N' tetramethylenediamine), pH 8.9 in 100 ml H_2O.

L_2: 45 gm acrylamide, 1.2 gm MBA (N,N' methylenebisacrylamide) in 100 ml H_2O.

A_p: 14 mg ammonium persulfate in 10 ml H_2O (made up fresh).

U_1: 10 gm acrylamide, 2.5 gm MBA in 100 ml H_2O.

U_2: 6.4 ml 1 M H_3PO_4, 1.425 gm Tris base pH 6.9 in 100 ml H_2O.

R: 12 gm Tris, 57.6 gm glycine, in 100 ml H_2O, pH 8.3.

Gels (7.5%) were made up by mixing 1 part of L_1, 0.87 part of L_2, 4 parts of A_p and 2.11 parts of H_2O. The solution was degassed with a water aspirator for 60 seconds, and 2-ml aliquots were allowed to gel at room temperature in 0.5 cm (i.d.) x 12 cm glass tubes. The stacking gels were made in similar manner by mixing equal volumes of solutions U_1, U_2, A_p, water and then degassing. Fifteen microliters of TEMED were added and 0.5-ml aliquots were allowed to gel on top of the running gel. The gels were stored at 4° for several hours before use. The reservoir buffer R was used in both the upper and lower chambers at a 10-fold dilution. The application buffer was made up of 10% glycerol, 0.01 M mercaptoethanol, and 1 µl 0.05% bromophenol blue in 10 ml of buffer U_2 which had been diluted 5-fold. In each case, 20-50 µg of the protein in 100 µl of the application buffer was loaded onto the gels. The gels were run vertically at 3 milliamperes per tube for approximately 2.5 hours. After electrophoresis, the gels

were removed immediately by means of a water-filled syringe with a long size 23 needle to loosen them from the glass tubing. They were then stained for protein and enzyme activity.

Protein was stained with 0.25% Coomassie Blue in methanol:acetic acid:water (5:1:5) for 20 minutes and then destained in 7.5% acetic acid, 5% methanol with 0.5 gm of amberlite-MB for two days. Dehydrogenase activities were located by incubating in the dark at 37° for 30 minutes in a mixture containing 20 ml of 0.09 M Tris-HCl pH 8.5, 0.1 M substrate, 1 mM NAD, 0.45 M nitroblue tetrazolium, 71 µM phenazine methosulfate. In each case, a control gel was incubated in the same solution without the substrate.

RESULTS

Survey of Plant Tissues for Glycolate Oxidase, Glycolate Dehydrogenase and Lactate Dehydrogenase

The distribution of these enzymes was run with the crude extracts of leaves and roots. A preparation was judged to contain glycolate oxidase if it oxidized glycolate and L-lactate but not D-lactate, and if this activity was not inhibited by 2 mM potassium cyanide. The presence of glycolate dehydrogenase was indicated by the oxidation of glycolate and D-lactate, and an inhibition by 2 mM potassium cyanide. A useful but less rigorous distinguishing criterion was whether or not inhibition of initial DCPIP reduction occurred when the assay was carried out aerobically (26). When glycolate oxidase was present, a greatly decreased rate of DCPIP reduction occurred aerobically because this enzyme also transferred hydrogen to O_2 instead of DCPIP (102). For glycolate dehydrogenase, which does not transfer electrons directly to molecular O_2, the rate is the same in nitrogen or in air.

Lactate dehydrogenase was judged to be present by the reduction of pyruvate, even though oxidation of either D- or L-lactate was not shown to occur. There was no lag in the initial rate and only the initial rate was measured to ensure that the oxidation of NADH was not from alcohol dehydrogenase acting on the acetaldehyde formed from pyruvate by pyruvate decarboxylase, which might be present in the crude extracts. Since lactate oxidation with NAD by lactate

dehydrogenase at high pH is much slower than the pyruvate reductase reaction, only the latter reaction was used in the surveys.

All the leaves of seedlings of higher plants examined had glycolate oxidase (Table I), and its specific activities ranged from 28 nmoles DCPIP/min/mg protein in the pea leaves to 1 nmole DCPIP/min/mg protein in sorghum leaves. None of the leaves had a glycolate dehydrogenase of the type found in green algae (81) or in marine diatom (84), in that none had a cyanide sensitive glycolate oxidation or a cyanide inhibited preferential utilization of D-lactate over L-lactate.

Activities with L-lactate were always present and varied from 50-130% of that with glycolate. All the activities were linked to molecular oxygen. L-Lactate oxidation was somewhat higher than that reported for the partially purified glycolate oxidase which generally oxidizes L-lactate at 1/4 to 1/2 the rate of glycolate (102). No significance is attributed to this difference in the C_3 plants examined. The greatest activity for L-lactate was in the extracts of the two C_4 plants, corn and sorghum, which oxidized L-lactate about as well as glycolate. Since C_4 plants have lower rate of photorespiration (glycolate metabolism) but possess microbodies, and since C_4 plants have extremely active pyruvate metabolism in the C_4 dicarboxylic acid cycle (39), my data suggest that the alpha-hydroxyacid oxidase in C_4 plants may be adapted to lactate oxidation.

A slight activity with D-lactate was detectable in the crude extracts. Except for corn leaves this low activity was not significant and might in part be attributed to impure D-lactate which may have contained as much as 5% of the L-isomer. The D-lactate activity

Table I. A survey of glycolate oxidizing enzymes in crude extracts of leaves of higher plants*

Plant	Relative Activity			Plus 2 mM Cyanide		
	Glycolate	L-lactate	D-lactate	Glycolate	L-lactate	D-lactate
			O_2 Electrode Assay			
Barley	100	42	0	150	63	0
Corn**	100	85	0	120	100	0
Peas	100	53	0	146	80	0
Oat	100	68	0	139	97	0
Sorghum**	100	80	0	120	90	0
Watercress	100	45	0	134	63	0
Wheat	100	60	0	150	92	0

Table I (continued)

Plant	Relative Activity			Plus 2 mM Cyanide			Plus Air or O_2		
	Glycolate	L-lactate	D-lactate	Glycolate	L-lactate	D-lactate	Glycolate	L-lactate	D-lactate
					DCPIP Assay				
Barley	100	80	10	110	93	0	0	0	0
Corn**	100	92	20	115	166	0	0	0	0
Peas	100	80	7	100	82	0	50	32	0
Oat	100	70	2	160	108	0	45	32	0
Sorghum**	100	130	5	142	210	0	8	33	0
Watercress	100	50	0	132	64	0	0	0	0
Wheat	100	90	11	103	90	0	41	15	0

*Relative activities are expressed as % on the basis of the assay with glycolate and O_2 in the O_2 electrode assay and on the basis of the assay with glycolate and DCPIP in the DCPIP assay. For assays in the presence of cyanide or O_2, the values are relative to activities in their absence. "0" indicates no or insignificant amounts of activity relative to activity with glycolate.

**C_4 plants.

in the C_4 corn plant should be reexamined in view of the possibility suggested above that the alpha-hydroxyacid oxidase in C_4 plants may be modified.

A final concentration of 2 mM cyanide stimulated enzymatic activity in all cases by both the DCPIP and the O_2 electrode assays. This has been observed since the discovery of glycolate oxidase and attributed to removal of the aldehyde or keto acid product which normally severely inhibits the utilization of the hydroxyacids.

In the roots of the same plants, the situation was entirely different. Except in the roots of two plants (wheat and watercress), glycolate oxidation was not detected by the same assay procedures. Even in the roots of watercress and wheat the levels of enzyme activities were very low, at least 50 times lower than the level found in the leaves (Table II). The alpha-hydroxyacid oxidase system in the crude extracts of roots of wheat and watercress oxidized L-lactate faster than glycolate, and also oxidized D-lactate to some extent. In these two plants at least, the system in the roots seemed to be modified from that in the leaves. Whereas the activity in the roots of watercress was stimulated by 2 mM potassium cyanide and inhibited by O_2 in the DCPIP assay, very much similar to the system in the leaves, the activity in the roots of wheat was inhibited by 2 mM cyanide and also by O_2 in a similar assay. Since the alpha-hydroxyacid oxidase activities in the crude extracts were very low, they had been concentrated from both root sources to confirm these observations by differential centrifugation.

To look for other enzymes of alpha-hydroxyacid oxidation, NAD-lactate dehydrogenase was assayed by using pyruvate as the substrate,

Table II. Oxidation of glycolate and lactate by extracts of wheat and watercress roots*

Plant	Relative Activity			Plus 2 mM Cyanide			Plus Air (O_2)		
	Glycolate	L-lactate	D-lactate	Glycolate	L-lactate	D-lactate	Glycolate	L-lactate	D-lactate
Watercress	100	167	30	111	185	0	0	0	0
Wheat	100	167	75	36	43	0	0	0	0

*Assayed by the anaerobic DCPIP method. Specific activity for watercress extract was 1.5 nmoles DCPIP/min/mg protein and for wheat extract was 0.5 nmoles DCPIP/min/mg protein. Activities are expressed as % of that in the anaerobic glycolate assay.

and hydroxypyruvate reductase or glycerate dehydrogenase was also assayed by using hydroxypyruvate as the substrate (Table III). Hydroxypyruvate reductase was extremely active in the leaves of C_3 plants, but only about 1/10 as active in the C_4 plants, corn and sorghum, as has been found previously (109). Lactate dehydrogenase was not present in the leaves and the active peroxisomal hydroxypyruvate reductase does not use pyruvate as a substrate. Some lactate dehydrogenase was present in the roots of the C_3 plants. Whether hydroxypyruvate reductase is present in the roots could not be resolved by this one assay on the crude homogenate, since lactate dehydrogenase can catalyze the reduction of both pyruvate and hydroxypyruvate (75). In the root extracts of two plants (i.e., corn and sorghum), neither pyruvate nor hydroxypyruvate reduction was observed, possibly due to inactivation of the enzyme during the grinding procedure.

Enzymes of Alpha-Hydroxyacid Oxidation from Watercress Roots

Since extracts of the roots of watercress showed activities for glycolate oxidase, hydroxypyruvate reductase, and lactate dehydrogenase, it was possible that there were one to three enzymes catalyzing these reactions and further experiments were conducted to test these possibilities. It was possible to separate the NAD requiring enzyme activities from that of glycolate oxidase by differential centrifugation. By method A described in the Materials and Methods, about half of the glycolate oxidase activity, measured in terms of DCPIP reduction with either glycolate or L-lactate as substrates, remained particulate, while 89% of the activity for

Table III. Distribution of hydroxypyruvate reductase and lactate dehydrogenase in extracts of tissues of higher plants

Plant	Tissue	Hydroxypyruvate reductase	Lactate dehydrogenase
Broad bean	Leaves	280	0
	Roots	26	13
Corn	Leaves	57	0
	Roots	0	0
Peas	Leaves	297	0
	Roots	11	3
Sorghum	Leaves	35	0
	Roots	0	0
Watercress	Leaves	136	0
	Roots	104	35
Wheat	Leaves	550	0
	Roots	25	15

hydroxypyruvate reduction and 86% of the activity for pyruvate reduction were in the supernatant (Table IV). The results indicated that lactate was being oxidized by at least two different enzymes, an alpha-hydroxyacid oxidase and a NAD-dehydrogenase.

Ammonium sulfate fractionation also indicated that there were different enzymes catalyzing these reactions. Most of the glycolate oxidase was lost during the fractionation, probably because of its particulate nature, but 16% of the hydroxypyruvate reduction and 50% of the pyruvate reduction were recovered in the 40% $(NH_4)_2SO_4$ pellet of the root homogenate. It was also possible to distinguish the glycolate oxidase from the NAD-linked dehydrogenases by their relative stabilities in root homogenates prepared by grinding in 50 mM potassium phosphate buffer at pH 7.5. In the homogenate, after centrifugation at 500 x g for 10 minutes to remove cell debris, glycolate oxidase activity, measured with L-lactate as substrate, remained stable over a period of at least several hours, while that of NADH pyruvate reductase and hydroxypyruvate reductase declined slowly (Figure 1). This experiment was performed with watercress roots that had been frozen for two months so this stability pattern for the enzymes may not be exactly the same with fresh tissues.

Some Properties of the Alpha-Hydroxyacid Oxidase Activity from Watercress Roots

The alpha-hydroxyacid oxidase activity from watercress roots differed from that of glycolate oxidase of the leaves in preferentially utilizing L-lactate, and in this respect was similar to that in the rice roots (108). The particulate fraction (37,000 x g pellet) of the root homogenates was used in further experiments.

Table IV. Distribution of glycolate oxidase, lactate dehydrogenase and hydroxypyruvate reductase in watercress roots following differential centrifugation

Enzymes	Substrates	Fraction	Total activity nmoles/min	Specific activity nmoles/min/mg protein	Distribution %
Alpha-hydroxyacid oxidase	Glycolate, DCPIP	270 x g supernatant	23	0.5	100
		37,000 x g supernatant	12	0.3	50
		37,000 x g pellet	12	1.0	53
	L-lactate, DCPIP	270 x g supernatant	76	1.7	100
		37,000 x g supernatant	41	1.2	53
		37,000 x g pellet	34	2.8	45
Hydroxy-pyruvate reductase	NADH, hydroxy-pyruvate	270 x g supernatant	4148	91	100
		37,000 x g supernatant	3688	109	89
		37,000 x g pellet	157	13	5
Lactate dehydro-genase	NADH, pyruvate	270 x g supernatant	1384	30	100
		37,000 x g supernatant	1184	35	86
		37,000 x g pellet	66	5	5
Catalase	H_2O_2	270 x g supernatant	400	9	100
		37,000 x g supernatant	333	27	66
		37,000 x g pellet	166	5	33

Figure 1. Relative stability of glycolate oxidase, lactate dehydrogenase and hydroxypyruvate reductase in an extract of watercress roots.

The roots were ground in 0.05 M potassium phosphate buffer at pH 7.5 containing 2% polyvinylpolypyrrolidone, and then centrifuged at 500 x g for 10 minutes. The 500 x g supernatant was assayed immediately (time zero) and at subsequent intervals during storage at 4°: glycolate oxidase (○—○), NADH-pyruvate reductase (△—▽), and NADH-hydroxypyruvate reductase (□—□).

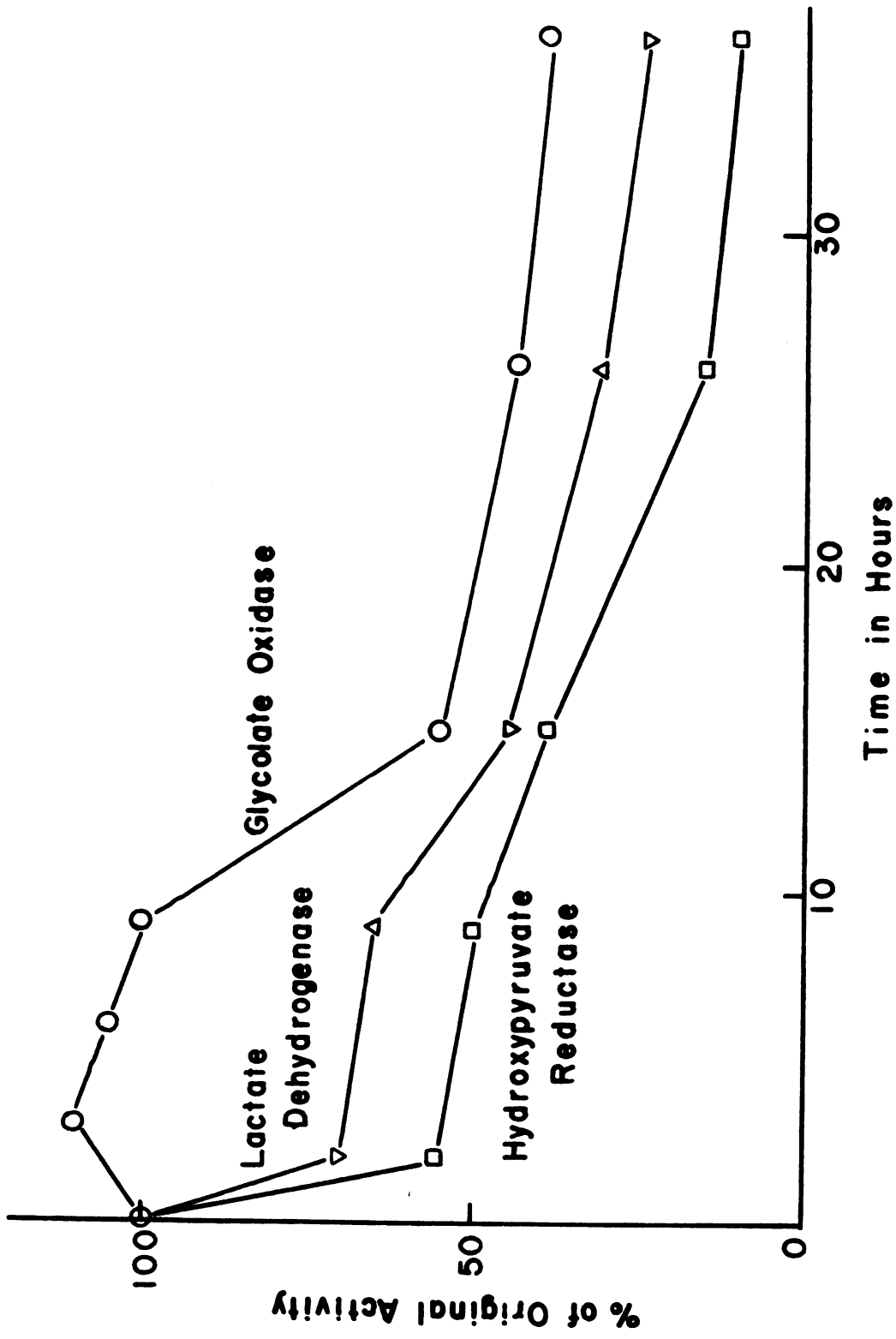
% of Original Activity
100
50
0
10
20
30
Time in Hours
Glycolate Oxidase
Lactate Dehydrogenase
Hydroxypyruvate Reductase

Figure 1

The addition of L-lactate resulted in the immediate utilization of molecular oxygen or the reduction of the artificial electron acceptor, DCPIP. The rates of reactions in both cases were slow. Oxygen consumption and dye reduction were not inhibited by 0.5 mM cyanide, 2 μg/ml antimycin A, or 10^{-6} M rotenone. The reactions were not inhibited by the sulfhydryl binding reagents, 10^{-5} M p-chloromercuribenzoic acid and 10^{-3} M N-ethylmaleimide. Besides activity with L-lactate, glycolate and some activity with D-lactate, the fraction catalyzed the oxidation of other alpha-hydroxyacids such as DL-alpha-hydroxyisocaprate and glyoxylate (Table V). Assays in which equal amounts of glycolate and lactate were added resulted in activity almost intermediate between that observed for the individual substrates, which suggested that a single enzyme was involved.

Separation of Hydroxypyruvate Reductase and L-Lactate Dehydrogenase from Pea Roots

Although homogenates of the roots of pea seedlings had no alpha-hydroxyacid oxidase activity, they readily catalyzed the reduction of pyruvate, hydroxypyruvate and glyoxylate. Since peas were easy to grow, the roots of pea seedlings were chosen to further study these dehydrogenase or reductase activities. Partial purification by methods described in the Materials and Methods is shown in Table VI for the L-lactate dehydrogenase activity and in Table VII for the hydroxypyruvate reductase activity with hydroxypyruvate as substrate. Upon passage of the protein fraction from 30-60% saturated $(NH_4)_2SO_4$ precipitation through the DEAE-cellulose column, the enzymes were well separated into two protein fractions (Figure 2). One protein, designated hydroxypyruvate reductase since it did not utilize lactate,

Table V. Substrate specificity of crude alpha-hydroxyacid oxidase from watercress roots

Rates of DCPIP reduction and oxygen consumption were expressed as the percentage of the rates with glycolate. Two enzyme preparations were used: one had specific activity of 1.3 nmoles DCPIP/min/mg protein,* the other 4.6 nmoles O_2/min/mg protein.** Final concentration of each substrate was 8 mM.

Substrates	Electron Acceptors	
	DCPIP %	Oxygen %
Glycolate	100*	100**
L-lactate	176	129
D-lactate	27	71
Glyoxylate	91	--
DL-glycerate	0	--
DL-alpha-hydroxy-isocaproate	82	--
Glycolate (8 mM) plus L-lactate (8 mM)	131	--

Table VI. Partial purification of lactate dehydrogenase from pea roots

Fraction	Protein (mg)	Total Activity µmoles NADH/ min	Specific Activity nmoles NADH/min/ mg protein	Purification (fold)	Recovery (%)
Crude	1201	3.86	3	1	--
30-60% $(NH_4)_2SO_4$	468	7.38	16	4.4	100
DEAE-52	153	6.67	44	13.6	89
Sephadex G-200	18	4.93	274	85.5	67

Table VII. Partial purification of hydroxypyruvate reductase from pea roots

Fraction	Protein (mg)	Total Activity µmoles NADH/ min	Specific Activity nmoles NADH/min/ mg protein	Purification (fold)	Recovery (%)
Crude	1201	38.9	32	1	100
30-60% $(NH_4)_2SO_4$	468	31.4	77	2	80
DEAE-52	59	11.1	189	6	28
Sephadex G-200	8.6	10.7	1240	40	27

Figure 2. Separation of lactate dehydrogenase and hydroxypyruvate reductase from homogenates of pea roots by DEAE-cellulose chromatography.

The column (DE-52, 2.5 cm x 30 cm) was washed with 5 mM potassium phosphate buffer containing 1 mM 2-mercaptoethanol and the protein was eluted at 30 ml per hour with a 600-ml gradient of potassium chloride (●—●) ranging from 0.1-0.5 M. Fractions of 6 ml were collected. Effluent fractions were monitored for protein at 280 nm (——) and also assayed for lactate dehydrogenase and hydroxypyruvate reductase as described in the Materials and Methods, using hydroxypyruvate (○—○), glyoxylate (□—□), and pyruvate (△—△) as substrates.

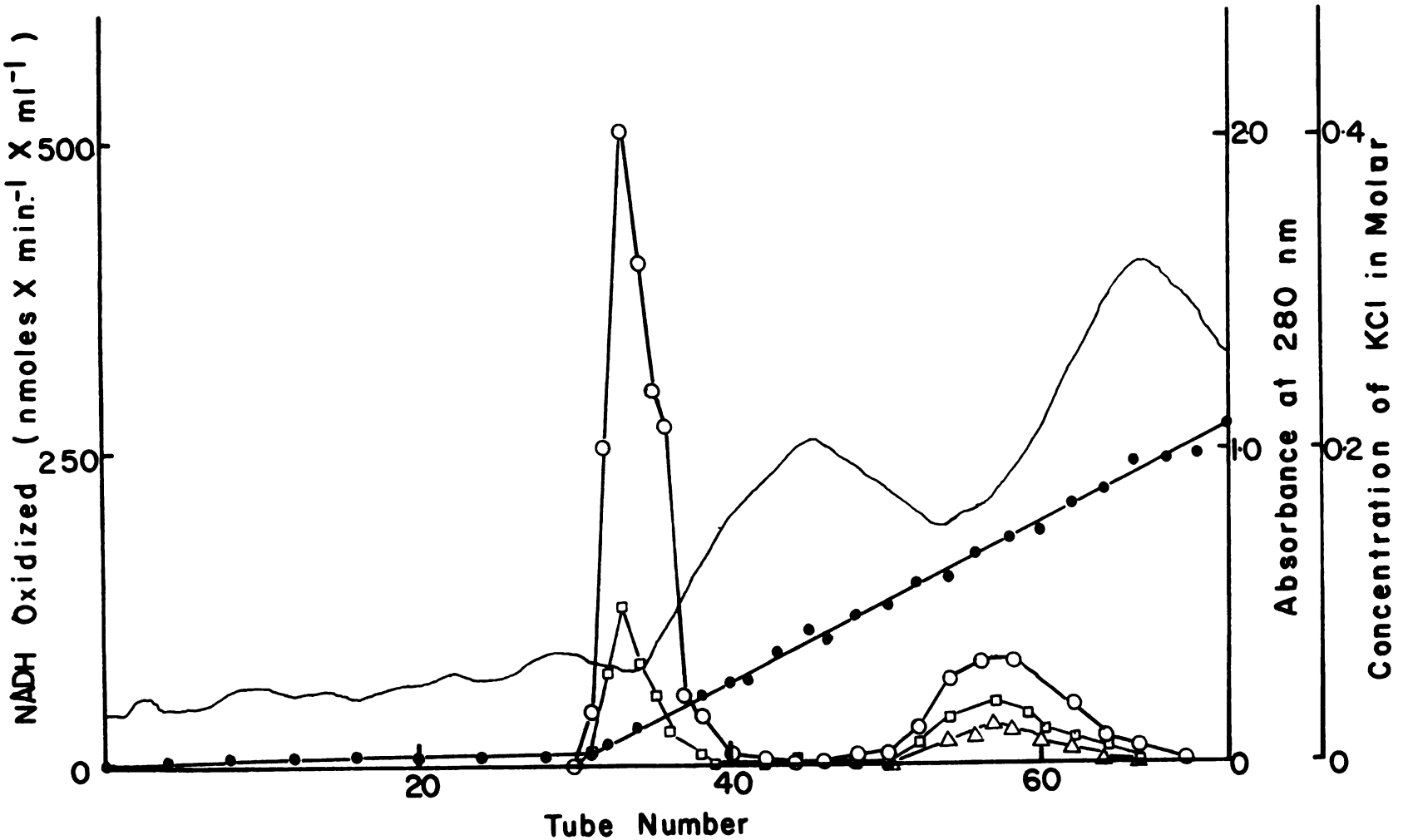
NADH Oxidized (nmoles X min.-1 X ml-1)
500
250
0
20
40
60
Tube Number
20
1·0
0
Absorbance at 280 nm
0·4
0·2
0
Concentration of KCl in Molar

Figure 2

was eluted at 0-0.05 M potassium chloride while the other protein, designated L-lactate dehydrogenase because it used L-lactate as well as the other two substrates, was eluted at 0.1-0.2 M potassium chloride. Each of these proteins was further purified 40- to 100-fold by being eluted through a Sephadex G-200 column separately (Figure 3). These partially purified enzymes (Tables VI and VII), though still not homogeneous, were free from each other (as shown by gel electrophoresis) and were used for further characterization studies in all of the subsequent sections.

Molecular Weight Estimations

The molecular weights of the lactate dehydrogenase and hydroxypyruvate reductase were estimated by using Sephadex G-200 molecular exclusion chromatography. Graphical estimation of the molecular weights of root lactate dehydrogenase and hydroxypyruvate reductase was obtained from a standard curve established by plotting the logarithms of the molecular weights of a series of protein standards versus K_{av} as described in Materials and Methods (Figure 4). The molecular weight of lactate dehydrogenase was found to be 160,000 daltons and that of hydroxypyruvate reductase was 68,000 daltons.

pH Optima of Hydroxypyruvate Reductase and Lactate Dehydrogenase from Pea Roots

Variation of enzymatic activities with pH was investigated with 0.1 M phosphate/pyrophosphate buffer over a pH range from 4.5-8.5 and with 0.1 M glycine-NaOH buffer over the range from 8.6-10.6. The pH values were adjusted with either NaOH or HCl. The final pH of the reaction mixture was measured with a Sargent single glass

Figure 3. Elution profiles of lactate dehydrogenase and hydroxypyruvate reductase from Sephadex G-200 column.

After the DEAE-cellulose column, 100 mg of the protein with lactate dehydrogenase activity and 60 mg of the protein with hydroxypyruvate reductase activity were separately developed on the Sephadex G-200 column (1.6 x 90 cm) with buffers as described in Materials and Methods. Activity of hydroxypyruvate reductase and that of lactate dehydrogenase was assayed using hydroxypyruvate (●- -●) and pyruvate (O—O), respectively, as substrates. Protein concentrations for hydroxypyruvate reductase (- - -) and for lactate dehydrogenase (———) were measured at 280 nm.

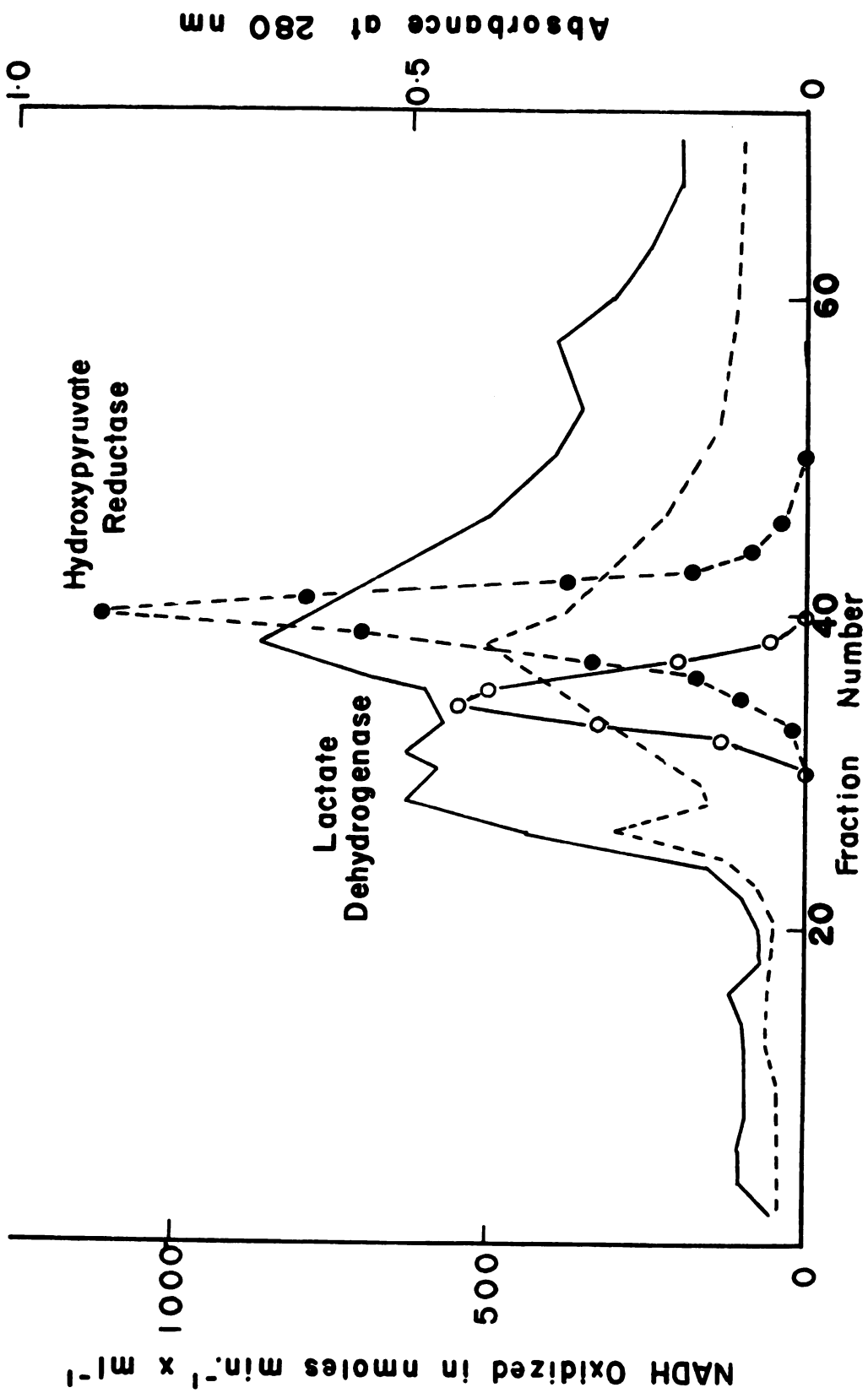
NADH Oxidized in nmoles min.⁻¹ x ml⁻¹
1000
500
0
Absorbance at 280 nm
1·0
0·5
0
Hydroxypyruvate Reductase
Lactate Dehydrogenase
20
40
60
Fraction Number

Figure 3

Figure 4. Molecular weights of lactate dehydrogenase and hydroxypyruvate reductase from pea roots.

The elution volume (V_e) of the protein standards was determined by monitoring absorbance at 280 nm. The void volume of the column (V_o) was determined with blue dextran and monitoring its elution at 640 nm. The bed volume (V_t) was determined by measuring the equivalent amount water the column could hold before it was packed. K_{av} was defined as $(V_e - V_o)/(V_t - V_o)$. The lactate dehydrogenase sample used for this determination contained 100 mg of enzyme after the DEAE-cellulose step in Table VI in a sample volume of 4 ml. The hydroxypyruvate reductase sample contained 60 mg of enzyme after the same step in Table VII. The protein standards contained 20 mg of each in a volume of 4 ml.

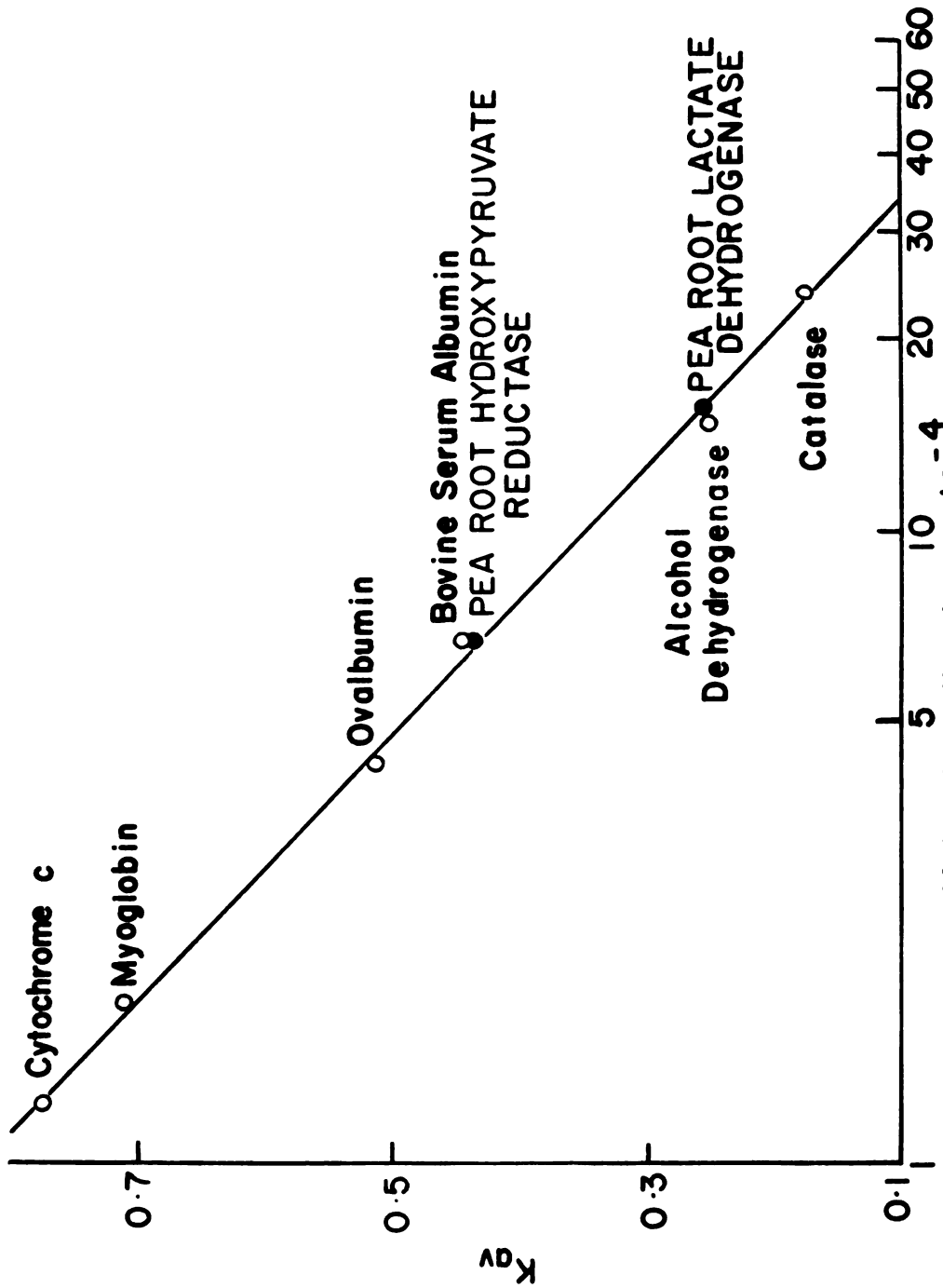
Cytochrome c
Myoglobin
Ovalbumin
Bovine Serum Albumin
PEA ROOT HYDROXYPYRUVATE REDUCTASE
Alcohol Dehydrogenase
PEA ROOT LACTATE DEHYDROGENASE
Catalase
0·7
0·5
0·3
0·1
K_{av}
5
10
20
30
40
50
60
Molecular Weight X 10^{-4}

Figure 4

electrode attached to a pH meter and the results are shown in Figure 5. Lactate dehydrogenase had a sharp pH optimum around pH 7.0 for all the substrates, pyruvate, hydroxypyruvate and glyoxylate. Hydroxypyruvate reductase had a broad pH optimum around 5.9. Both enzymes were the most active by far with hydroxypyruvate and both enzymes were able to reduce glyoxylate but at a slower rate. The rate of the reverse reaction at alkaline pH was also determined by the standard assays as described in the Materials and Methods, and the results are shown in Figure 6. The pH optimum of L(+)-lactate oxidation by lactic dehydrogenase was 9.6, and the optimum for glycerate oxidation by hydroxypyruvate reductase was at pH 9.1.

Substrate Specificities of L-Lactate Dehydrogenase and Hydroxypyruvate Reductase from Pea Roots

The lactate dehydrogenase from pea roots catalyzed NADH-dependent conversion of pyruvate presumably to L(+)-lactate because it oxidized L(+)-lactate at a much greater rate than the D(-)-isomer. Therefore, it is probably similar to L(+)-lactate dehydrogenase from other tissues, which also reduces hydroxypyruvate and glyoxylate. Hydroxypyruvate reductase, on the other hand, did not use pyruvate and was active only with hydroxypyruvate and glyoxylate. These results are summarized in Table VIII. Both enzymes were specific for NADH and could not utilize NADPH. Both enzymes were able to catalyze the reverse reaction at high pH at a much slower rate than the forward reaction. Lactate dehydrogenase was able to oxidize glyoxylate as well as to reduce it, similar to the lactate dehydrogenase found in animals (24). The stereoisomerism of the substrates that can be

Figure 5. pH Curves for the reduction of pyruvate, hydroxypyruvate and glyoxylate by lactate dehydrogenase and hydroxypyruvate reductase from pea roots.

Incubation mixtures contained: substrates 10 mM, NADH 0.12 mM, appropriate amount of the partially purified enzymes from Sephadex G-200 and 0.1 M potassium phosphate/pyrophosphate buffers in a total volume of 1 ml. Reduction of pyruvate (△—△), glyoxylate (□—□) and hydroxypyruvate (○—○) is denoted by a thin line for lactate dehydrogenase and a heavy line for hydroxypyruvate reductase.

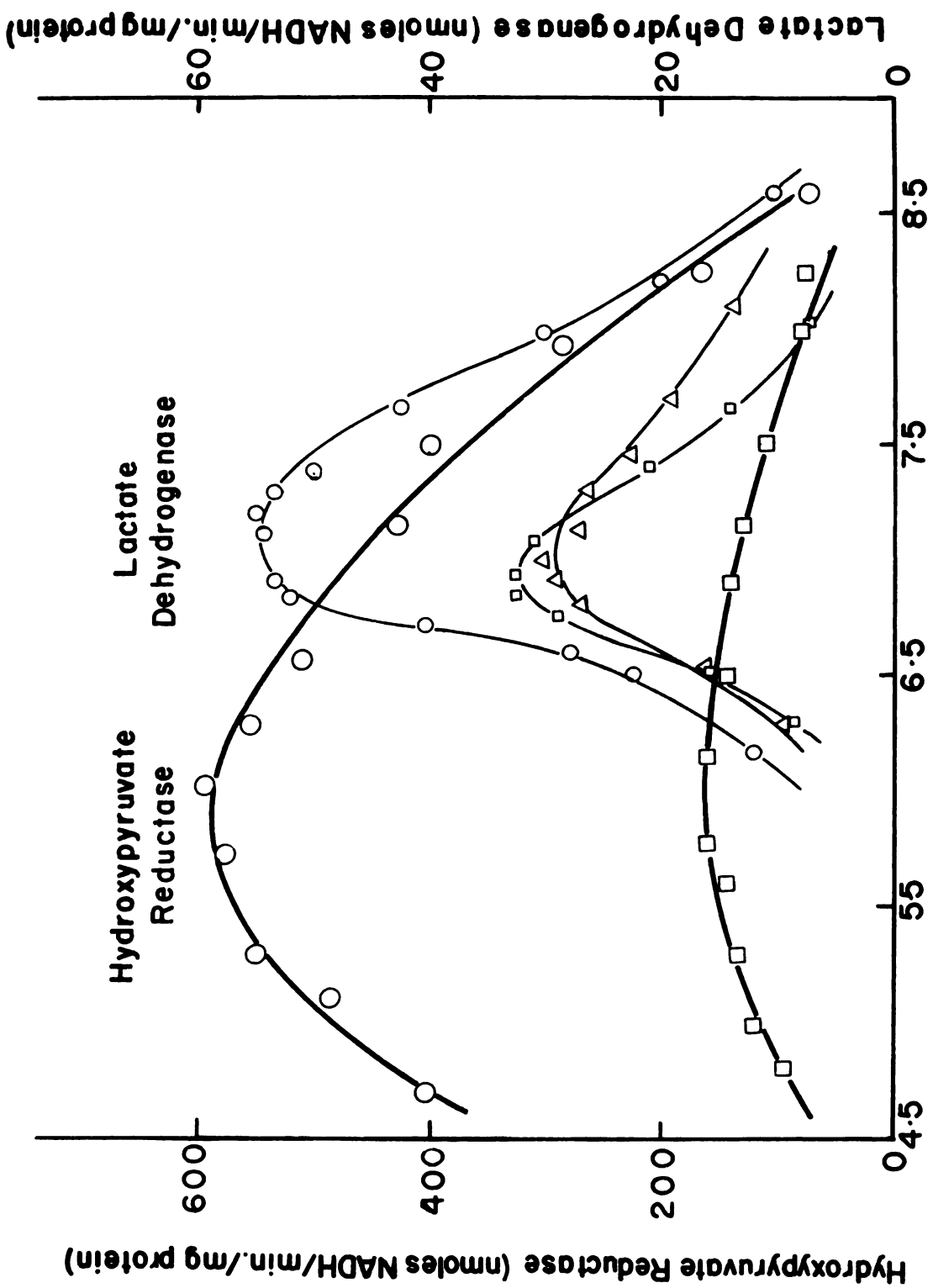
Hydroxypyruvate Reductase (nmoles NADH/min./mg protein)
Lactate Dehydrogenase (nmoles NADH/min./mg protein)
Hydroxypyruvate Reductase
Lactate Dehydrogenase
pH
600
400
200
0
60
40
20
0
4·5
5·5
6·5
7·5
8·5

Figure 5

Figure 6. Effect of pH upon the oxidation of L-lactate by lactate dehydrogenase and the oxidation of DL-glycerate by hydroxypyruvate reductase from pea roots.

Incubation mixture contained: substrate 20 mM, NAD 3 mM, appropriate amount of the partially purified enzymes from Sephadex G-200 and 0.1 M NaOH-glycine buffer in a total volume of 1 ml. Substrates used were L-lactate (□—□) and DL-glycerate (○—○) for lactate dehydrogenase and hydroxypyruvate reductase, respectively.

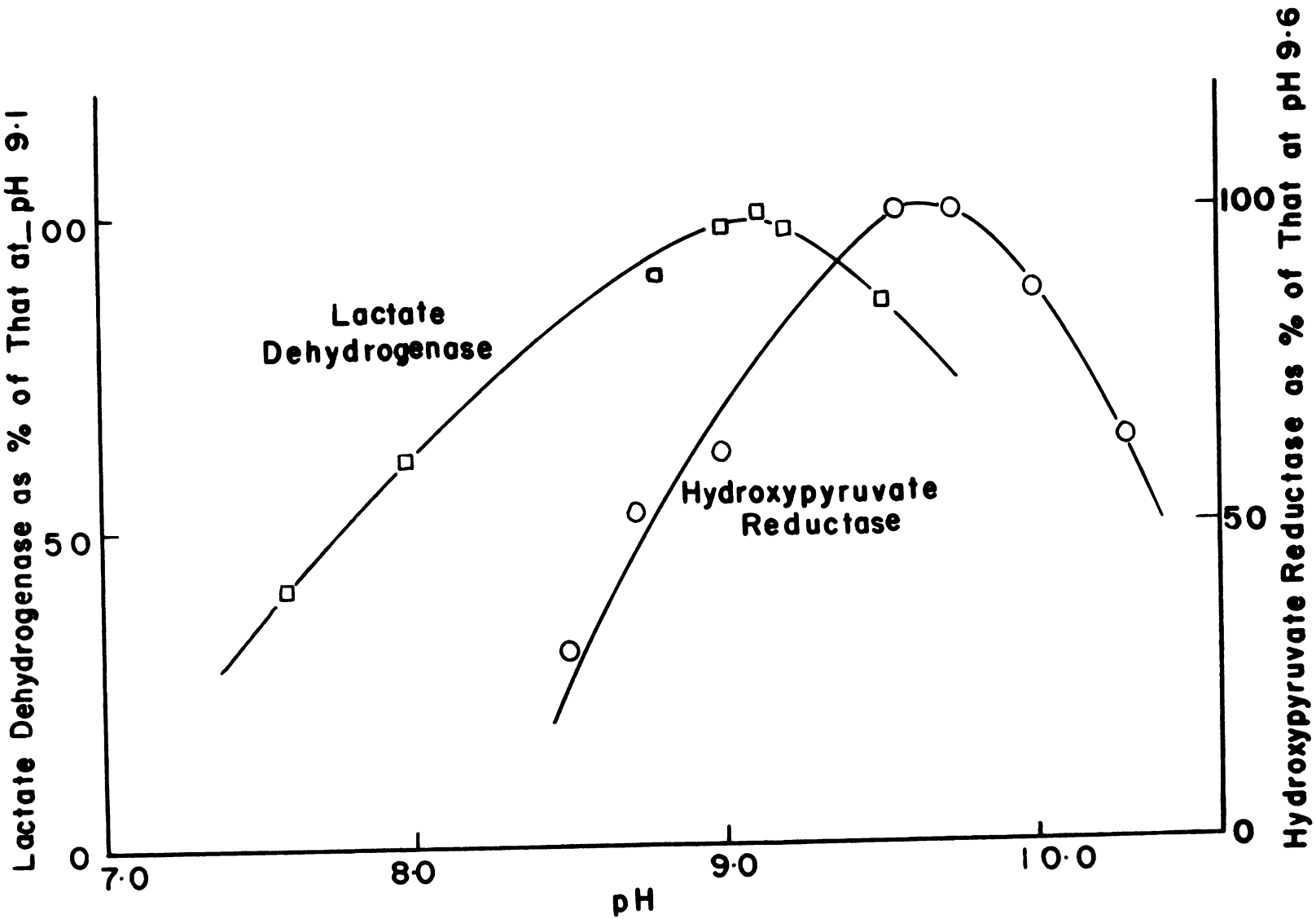
Lactate Dehydrogenase as % of That at pH 9·1
100
50
0
Lactate Dehydrogenase
Hydroxypyruvate Reductase
7·0
8·0
9·0
10·0
pH
100
50
0
Hydroxypyruvate Reductase as % of That at pH 9·6

Figure 6

Table VIII. Substrate and coenzyme specificity of hydroxypyruvate reductase and lactate dehydrogenase from pea roots

Each incubation mixture contained 4-10 µg protein (the fractions eluted from the Sephadex G-200 column in Table VII for hydroxypyruvate reductase and in Table VI for lactate dehydrogenase), 0.12 mM NADH or NADPH, 10 mM substrate, and buffer plus water in a total volume of 1 ml in a 1-ml quartz cuvette with pathlength of 1 cm. The buffers used were 50 mM potassium phosphate at pH 7.0 for lactate dehydrogenase and at pH 6.2 for hydroxypyruvate reductase. The substrate-dependent reduction of NAD or NADP by the enzyme was also monitored in 1-ml reaction mixtures containing 0.1 M NaOH-glycine at pH 9.5, 7.5 mM NAD or NADP, 20 mM substrate, 4-10 µg protein, and water.

Substrates	Lactate dehydrogenase	Hydroxypyruvate reductase
	nmoles/min/mg protein	
Pyruvate plus NADH	104	0
Pyruvate plus NADPH	0	0
Hydroxypyruvate plus NADH	243	676
Hydroxypyruvate plus NADPH	0	0
Glyoxylate plus NADH	126	250
Glyoxylate plus NADPH	0	0
Alpha-ketobutyrate plus NADH	60	0
Alpha-ketoglutarate plus NADH	10	0
L-lactate plus NAD	46	0
L-lactate plus NADP	0	0
D-lactate plus NAD	12	0
D-lactate plus NADP	0	0
Phenylpyruvate plus NADH	0	0
DL-glycerate plus NAD	0	11
DL-glycerate plus NADP	0	0
Glyoxylate plus NAD	10	0

utilized by hydroxypyruvate reductase has not been investigated, but all previous work on the enzyme from other sources indicated that it was specific for the D-isomers (55,95,120).

Inhibition of the Root Lactate Dehydrogenase and Hydroxypyruvate Reductase

p-Chloromercuribenzoate (PCMB) completely inhibited hydroxypyruvate reductase at 0.1 mM final concentration, but it had little effect on lactate dehydrogenase (Table IX). The approximate 20% inhibition of lactate dehydrogenase by PCMB was not prevented by addition of 5 mM dithiothreitol, cysteine, mercaptoethanol, or glutathione, which by themselves had no effect on the enzyme. Both enzymes were inhibited by 10 mM oxamate, 10 mM oxalate and 10 mM o-phenanthroline, though to a different extent. It was surprising that the lactate dehydrogenase was completely inhibited by o-phenanthroline, since no metal requirement is known for this enzyme. Cu^{++}, Zn^{++}, Mg^{++}, Fe^{+++}, Mn^{++} at 1 mM did not affect the activities of either enzyme.

Thermal Stability

Aliquots of the partially purified enzymes were incubated at various temperatures in a water bath for 20 minutes. After centrifugation to remove precipitated proteins, the supernatants were assayed for enzyme activities at 25°. The activity of hydroxypyruvate reductase remained constant up to 40° (Figure 7), and half the activity was lost in 20 min at a temperature of about 55°. Lactate dehydrogenase was stable for 20 minutes up to a temperature of 76°. This is a particularly high temperature stability for a plant enzyme,

Table IX. Inhibitors of root lactate dehydrogenase and hydroxypyruvate reductase

The two enzymes were assayed by measuring the rate of oxidation of NADH in the presence of hydroxypyruvate after prior incubation at 25° for 5 minutes with the inhibitory compounds.

Added compound	Relative Rate of Reaction*	
	Hydroxypyruvate reductase	Lactate dehydrogenase
None	100	100
PCMB 0.0125 mM	88	100
PCMB 0.05 mM	68	89
PCMB 0.1 mM	0	81
PCMB 0.25 mM	0	68
Dithiothreitol 5 mM	100	100
Dithiothreitol (5 mM) plus PCMB (0.1 mM)	100	81
Potassium cyanide 10 mM	100	100
Sodium arsenite 10 mM	94	88
Oxamate 10 mM	22	55
Oxalate 10 mM	9	82
EDTA 10 mM	105	100
o-phenanthroline 10 mM	55	0
Iodoacetamide 2 mM	89	67
Ethylmaleimide 1 mM	34	95

*100% corresponded to 84 nmoles NADH/min/mg protein for hydroxypyruvate reductase and 87 nmoles NADH/min/mg protein for lactate dehydrogenase.

Figure 7. Heat stability of L-lactate dehydrogenase and hydroxypyruvate reductase from pea roots.

The partially purified enzymes were incubated at various temperatures in a water bath for 20 minutes. After centrifugation to remove the precipitated proteins, the supernatants were assayed for activities with NADH and pyruvate for lactate dehydrogenase (■—■) and with NADH and hydroxypyruvate for hydroxypyruvate reductase (O—O). Activities are expressed as % of that at 25°.

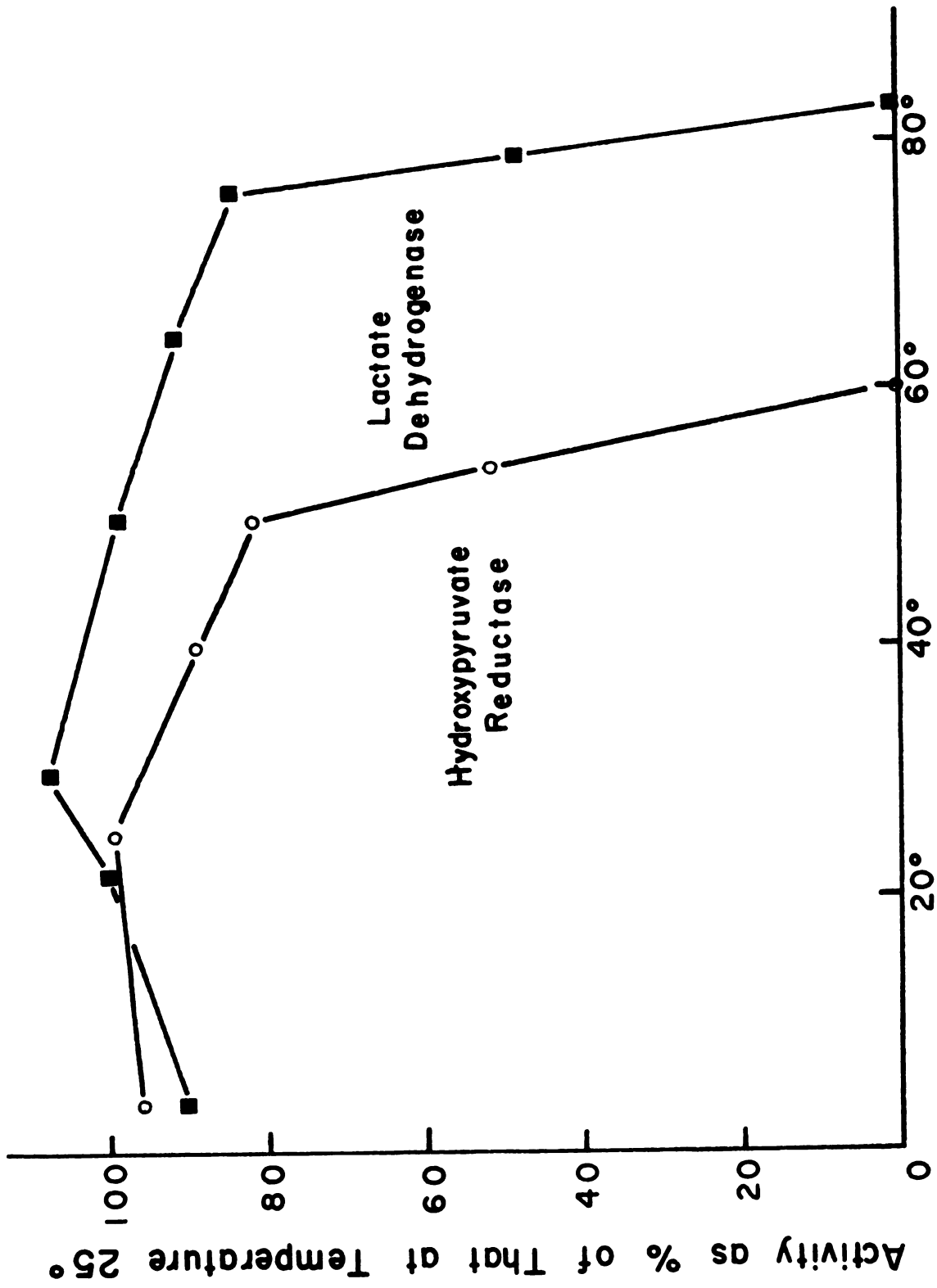
Activity as % of That at Temperature 25°
0
20
40
60
80
100
20°
40°
60°
80°
Temperature of Pre-incubation
Lactate Dehydrogenase
Hydroxypyruvate Reductase

Figure 7

which are generally inactivated above 35-45°. In this respect the lactate dehydrogenase from roots is similar to the enzyme in cotyledons which also have a high thermal stability (50).

Kinetic Properties of the Root Lactate Dehydrogenase and Hydroxypyruvate Reductase

Effect of substrate concentration on the initial rate was determined at pH 7.0 for lactate dehydrogenase and at pH 6.2 for hydroxypyruvate reductase in the presence of a constant amount of enzyme. Both enzymes showed normal Michaelis-Menten kinetics and the Michaelis constants for the substrates and coenzyme are shown in Table X. Both enzymes were inhibited to some extent by high concentration of substrates and NADH. Pyruvate concentration greater than 10 mM and NADH concentration greater than 0.125 mM were inhibitory for root lactate dehydrogenase. Hydroxypyruvate concentration greater than 8 mM was inhibitory for hydroxypyruvate reductase.

Hydroxypyruvate reductase from pea roots utilizes hydroxypyruvate at a K_m of 4.8×10^{-4} M but the K_m for glycerate at 2.4×10^{-2} M is so high that the dehydrogenase reaction seems hardly possible *in vivo*. The same concern is noted for the K_m (lactate) of 5.3×10^{-2} M for lactate dehydrogenase. It is presumed in the liver that lactate oxidation occurs by this enzyme because of immense amounts of enzyme and a high concentration of lactate, but neither of these factors is present in the roots of plants to favor the dehydrogenase reactions.

Subcellular Localization of Hydroxypyruvate Reductase in Pea Roots

Lactate dehydrogenase is considered to be a soluble or cytoplasmic enzyme from work with animal tissues (74). However, hydroxypyruvate

Table X. Michaelis constants for the root lactate dehydrogenase and hydroxypyruvate reductase

Enzymes	pH	Variable Substrate	Constant Substrate	K_m (molar)
Hydroxypyruvate reductase	6.2	Hydroxypyruvate (Li-)	0.12 mM NADH	4.8×10^{-4}
Hydroxypyruvate reductase	6.2	Glyoxylate (Na-)	0.12 mM NADH	1.8×10^{-3}
Hydroxypyruvate reductase	6.2	NADH	10 mM hydroxypyruvate	7.6×10^{-6}
Hydroxypyruvate reductase	9.2	DL-glycerate (Ca-)	3 mM NAD	2.4×10^{-2}
Lactate dehydrogenase	7.0	Pyruvate (K-)	0.12 mM NADH	3.4×10^{-4}
Lactate dehydrogenase	7.0	Hydroxypyruvate (Li-)	0.12 mM NADH	9.4×10^{-4}
Lactate dehydrogenase	7.0	NADH	10 mM hydroxypyruvate	6.1×10^{-6}
Lactate dehydrogenase	9.2	L-lactate (Li-)	3 mM NAD	5.3×10^{-2}
Lactate dehydrogenase	9.2	NAD	20 mM L-lactate	4.5×10^{-4}

reductase was found in the peroxisomes in the leaves (111), although it has not been found in the peroxisomes of the rat liver (74). Therefore, the subcellular distribution of hydroxypyruvate reductase in pea roots was examined. In an experiment by differential centrifugation in 0.5 M sucrose, as described in method B, 40% of the catalase and 88% of the cytochrome c oxidase was found in the particulate fraction but only 9% of the hydroxypyruvate reductase was in the same fraction and the rest was in the supernatant. The specific activity of hydroxypyruvate reductase in this fraction was the same as that in the crude extract, while its specific activity in the supernatant increased due to particle removal by centrifugation (Table XI). In another experiment, the root homogenate was centrifuged over a continuous linear sucrose density gradient as described in Materials and Methods. The distribution of specific marker enzymes for organelles as well as for the hydroxypyruvate reductase was determined (Figure 8). The peroxisomal marker enzyme, catalase, and the mitochondrial marker enzyme, cytochrome c oxidase, overlapped at an equilibrium density of about 1.21 g/cm^3, while hydroxypyruvate reductase was found only at the low density part of the gradient, i.e., the supernatant. Although the gradient failed to separate the organelles in the root homogenate, it was good enough to show that the hydroxypyruvate reductase was present in the cytosol of the cell. It is possible that one of the isoenzymes of hydroxypyruvate reductase is present in the microbodies of the root in too low a level for detection. This possibility is not likely, since in the next section it is shown that in the disc electrophoretic patterns of the isoenzymes of the root hydroxypyruvate reductase and leaf hydroxypyruvate

Table XI. Distribution of hydroxypyruvate reductase in subcellular fractions of pea root homogenates

Differential centrifugation in 0.5 M sucrose was performed as described in method B.

Fraction	Catalase		Cytochrome c Oxidase		Hydroxypyruvate Reductase	
	µmoles/min/mg protein	Distribution (%)	nmoles/min/mg protein	Distribution (%)	nmoles/min/mg protein	Distribution (%)
Crude	45	100	27	100	21	100
270 x g pellet	58	5	36	0.5	13	1
6000 x g pellet	160	40	209	88	28	9
37,000 x g pellet	65	13	31	10	15	4
37,000 x g supernatant	38	57	2	5	45	86
Total recovery*		115		104		100

*As % of total homogenate activity.

Figure 8. Distribution of microbody and mitochondrial marker enzymes and hydroxypyruvate reductase from pea roots in a continuous linear sucrose density gradient.

Peroxisomal marker is indicated by catalase and mitochondria by cytochrome c oxidase. One unit is defined as the amount of enzyme catalyzing the disappearance of 1 nmole of substrate per minute.


Hydroxypyruvate reductase in nmoles NADH oxidized per minute per ml gradient.


Cytochrome c oxidase in nmoles cytochrome c oxidized per minute per ml gradient.


Catalase in μmoles H_2O_2 disappeared per minute per ml gradient.

Sucrose density in gm per cm^3.

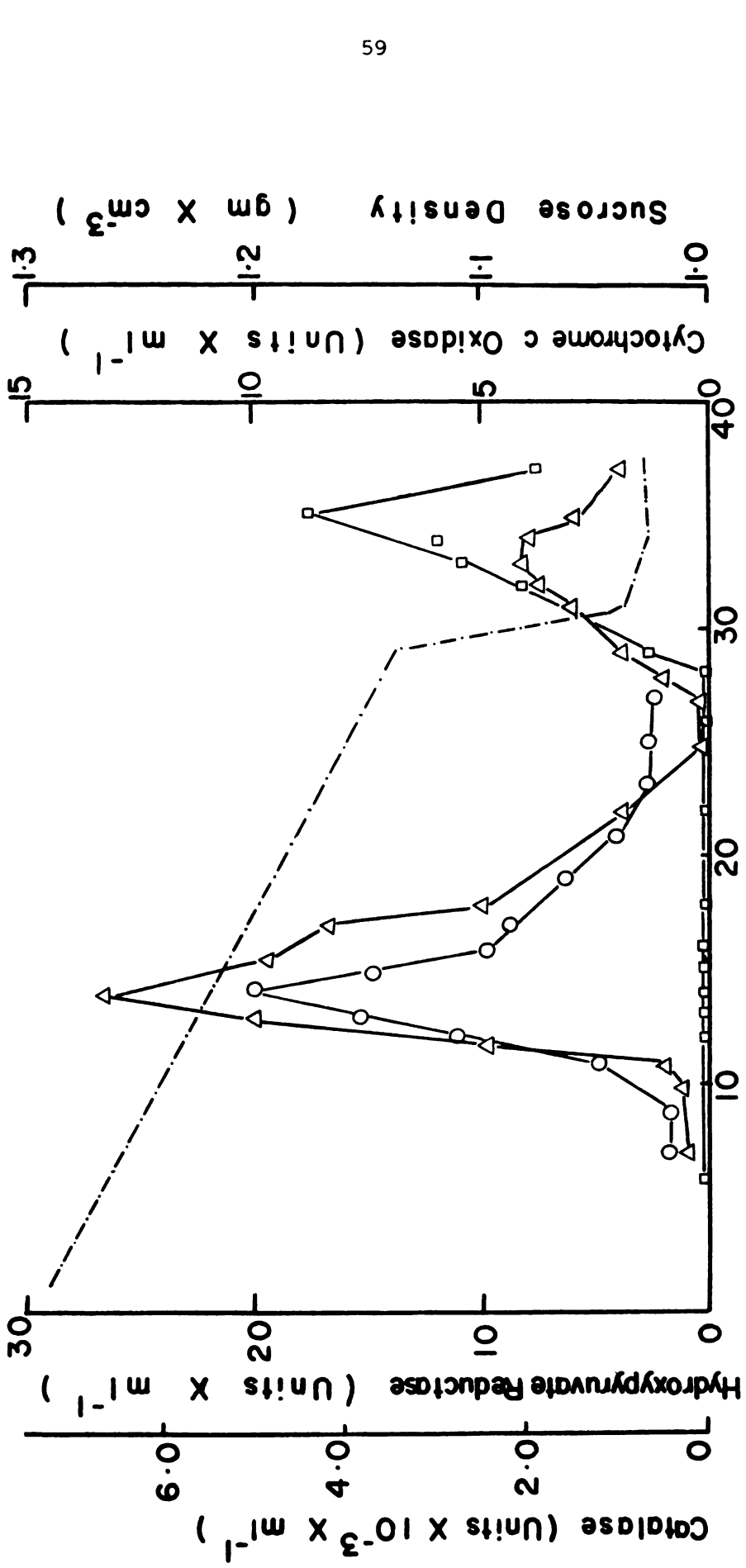
Catalase (Units x 10^{-3} x ml^{-1})
Hydroxypyruvate Reductase (Units x ml^{-1})
Cytochrome c Oxidase (Units x ml^{-1})
Sucrose Density (gm x cm^{-3})
Fraction Number
0
2.0
4.0
6.0
10
20
30
5
15
40
1.0
1.1
1.2
1.3

Figure 8

reductase, the root enzyme pattern had one band less than that of the leaf enzyme, raising the possibility that this missing band might be the isoenzyme present in the microbodies.

Polyacrylamide Gel Disc Electrophoresis

In an experiment to determine whether isoenzymes of both lactate dehydrogenase and hydroxypyruvate reductase in the roots existed, the crude as well as the partially purified enzyme extracts were subjected to acrylamide gel disc electrophoresis. Only one protein band contained lactate dehydrogenase activity when specifically stained for the enzyme with either L- or D-lactate in both the crude and the partially purified preparations, even though several protein bands were present. In similar polyacrylamide gel disc electrophoresis, commercial rabbit muscle lactate dehydrogenase (Sigma) was composed of the five well known bands derived from a tetramer of 2 different subunits. The distal 4 bands had R_f values of 0.11, 0.16, 0.27, and 0.33, respectively. The single lactate dehydrogenase band from pea roots had an R_f value of 0.29, closely resembling one of the slower moving rabbit muscle isoenzymes.

Hydroxypyruvate reductase from pea roots was resolved by polyacrylamide gel disc electrophoresis into three active bands at R_f values of 0.152, 0.168, and 0.179.

Commercial hydroxypyruvate reductase from spinach leaves was composed of four bands with R_f values of 0.185, 0.201, 0.228, and 0.271. Thus it appears that hydroxypyruvate reductase from roots has 3 isoenzymes while that in the leaf has 4. Whether these multiple bands were truly isoenzymes, or merely the degraded form of the native enzyme or experimental artifacts is not known.

DISCUSSION

Alpha-Hydroxyacids Oxidase in Roots of Higher Plants

Although the number of plants surveyed for both alpha-hydroxyacid oxidase and lactate dehydrogenase was limited, it is obvious that a glycolate oxidase is not of universal distribution in the roots of the plants, confirming the reports of other workers (80,106). The algal glycolage dehydrogenase was not detected in either roots or leaves of the higher plant. The facts that an alpha-hydroxyacid oxidase was present in the roots of only a limited number of plants and, even if present, the level of activity was very low raise the question of its physiological significance in the roots. It is known that glycolate oxidase is active and of importance in green tissues because it is needed to metabolize glycolate, one of the early products of photosynthesis. However, in the roots there is no photosynthesis so that there is no obvious source of glycolate, although a small amount of both glycolic acid and glyoxylic acids were reported to be present in the roots (32,58). It is possible that the enzyme really functions as lactate oxidase in the roots, as suggested by Tolbert *et al.* (108), since in all cases the enzyme in the roots oxidizes lactate better than glycolate. For this reason the enzyme in the roots is being referred to as an alpha-hydroxyacid oxidase. If such is the case, then it can act, together with lactate dehydrogenase, as a possible

terminal oxidase system in the roots. In this proposed scheme, lactate dehydrogenase functions as a reductase to form lactate from pyruvate and the alpha-hydroxyacid oxidase transfers the reducing equivalents to O_2. Since the alpha-hydroxyacid oxidase in the crude extracts of roots of wheat is inhibited by cyanide, it might be indirectly linked to O_2 by some intermediate electron acceptors of the respiratory chain. The oxidase in watercress roots is not inhibited by cyanide, sulfhydryl binding reagents, or inhibitors of the respiratory electron transport chain, so that it could be a typical terminal oxidase system like that for glycolate oxidase in leaf peroxisomes. In all cases it is possible that the alpha-hydroxyacid oxidase functions to remove excess reducing power or plays a regulatory role in glycolysis.

The low activity of the alpha-hydroxyacid oxidase in the root extracts (assuming the enzyme has not been changed or inactivated during the grinding procedure, and no natural inhibitor is present) indicates that one must still consider that the enzyme arises as the consequence of the root being exposed to light, as reported by Mothes and Wagner (80). In the present experiments, both plants (watercress and wheat) were grown in nutrient solution culture in the presence of light, making it possible that the proplastids in the roots were activated to produce a small photosynthetic system as well as a small amount of alpha-hydroxyacid oxidase. No roots used in the experiments showed any sign of green color, however, and whether the above possibility is true or not has not been investigated. However, in view of the different properties of the alpha-hydroxyacid oxidase from roots as compared to glycolate oxidase in leaves, this hypothesis is not favored.

The major significant difference between the leaf oxidase and the root oxidase found in this report has been that the root oxidase can utilize L-lactate more readily than glycolate. This has been found true for the alpha-hydroxyacid oxidase from rice roots (108) and from leaves of carrot, rhubarb, grape, dogwood, lilac (82), sorghum (Table I), and probably other plants wherever low concentration of 'glycolate oxidase' occurs. The reason for this is not known, but it is possible that in these tissues the glycolate oxidase is modified, as mentioned in the results section. In tissues such as the roots (44) and the mesophyll cell of the sorghum leaf (43), the microbodies are of nonspecialized type and glycolate metabolism is almost nil.

My results and those in the literature (80) suggested that glycolate is not an active metabolite in the roots as it is in the leaves. Some glycolate that was reported in the roots (32,58) might arise from glyoxylic acid by the action of either lactate dehydrogenase or hydroxypyruvate reductase which catalyzed the NADH-dependent reduction of glyoxylate. The reaction is not reversible, however, so that the glycolate would be the metabolic end product, but it should be transportable to the leaves. Glyoxylate may arise from the enzymatic hydrolysis of allantoin (58), which occurs widely in the root tissues of plants (78,79).

Root Lactate Dehydrogenase

L-Lactate dehydrogenase was found to be widely distributed in the roots of young plants. The properties of lactate dehydrogenase found in the roots of pea seedlings are in general consistent with

the properties of this enzyme from other sources (Table XII), but there are notable differences between the heart enzyme and the enzyme from roots or cotyledons. Although activity with pyruvate is lower than that with hydroxypyruvate, the K_m (pyruvate) is lower so that at physiological concentration of the metabolites, pyruvate is a readily available substrate. The control of the root lactate dehydrogenase in relation to fermentation has not been studied. In microorganisms, it has been proposed that one way in which glycolysis is controlled is by regulation of the enzyme which catalyzes the terminal step, namely lactate dehydrogenase. Thus ATP (99,121), GTP (63), NADH (47), and the glycolytic intermediates fructose 1,6-diphsophate (9) have been found to be effectors for a number of lactate dehydrogenases. For potato tuber lactate dehydrogenase (21), ATP was a potent inhibitor at low pH but a weak competitive inhibitor at high pH. The root lactate dehydrogenase is inhibited by high concentration of pyruvate. In this respect it has the properties resembling the animal heart lactate dehydrogenase, which is also inhibited by high concentration of pyruvate (118). However, the high concentration of pyruvate (greater than 10 mM) that is inhibitory to the root lactate dehydrogenase makes the physiological significance of this inhibition questionable. Probably under partial anaerobic conditions, the root needs a lactate dehydrogenase to maintain glycolysis. The root lactate dehydrogenase also resembles animal lactate dehydrogenase in being sensitive to sulfhydryl inhibitors and substrate analogs such as oxamate and oxalate. The root enzyme and also the soybean cotyledon enzyme are different from the animal enzyme in having no isoenzymic forms. The molecular significance of this needs further investigation.

Root Hydroxypyruvate Reductase

The properties of the hydroxypyruvate reductase from the roots are in general more similar to the same enzyme from animals than the one from the leaves (Table XIII). The enzymes from both animals (89,90,96,120) and leaves (54,55,111) have been studied in detail. Hydroxypyruvate reductase from roots is similar to the liver enzyme in molecular weight, intracellular localization and sensitivity to sulfhydryl reagents. The role of the hydroxypyruvate reductase in the roots would be expected to be the same as that found in animals or in the leaves, namely to function in the interconversion between glycerate and serine. The enzyme in beef liver is inhibited by NADH and other glycolytic intermediates, indicative of feedback inhibition; this has been pointed out as evidence for its function in the catabolic conversion of L-serine to glycolytic intermediates (96). In the leaves, there is an immense pool of serine as a result of photorespiration and therefore much higher level of hydroxypyruvate reductase, and in leaves the interconversion between glycerate and serine has been demonstrated by ^{14}C-labeling experiments (40,45).

Root Microbodies

Huang and Beever (44) have reported that root microbodies contained glycolate oxidase. The fact that this enzyme, which we prefer to call alpha-hydroxyacid oxidase, is present in low level of activity, if at all, in root tissues and that hydroxypyruvate reductase, even if present, is not located in the microbodies of the roots, reinforce the suggestion that the microbodies in the roots are of nonspecialized type. This means that their composition

Table XII. Comparison of lactate dehydrogenase from pea roots, heart (human) and soybean cotyledon

Properties	Root	Soybean Cotyledon*	Heart*
Molecular weight	160,000	not determined	135,000
Substrates	Hydroxypyruvate Pyruvate Glyoxylate L-lactate	Hydroxypyruvate Pyruvate Glyoxylate L-lactate	Hydroxypyruvate Pyruvate Glyoxylate L-lactate
Coenzyme	NAD/NADH	NAD/NADH NAD analogs	NAD/NADH NAD analogs
Substrate inhibition (pyruvate)	yes	no	yes
K_m values:			
Pyruvate	3.4×10^{-4} M (pH 7.0)	2.96×10^{-4} M (pH 7.0)	1.18×10^{-4} M (pH 7.4)
L-lactate	5.3×10^{-2} M (pH 9.2)	not determined	4.4×10^{-3} M (pH 8.7)
pH Optima:			
Pyruvate	7.0	7.0	7.4
L-lactate	9.1	9.2	8.7
Isoenzymic form	1	1	5
Electrophoretic mobility (R_f values)	0.29	0.27	0.27, 0.33**
Sensitivity to PCMB	inhibited	not inhibited	inhibited
Thermal stability***	78°	73°	65°

* Data taken from reference 50 for enzyme from soybean cotyledon and 118 for heart enzyme.

** Fastest moving isoenzymes of the 5 isoenzymes of commercial rabbit muscle lactate dehydrogenase toward anode when subjected to polyacrylamide gel electrophoresis under the same conditions as used for the root enzyme.

*** Temperature at which the activity of pyruvate reduction is inhibited by 50% when incubated for 20-30 minutes.

Table XIII. Comparison of hydroxypyruvate reductase from pea roots, spinach leaves and beef liver

Properties	Root	Leaf	Ref.	Beef liver	Ref.
Molecular weight	68,000	97,500	53	72,000 65,000-70,000	96 89
Substrate specificity	Hydroxypyruvate	Hydroxypyruvate	42,	Hydroxypyruvate	90,96
	Glyoxylate	Glyoxylate	111,	Glyoxylate	
	DL-glycerate	D-glycerate	122	D-glycerate	
Coenzyme specificity	NADH/NAD	NADH/NAD	111	NADH/NAD	
	Not NADP	NADPH/NADP		NADPH/NADP	90,96
Substrate inhibition (hydroxypyruvate)	yes	not reported		yes	96
K_m values:					
D-glycerate	2.4×10^{-2} M	--		1.2×10^{-3} M	90
Glyoxylate	1.8×10^{-3} M	1.4×10^{-2}	42	1.4×10^{-4} M	
Hydroxypyruvate	4.8×10^{-4} M	1.2×10^{-4}		4.5×10^{-5} M	

Table XIII (continued)

Properties	Root	Leaf	Ref.	Beef liver	Ref.
pH Optima:					
Hydroxypyruvate reduction	5.9 (broad)	6.0-6.6	111	6.8-7.0	90,96
Glycerate oxidation	9.6	9.2		9.3	96
Activation by anions	not examined	yes	42,122	yes	90,96
Number of isoenzymatic forms	3	3-4	53	1	89
Electrophoretic mobilities of isoenzymes	0.152, 0.168, 0.179	0.170, 0.190, 0.220, 0.270	53	0.140	89
Sensitivity to PCMB:					
% inhibition (concentration of PCMB)	100 (0.1 mM)	55 (5 μM)	53,42	40 (19 μM)	90
Intracellular location	cytosol	peroxisome	111	cytosol	74

and function remain to be determined. So far, only catalase (103), glycolate oxidase (19,44), urate oxidase and one transaminase (44, 100) have been reported in the root microbodies of plants and only traces of the latter three enzymes are present. Other microbody enzymes, such as malate dehydrogenase, are either not found (127) or have not been investigated. The physiological significance of these organelles with limited enzyme composition must await further research.

LIST OF REFERENCES

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1. Armstrong, J. McD., The molar extinction coefficient of 2,6 dichlorophenol-indophenol. Biochim. Biophys. Acta 86, 194-198 (1968).

2. Avers, C. J., Peroxisomes of yeast and other fungi. Sub-Cell. Biochem. 1, 25-37 (1957).

3. Barber, D. A., Lactic acid formation and carbon dioxide fixation. Nature 180, 1053 (1957).

4. Barker, J. and L. W. Mapson, Studies in the respiratory and carbon-hydrate metabolism of plant tissues. VI. Analysis of the interrelationship between the rate of CO_2 production and the changes in the content of lactic acid. Proc. Roy. Soc. (London) B 141, 338-361 (1950).

5. Barker, J. and E. Saifi, Studies in the respiratory and carbon-hydrate metabolism of plant tissues. I. Experimental studies of formation of CO_2, lactic acid and other products in potato under anaerobic conditions. Proc. Roy. Soc. (London) B 140, 362-384 (1952).

6. Barron, E. S. S., G. K. Link and R. M. Klein, The metabolism of potato slices. Biochim. Biophys. Acta 28, 377-398 (1950).

7. Bourne, D. T. and S. L. Ranson, Respiratory metabolism in detached rhododendron leaves. Plant Physiol 40, 1178-1190 (1965).

8. Bridgers, W. F., Biosynthesis of serine in mouse brain extracts. J. Biol. Chem. 240, 4591-4597 (1965).

9. Brown, A. T., C. P. Christian and R. L. Eifert, Purification, characterization and regulation of a NAD-lactate dehydrogenase from *Actinomyces viscosus*. J. of Bact. 122, 1126-1135 (1975).

10. Cammack, R., Assay, Purification and properties of mammalian D-2-hydroxyacid dehydrogenase. Biochem. J. 115, 55-64 (1969).

11. Cameron, D. S. and E. A. Cossins, Studies of intermediate metabolism in germinating pea cotyledon. Biochem. J. 105, 323-331 (1967).

12. Campbell, W. H., M. P. Ko and N. E. Tolbert, Isolation of dihydroxy-acetonephosphate reductase from higher plants (manuscript in preparation).

13. Cartwright, L. N. and R. P. Hullin, Purification and properties of two glyoxylate reductase from a species of *Pseudomonas*. Biochem. J. 101, 781-791 (1966).

14. Clagget, C. O., N. E. Tolbert and R. H. Burris, Oxidation of alpha-hydroxy acids by enzymes from plants. J. Biol. Chem. 178, 977-987 (1949).

15. Codd, B. A., J. M. Lord and M. J. Merrett, The glycolate oxidizing enzymes of algae. FEBS 5, 341-342 (1969).

16. Collins, N. and M. J. Merrett, Localization of glycolate pathway enzymes in *Euglena*. Biochem. J. 148, 321-328 (1975).

17. Cossins, E. A., Formation and metabolism of lactic acid during germination of pea seedlings. Nature 203, 989-990 (1964).

18. Cossins, E.A., L. C. Kopala, B. Blowacke and A. M. Sprock, Some properties of higher plant alcohol dehydrogenase. Phytochemistry 7, 1125-1134 (1968).

19. Dalling, M. J., N. E. Tolbert and R. H. Hageman, Intracellular location of nitrate reductase and nitrite reductase. II. Wheat roots. Biochim. Biophys. Acta 283, 505-512 (1972).

20. Davies, B. J., Disc electrophoresis. II. Method and application to human serum protein. Ann. N.Y. Acad. Sci. 121, 404-427 (1964).

21. Davies, D. D. and S. Davies, Purification and properties of L-lactate dehydrogenase from potato tubers. Biochem. J. 129, 831-839 (1972).

22. Dennis, D. and N. O. Kaplan, D- and L-lactic dehydrogenase from *Lactobacillus plantarum*. J. Biol. Chem. 235, 810-818 (1960).

23. Duffus, J. H., Alcohol dehydrogenase in barley embryo. Phytochemistry 7, 1135-1137 (1968).

24. Duncan, R. J. and K. F. Tipton, The oxidation and reduction of glyoxylate by lactate dehydrogenase. Europ. J. Bioch. 11, 58-61 (1968).

25. Effer, W. R. and S. L. Ranson, Respiratory metabolism in buckwheat seedlings. Plant Physiol. 42, 1042-1052 (1967).

26. Federick, S. E., P. J. Gruber and N. E. Tolbert, Occurrence of glycolate dehydrogenase and glycolate oxidase in green plants. Plant Physiol. 52, 318-323 (1973).

27. Feieraband, J. and H. Beever, Development studies on microbodies in wheat leaves. I. Conditions influencing enzyme development. Plant Physiol. 49, 28-32 (1972).

28. Franke, W. and Schulz, Oxydations Fermente aus Schimmelpilzen und Hefen. I. Uber die Glykolico-dehydrase der Schimmilpilze. Hoppe-Seylers Z. Physiol. Chem. 303, 30-57 (1956).

29. Frigerio, N. A. and H. A. Harbury, Preparation and some properties of crystalline glycolic acid oxidase of spinach. J. Biolog. Chem. 231, 135-157 (1958).

30. Garland, R. C. and N. O. Kaplan, Salt induced alternation of D-lactate dehydrogenase from *Polysphondylium palledium*. Biochem. Biophys. Res. Comm. 26, 679-685 (1967).

31. Gleason, F. H., R. A. Nolan, A. C. Wilson and R. Emerson, D-lactate dehydrogenase in lower fungi. Science 152, 1272-1273 (1966).

32. Graf, G. E. and S. Aronoff, Carbon dioxide fixation by roots. Science 121, 211-212 (1955).

33. Graves, L., R. H. Trelease and W. M. Becker, Particulate nature of glycolate dehydrogenase in *Euglena:* possible localization in microbodies. Biochem. Biophys. Res. Comm. 44, 280-286 (1971).

34. Gregolin, C. and T. P. Singer, The D-lactate cytochrome reductase of yeast: its chemical nature, origin and relation to respiratory chain. Biochem. Biophys. Res. Comm. 4, 189-194 (1961).

35. Grodzinski, B. and B. Colman, Glycolic acid oxidase activity in cell free preparation of blue green algae. Plant Physiol. 45, 735-737 (1970).

36. Gruber, P. J., S. E. Frederick and N. E. Tolbert, Enzymes related to lactate metabolism in green algae and lower land plants. Plant Physiol. 53, 167-170 (1974).

37. Hadacova, V. and J. Svachulova, Isoenzymes of lactate dehydrogenase in root growth zones of *Vicia faba* L. Biologia Plant. 14, 170-172 (1972).

38. *Handbook of Chemistry and Physics* (42nd Ed.), The Chemical Rubber Publishing Co., Cleveland, Ohio (1960), p. 1707.

39. Hatch, M. D. and C. R. Slack, The C_4 dicarboxylic acid pathway of photosynthesis. In: L. Reinhold and Y. Liwschitz, eds., *Progress in Phytochemistry*, Vol. 2. Interscience, London (1970), pp. 35-106.

40. Hess, J. L. and N. E. Tolbert, Glycolate, glycine and glycerate formation during photosynthesis in tobacco leaves. J. Biolog. Chem. 241, 5705-5711 (1966).

41. Hoagland, D. R. and D. I. Arnon, The water culture method without soil. University of California Agricultural Experimental Station Circular 347 (1938).

42. Holzer, H. and A. Holldorf, Isolierung von D-Glycerate dehydrogenase, einige Eigenschaften des Enzymes und seine Verwendung zur enzymatisch-optischen Bestimmung von Hydroxypyruvat neben Pyruvat. Biochem. Z. 239, 292-312 (1957).

43. Huang, H. C. and H. Beevers, Microbody enzymes and carboxylases in sequential extracts from C_4 and C_3 leaves. Plant Physiol. 50, 242-248 (1972).

44. Huang, H. C. and H. Beevers, Isolation of microbodies from plant tissues. Plant Physiol. 48, 637-641 (1971).

45. Jimenez, E., R. G. Baldwin, N. E. Tolbert and W. A. Wood, Distribution of ^{14}C in sucrose from glycolate ^{14}C and serine-3-^{14}C in soybean and wheat. Arch. Biochem. Biophys. 98, 172-175 (1962).

46. Kaback, H. R., Transport across isolated bacteria cytoplasmic membranes. Biochim. Biophys. Acta 265, 367-416 (1972).

47. Kalonstian, H. D. and N. O. Kaplan, Lactate dehydrogenase of lobster (*Homarus americanis*) tail muscle. J. Biolog. Chem. 244, 2902 (1969).

48. Kemp, M. B., D- and L-lactate dehydrogenase of *Pseudomonas aeruginosa*. Biochem. J. 130, 307-309 (1972).

49. Kenten, R. H. and P. J. G. Mann, H_2O_2 formation in oxidation catalyzed by plant alpha-hydroxy oxidase. Biochem. J. 52, 130-134 (1952).

50. King, J., The isolation, properties and physiological role of lactic dehydrogenase from soybean cotyledon. Can. J. Bot. 48, 533-540 (1970).

51. Köhe, J. G., Zur Bestimmung von Enzymaktivitaten Pflanzlicher geweb. Flora A 160, 253-257 (1969).

52. Kolesmkov, P. A., Glycolic acid oxidase in green plants. Usp. Sovrem Biol. 38, 133-142 (1954). Cited from Chem. Abstr. 49, 5600d (1955).

53. Kohn, L. O. and W. B. Jakoby, Hydroxypyruvate reductase from *Pseudomonas*. In: S. P. Colowick and N. O. Kaplan, eds., *Methods in Enzymology*, Vol. 9, Academic Press, New York (1966), pp. 229-232.

54. Kohn, L. D. and W. A. Warren, The kinetic properties of spinach leaf glyoxylic acid reductase. J. Biolog. Chem. 245, 3831-3839 (1970).

55. Kohn, L. D., W. A. Warren and W. R. Carroll, Structure, properties of spinach leaf glyoxylic acid reductase. J. Biolog. Chem. 245, 3821-3830 (1970).

56. Kornberg, H. L. and A. M. Gotton, Biosynthesis of cell constituents from C_2 compounds. Nature 179, 1-93 (1959).

57. Kowallik, W. and G. H. Schmid, On the oxidation of glycolate by unicellular green algae. Planta 96, 224-237 (1971).

58. Krupka, R. M. and G. H. N. Towers, Studies of keto acids of wheat. II. Glyoxylic acid and its relation to allantoin. Can. J. Bot. 36, 179-185 (1958).

59. Kuczmak, N. and N. E. Tolbert, Glycolate oxidase formation in greening leaves. Plant Physiol. 37, 729-734 (1962).

60. Kun, E., I. M. Dechary and H. C. Pittot, The oxidation of glycolic acid by a liver enzyme. J. Biol. Chem. 210, 269-280 (1954).

61. Laudahn, G., Vergleichende untersuchungen zum Umsatz von Pyruvat, Hydroxypyruvat und Glyoxylat durch die NAD-Oxydoreduktasen Lactat-dehydrogenase, Glyoxylsäure-Reduktase und D-glycerat-dehydrogenase. Biochem. Z. 337, 449-461 (1963).

62. Lawrence, A. J. and J. R. Quayle, An alternative assimulation pathway in methane utilization bacteria. J. Gen. Micro. 63, 371-374 (1970).

63. LeJohn, H. B., D-lactate dehydrogenase in fungi. J. Biolog. Chem. 246, 2116-2126 (1971).

64. Leblova, S., I. Zimakova, D. Safrova, and I. Barthova, Occurrence of ethanol in pea plants in the course of growth under normal and anaerobic conditions. Biologia Plant. 11, 417-423 (1969).

65. Leblova, S., E. Sinecka and V. Vanickova, Pyruvate metabolism in germinating seeds during natural anaerobiosis. Biologia Plant. 16, 406-411 (1974).

66. Leblova, S., I. Zimakova, J. Barthova and D. Etheicohova, On alcohol dehydrogenase. Biologia Plant. 13, 33-42 (1971).

67. Long, G. L. and N. O. Kaplan, D-lactic acid specific pyridine nucleotide lactate dehydrogenase in animals. Science 162, 685-686 (1968).

68. Lord, J. M., Glycolate oxidoreductase in *E. coli*. Biochim. Biophys. Acta 267, 227-237 (1972).

69. Lord, J. M. and M. J. Merrett, Glycolate oxidase in *Chlorella pyrenoidosa*. Biochim. Biophys. Acta 159, 543-544 (1968).

70. Lowe, C. and W. O. James, Alcohol dehydrogenase in carrot tissues. New Phytologist 59, 288-291 (1960).

71. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall, Protein measurement with the Folin phenol reagent. J. Biolog. Chem. 193, 265-275 (1951).

72. Lück, H., Catalase. In: H. U. Bergmeyer, ed., *Method of Enzymatic Analysis*, Academic Press, New York (1965), pp. 885-894.

73. Massey, V., The microestimation of succinate and extinction coefficient of cytochrome c. Biochim. Biophys. Acta 34, 255-256 (1959).

74. Mcgroarty, E., B. Hsieh, D. M. Wied, R. Gee and N. E. Tolbert, Alpha hydroxy acid oxidation by peroxisomes. Arch. Biochem. Biophys. 161, 194-210 (1974).

75. Meister, A., Reduction of α-γ diketo and α-keto acids catalyzed by muscle preparations and by crystalline lactic dehydrogenase. J. Biolog. Chem. 161, 117-129 (1950).

76. Merrett, M. J. and J. M. Lord, Glycolate formation and metabolism by algae. New Phytologist 72, 751-767 (1973).

77. Mitsui, S., T. Horiguchi and K. Kumazawa, Comparison of glycolic acid oxidase activity in the roots of lowland (swamp) plant and upland (non-swamp) plants. Japanese J. of Soil and Fert. 32, 455-456 (1961).

78. Mothes, K. and L. Engelbreckt, Uber Allantoinsäure and Allantoin. I. Ihre Rolle als Wanderforms des Stickstoffs und ihre Beziehungen zum Eiweisstowechsel des Ahorns. Flora (Jena) 139, 586-616 (1952).

79. Mothes, K. and L. Engelbrecht, Uber Allantoinsäure und Allantoin. II. Ihre Verhalten in den Speigfherwurzein von Symphytum officine. Flora (Jena) 141, 356-378 (1954).

80. Mothes, K. and A. N. Wagner, The activity of glycolic acid dehydrogenase with a particular reference to its activity in roots. Biochimya 22, 171-177 (1957).

81. Nelson, E. B. and N. E. Tolbert, Glycolate dehydrogenase in green algae. Arch. Biochem. Biophys. 141, 102-110 (1970).

82. Noll, G. R. Jr. and R. H. Burris, Nature and distribution of glycolic acid oxidase in plants. Plant Physiol. 29, 261-265 (1954).

83. Pattee, H. E. and H. E. Swaisgood, Peanut alcohol dehydrogenase. I. Isolation and purification. J. Food Sci. 33, 250-253 (1968).

84. Paul, J. S. and B. B. Volcani, Photorespiration in diatom. I. Oxidation of glycolic acid in *Thallessioira pseudonana*. Arch. Microbiol. 101, 115-120 (1974).

85. Paul, J. S., C. W. Sullivan and B. E. Volcani, Photorespiration in diatoms: Mitochondrial glycolate dehydrogenase in *Cylindrotheca fusiformis* and *Nitzschia alba*. Archiv. Biochem. Biophys. 169, 152-159 (1975).

86. Pizer, L. I., Enzymology and regulation of serine biosynthesis in cultured human cells. J. Biolog. Chem. 239, 4219-4226 (1964).

87. Purohit, K. and G. Turian, D-lactate dehydrogenase from *Allomyces*. Partial purification and allosteric properties. Arch. Mikro. Biol. 84, 287-300 (1972).

88. Rabson, R., N. E. Tolbert and P. C. Kearny, Formation of serine and glycine by the glycolate pathway. Arch. Biochem. Biophys. 98, 154-163 (1962).

89. Roseblum, I. Y., D. H. Antkowick, H. J. Sallack, L. E. Flanders and L. A. Fahien, Purification and regulatory properties of beef liver D-glycerate dehydrogenase. Arch. Biochem. Biophys. 144, 375-383 (1971).

90. Sallach, J. H., D-glycerate dehydrogenase of liver and spinach. In: W. A. Wood, ed., *Methods in Enzymology*, Vol. 9, Academic Press, New York (1966), pp. 221-228.

91. Sallach, H. J. and R. D. Feld, Comparison of D-glycerate dehydrogenase from beef liver, beef spinal cord and hog spinal cord. Arch. Biochem. Biophys. 166, 417-425 (1975).

92. Scheid, M. J. and J. Awapara, Stereospecificity of some invertebrate lactate dehydrogenase. Comp. Biochem. Physiol. 43B, 619-626 (1972).

93. Sherwin, T. and E. W. Simon, The appearance of lactic acids in *Phaseolus* seeds germinating under wet conditions. J. Exp. Bot. 20, 776-785 (1969).

94. Stabenau, H., Localization of enzymes of glycolate metabolism in the algae *Chlorogonium elongatum*. Plant Physiol. 54, 921-924 (1974).

95. Stafford, H. A., A. Magaldi and B. Vennesland, The enzymatic reduction of hydroxypyruvate to D-glyceric acid in higher plants. J. Biolog. Chem. 207, 621-629 (1954).

96. Sugimto, E., Y. Kitagawa, K. Nakanishi and H. Chiba, Purification and properties of beef liver G-glycerate dehydrogenase. J. Biochem. 72, 1307-1315 (1972).

97. Szabo, A. S. and C. J. Avers, Some aspects of regulation of peroxisomes and mitochondria in yeast. Ann. N.Y. Acad. Sci. 168, 302-312 (1969).

98. Tanner, W. H. and H. Beevers, Glycolic acid oxidase in castor bean endosperm. Plant Physiol. 40, 971-976 (1965).

99. Tarmy, E. M. and N. O. Kaplan, Chemical characterization of D-lactate dehydrogenase from *E. coli*. J. Biolog. Chem. 243, 2579-2586 (1972).

100. Theimer, R. R. and H. Beevers, Uricase and allantoinase in glyoxisomes. Plant Physiol. 147, 246-251 (1971).

101. Tolbert, N. E., Glycolate pathway. Natl. Acad. Sciences - Natl. Res. Counc. Publ. No. 1145 (1963).

102. Tolbert, N. E., Isolation of leaf peroxisomes. Methods in Enzymology 13A, 665-682 (1971).

103. Tolbert, N. E., Microbodies - peroxisomes and glyoxisomes. Ann. Rev. of Plant Physiol. 220, 45-74 (1971).

104. Tolbert, N. E., Glycolate biosynthesis. Current Topics in Cellular Regulation 7, 21-46 (1973).

105. Tolbert, N. E., Photorespiration. In: W. D. P. Stewart, ed., *Algal Physiology and Biochemistry*, Blackwell Scientific Publications, Oxford (1973), pp. 474-504.

106. Tolbert, N. E. and R. H. Burris, Light activation of plant enzyme which oxidizes glycolic acid. J. Biolog. Chem. 186, 791-804 (1950).

107. Tolbert, N. E. and M. S. Cohan, Activation of glycolic acid oxidase in plants. J. Biolog. Chem. 204, 639-648 (1953).

108. Tolbert, N. E., M. P. Ko and B. Juliano, Alpha-hydroxyacid oxidase in rice plants (manuscript in preparation).

109. Tolbert, N. E., A. Oeser, R. H. Yamazaki, R. H. Hageman and T. Kisaki, A survey of plant for leaf peroxisomes. Plant Physiol. 44, 135-147 (1969).

110. Tolbert, N. E., A. Oeser, T. Kisaki, R. H. Hageman and R. K. Yamazaki, Peroxisomes from spinach leaves containing enzymes related to glycolate metabolism. J. Biolog. Chem. 243, 5179-5184 (1968).

111. Tolbert, N. E., R. K. Yamazaki and A. Oeser, Localization and properties of hydroxypyruvate and glyoxylate reductase in spinach leaf particles. J. Biolog. Chem. 245, 5120-5136 (1970).

112. Tubbs, P. K. and G. D. Greville, Dehydrogenation of D-lactate by a soluble enzyme from kidney mitochondria. Biochim. Biophys. Acta 34, 290-291 (1959).

113. Tubbs, P. K. and G. D. Greville, The oxidation of D-alpha-hydroxy acids in animal tissues. Biochem. J. 81, 104-114 (1961).

114. Ushijima, Y., Identity of L-alpha-hydroxy acid oxidase and glycolate oxidase from rat liver. Arch. Biochem. Biophys. 155, 361-367 (1973).

115. Walsh, D. A. and H. J. Sallach, Comparative studies on pathways for serine biosynthesis in animal tissues. J. Biolog. Chem. 241, 4068-4076 (1966).

116. Wang, D. and R. H. Burris, Carbon metabolism of ^{14}C-labelled amino acids in wheat leaves. II. Serine and its role in glycine metabolism. Plant Physiol. 38, 430-439 (1963).

117. Warburg, O., H. S. Gewitz and W. Volker, D-Milchsaure in *Chlorella*. Z. Naturforsch 12b, 722-726 (1957).

118. Wilkinson, J. H., *Isoenzymes*. E. and F. N. Spon Ltd., London (1965), pp. 43-79.

119. Williams, D. E. and R. A. Reisfeld, Disc electrophoresis in polyacrylamide gels: extension to new conditions of pH and buffer. Ann. N.Y. Acad. Sci. 121, 373-381 (1964).

120. Willis, J. E. and H. J. Sallach, Evidence for a mammalian D-glycerate dehydrogenase. J. Biolog. Chem. 237, 910-915 (1962).

121. Wittenberger, C. L., Kinetic studies on the inhibition of D-lactate dehydrogenase by adenosine triphosphate. J. Biolog. Chem. 243, 3067-3075 (1968).

122. Zelitch, I., Oxidation and reduction of glycolic and glyoxylic acid in plants. J. Biolog. Chem. 201, 719-726 (1953).

123. Zelitch, I., The isolation and action of crystalline glyoxylic acid reductase from tobacco leaves. J. Biolog. Chem. 216, 553-575 (1955).

124. Zelitch, I. and G. A. Barber, Oxidative phosphorylation and glycolate oxidation by particles from spinach leaves. Plant Physiol. 35, 205-209 (1960).

125. Zelitch, I. and P. R. Day, Glycolate oxidase activity in algae. Plant Physiol. 43, 289-291 (1968).

126. Zelitch, I. and S. Ochoa, Glycolic and glyoxylic acid in plants. I. Glycolic acid oxidase. J. Biolog. Chem. 201, 707-718 (1953).

127. Zschoche, W. C. and I. P. Ting, Malate dehydrogenase of *Pisum sativum:* tissue distribution and properties of the particulate forms. Plant Physiol. 51, 1076-1081 (1973).