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A STUDY OF THE GLYCOLYTIC ENZYMES IN

THE AQUATIC SNAIL PHYSA HALEI LEA

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CHAPTER I

INTRODUCTION

Recent studies of intermediary carbohydrate catabolism of invertebrates have pointed to a general concept of biochemical unity. At the same time these studies have shown that manifold deviations from classic metabolic processes do exist. Among invertebrates, carbohydrate catabolism has been most intensively studied in the parasitic protozoans, parasitic helminthes, and the insects.

Reviews of these studies (Hutner and Lwoff, 1955; von Brand, 1957; and Drummond and Black, 1960) have pointed out metabolic deviations in the excretion of volatile fatty acids instead of lactic acid, and, in the partial or complete absence of a Krebs tricarboxylic acid cycle in many parasitic forms. In insect muscle a-glycerophosphate, instead of lactic acid, is the excretory product of anaerobic catabolism,

and a-glycerophosphate dehydrogenase is thought to replace lactic dehydrogenase in reoxidizing reduced diphosphopyridine nucleotide (DPNH) (Drummond and Black, 1960). The heterogeneity of individual enzymes derived from different animal sources has recently been discussed by Kaplan, et al. (1960). They review the similarities and dissimilarities detected in enzymes by immunological and chromatographic techniques as well as by physical properties. Data are presented on comparative studies of the activity measurements of lactic dehydrogenase from a variety of taxonomic groups. These activity measurements show variation from group to group in the catalytic ability of this enzyme. Variations in the cofactor sensitivity of lactic dehydrogenase are also presented, and with the arthropods, for example, the greatest activity of the enzyme was obtained with 3-acetyl pyridine diphosphopyridine nucleotide, a synthetic analog of diphosphopyridine nucleotide. The variations in carbohydrate metabolism which are known to exist and the heterogeneity of enzymes both point to the need for detailed studies of the various taxonomic groups of invertebrates.

Studies have been conducted only very recently on isolated and partially purified enzyme systems of

invertebrates and most of these studies have been made on parasitic forms. As von Brand (1957) points out in a recent review, these studies are positive contributions to comparative biochemistry and provide a rational approach to parasite chemotherapy. It seems reasonable to assume that comparable contributions to theoretical and practical knowledge can also be obtained from studies with free-living invertebrate forms.

The phylum Mollusca is the second largest group of invertebrates in terms of numbers of described species. It is of economic importance in agriculture and commerce and, since it includes intermediate hosts for many parasitic organisms, is of public health significance in some regions. Nevertheless, our information on the intermediary metabolism of this group is quite limited. Studies which have been conducted on the intermediary carbohydrate metabolism of the Mollusca are reviewed in the following chapter. These studies include measurements which strongly support the existence of an aerobic cycle in molluscs that is similar to the Krebs cycle of vertebrate tissue and yeast. Overall glycolysis has been demonstrated in many molluscs but, with aquatic snails, observations such as propionic acid excretion under anaerobic conditions, and the failure of fluoride to

inhibit pyruvate formation indicate possible metabolic deviation from the glycolytic scheme as it is known for vertebrate tissue and yeast. Further, it must be pointed out that in none of the studies of glycolysis have the individual enzymes responsible for catalyzing the chemical reactions been characterized. The preceding paragraphs of this chapter have pointed out the need for information on the characteristics of the various enzymes and the value such information will contribute to our overall knowledge. This paper is a study which will extend our knowledge of the chemical sequence of glycolysis in one species of the Mollusca, the aquatic snail Physa halei Lea, and describe the characteristics of some of the enzymes catalyzing these reactions.

CHAPTER II

REVIEW OF THE LITERATURE

Aerobic Metabolism

Of the classes of Mollusca studied in connection with oxidative carbohydrate metabolism, the least investigated is the Cephalopoda. Slices of squid heart, Loligo pealii, have been shown to metabolize acetate and oxalacetate, suggesting a mechanism similar to the Krebs cycle (Barron, et al., 1953). In Barron's study citric acid was observed to markedly stimulate respiration. There have been some studies of the terminal pathways of respiration of cephalopods. In view of the intimate relationship which exists in vertebrate tissues between the cytochromes of terminal respiration and aerobic carbohydrate catabolism, evidence for the existence of cytochromes in the cephalopods, and in the other molluscs, is included in this review. Cytochromes a, b, and c, succinic dehydrogenase and cytochrome oxidase have been reported (Ball and Meyerhof, 1940) in tissue slices

(heart, head and neck muscle) of the squid. These pigments and enzymes have also been reported in the nervous tissue of L. pealii (Cooperstein and Lazarow, 1951; Nachmansohn, et al., 1942, 1943). The presence of succinic dehydrogenase, cytochrome oxidase, and cytochromes a, b, and c was strongly suggested by measurements made with extracts of Octopus vulgaris (Ghiretti-Magaldi, et al., 1957). In a subsequent and more detailed study with a highly purified extract, Ghiretti-Magaldi, et al. (1958) have obtained more complete evidence for the presence of succinic dehydrogenase and the cytochromes in O. vulgaris. There now seems little question that the terminal pathway of respiration in cephalopods functions in a manner quite similar to that of vertebrates. Although this similarity between the two groups does exist and the presence of the various cytochromes is indirect evidence for aerobic carbohydrate catabolism, it must be pointed out that direct evidence is available for only one of the several chemical reactions which comprise the Krebs cycle.

There is more information on the carbohydrate metabolism of pelecypods than of cephalopods. Measurements of cytochromes a, b, and c and succinic dehydrogenase have been reported (Ball and Meyerhof, 1940) in slices of the heart muscle of the clam, Venus mercenaria. Homogenates of muscle

tissue from the oyster, Saxostrea commercialis, contain a succinoxidase system (Humphrey, 1947). In the latter study succinate definitely stimulated oxygen consumption. However, excess malonate was observed to inhibit this increase by only 40 per cent. Cyanide and azide both inhibited oxygen consumption and this inhibition was reversed upon addition of methylene blue to the mixture. Jodrey and Wilber (1955), using homogenates and acetone precipitates of mantle tissue of the oyster, Crassostrea virginica, demonstrated the presence of succinic dehydrogenase, iso-citric dehydrogenase, malic dehydrogenase, oxalacetic decarboxylase, and cytochrome oxidase. It is interesting to note that they were not able to demonstrate the presence of aconitase. Cyanide was found to markedly inhibit oxygen consumption, but methylene blue (1×10^{-5} M.) did not have an appreciable effect in averting or suppressing the inhibition. They suggest that the cytochrome system perhaps plays only a minor role in the respiration of oyster tissue. Kawai (1958) has recently reported measurements on the cytochromes and cytochrome oxidase in the oyster, Crassostrea gigas. His measurements confirm those of Jodrey and Wilber (1955) although he found that methylene blue in concentrations of 6×10^{-5} M. did reverse cyanide inhibition by approximately 40 per cent. In a

subsequent study on the significance of the cytochrome system in respiration, Kawai (1959) obtained measurements on the oyster, Crassostrea gigas, the pearl oyster, Pinctada martensii, and the mussel, Mytilus crassitesta, which indicated that active cytochrome oxidase and cytochromes a, b, and c were present in each species. By studying the effect of carbon monoxide upon the cytochrome oxidase and upon the total respiration, he calculated that the cytochrome system accounts for approximately 80 per cent of the total respiration.

Hammen and Wilber (1959) used radioactive sodium bicarbonate and chromatographic techniques to follow the incorporation of carbon dioxide into organic acids of isolated strips of mantle tissue from the oyster, Crassostrea virginica. They found that radioactive carbon appeared first in succinic acid, then in fumaric acid, and last in malic acid. This suggests the presence of succinic dehydrogenase, malic dehydrogenase, and fumarase.

In the Gastropoda the aerobic phase of catabolism has been investigated by Baldwin (1938) using slices of the hepatopancreas from Helix pomatia. Oxygen consumption was stimulated by succinate and inhibited by malonate. Rees (1953) confirmed this observation and obtained additional

evidence for a functional Krebs cycle. He used a suspension of the hepatopancreas of H. pomatia and observed increased oxygen consumption upon addition of each intermediate of the Krebs cycle. Eckstein and Abraham (1959) measured succinic dehydrogenase in homogenates of the hepatopancreas of the snail, (Helix) Levantina hierosolyma, and observed increases in the activity of the enzyme as the animal came out of estivation. Minced tissue of the aquatic snail, Australorbis glabratus, was used by Weinbach (1953) in studies of the Krebs cycle. Oxygen uptake was increased upon addition of citrate, fumarate, succinate, malate, and a-ketogluterate. Weinbach was not able to show any marked inhibition of oxygen consumption upon addition of fluoroacetate, trans-aconitate, or malonate. The phosphorylation which accompanies oxidative catabolism has been demonstrated in the albumen gland of the aquatic snail, Lymnaea stagnalis (Weinbach, 1956). In investigations using tissue fluids of the marine snail, Busycon canaliculatum, Ball and Meyerhof (1940) demonstrated cytochromes a, b, and c spectrophotometrically. They also demonstrated succinic dehydrogenase by following the anaerobic reduction of methylene blue in the presence of added succinate. Person, et al. (1959) reported the presence of cytochrome oxidase in the odontophore of the snail, B. canaliculatum.

The cytochrome system of two species of sea hares, Aplysia depilaris and A. limacina, has been investigated by Ghiretti, et al. (1959). They reported the presence of cytochromes a, b, c, c_1, and a_3 with cytochrome a_3 functioning as the terminal oxidase. They also offered evidence for a Krebs cycle by showing increased respiration upon addition of succinate, malate, or citrate to slices of gizzard muscle and inhibition of succinate-stimulated respiration upon addition of malonate.

These studies with terminal respiratory pigments in mollusca point to a system very similar to that described for vertebrate tissue. However, the data on the individual steps of the Krebs cycle suggest that the system in mollusks lacks at least one of the enzymes of the vertebrate cycle. It seems quite possible that the aconitase step is missing. The absence of this enzyme has been suggested previously by Jodrey and Wilber (1955), and they proposed that the first compound formed may be iso-citrate rather than citrate.

Anaerobic Metabolism

Von Brand (1946) has reviewed the literature on anaerobic metabolism in mollusks up to early 1945. Most of the early studies involved subjecting various species to fatigue or anaerobic conditions and measuring carbon dioxide

and acid production. While the production of acid suggests a glycolytic pathway similar to the one in vertebrates, no measurements were made that would demonstrate individual steps of the scheme. In fact, in most instances the acid produced was not identified but was assumed to be lactic acid. Mono-bromoacetic acid had been observed to inhibit acid production by oyster tissue, and iodoacetic acid had been employed to produce similar effects on slices of mussel tissue. These compounds are classic inhibitors of glycolysis in vertebrate tissue, inhibiting the oxidation of glyceraldehyde-3-phosphate. Thus, inhibition of acid production by these agents is contributory evidence for the presence of the enzyme.

Humphrey (1944) reported an increased lactic and pyruvic acid formation in extracts of oyster muscle, Saxostrea commercialis, subjected to anaerobic conditions. In these investigations he observed inhibition of acid production upon addition of fluoride or iodoacetic acid. The gill epithelium of the fresh water mussel was used by Wernstedt (1944) in a study of carbohydrate metabolism of the animal. Volatile fatty acids were detected as products of anaerobic metabolism. Wernstedt reported that ciliary movement was inhibited by addition of fluoride to the reaction mixture. Rees (1953)

conducted investigations on the glycolytic pathway of the snail, Helix pomatia. He reported that the addition of galactose, fructose-6-phosphate, fructose diphosphate, phosphoglyceric acid or glucose increased the oxygen consumption of a suspension of H. pomatia hepatopancreas. He was also able to demonstrate glycolytic phosphorylation in homogenates of the hepatopancreas.

Overall catabolism of carbohydrates has been demonstrated in intact aquatic snails by von Brand, et al. (1950). In this study, which included eighteen species, only two species were found to produce lactic acid as the quantitative end product of anaerobic metabolism. In further investigation of these observations, Mehlman and von Brand (1951) found volatile fatty acids, identified as acetic and propionic, to be among the end products of anaerobic metabolism. Weinbach (1953) employed minced tissue and homogenates in a study on carbohydrate metabolism of the snail, Australorbis glabratus. The minced tissue accumulated lactic and volatile acids under anaerobic conditions, as did intact snails. When the effect of added glucose, mannose, galactose, glucose-1-phosphate, and fructose diphosphate on endogenous oxygen consumption and pyruvate production was studied with minced tissue, only fructose diphosphate produced a measurable

increase in activity. Pyruvate production increased 50 per cent with the addition of fructose diphosphate. Why fructose diphosphate alone of the several carbohydrates employed stimulated pyruvate production is not immediately apparent. It does not seem probable that fructose diphosphate is the only compound of the group which can penetrate intact cells of this species. Iodoacetamide inhibited both oxygen consumption and pyruvate production although fluoride in 0.01 M concentrations had no inhibitory effect. In vertebrate tissue and yeast, fluoride inhibits glycolysis, and hence pyruvate formation, via its action on enolase. The lack of sensitivity of snail mince to fluoride suggests either an enolase quite different from the one in mammalian tissue or a pathway for the formation of pyruvate which does not involve enolase. The inhibitory action of iodoacetamide suggests a glycolytic enzyme in snail tissue comparable to glyceraldehyde-3-phosphate dehydrogenase of muscle and yeast. The presence of such an enzyme in snails is further suggested by the occurrence of glycolytic phosphorylation in the albumen gland of Lymnaea stagnalis (Weinbach, 1956). In none of the studies with aquatic snails (or any mollusks), however, have the individual steps of glycolysis been characterized. A detailed study of glycolysis in these organisms would

provide better understanding of the apparent metabolic deviations described above.

CHAPTER III

MATERIALS AND METHODS

Snails

Snails used in this study were identified by Branley A. Branson of Oklahoma State University as *Physa halei* Lea. Both laboratory-reared snails and snails collected from the University of Oklahoma golf course pond were used in this study. The laboratory-reared colony was started from eggs laid by snails that had been collected from the golf course pond.

The snails were maintained at 20° C. in all-glass aquaria containing tap water to which small amounts of $CaCO_3$ had been added. The snails were fed *ad libitum* with lettuce leaves and fish food. Contents of the individual aquaria were changed twice each week. Only snails weighing more than 50 mg. were used in the experiments.

Enzyme Preparation

The snails were placed in a Petri dish with a small volume of water and chilled by placing the dish on ice cubes.

After five minutes the snails were removed individually from the dish, their shells cracked open, and the soft parts removed and placed in a small volume of chilled homogenizing solution (0.154 M KCl made alkaline with 8 ml./ liter of 0.02 M $KHCO_3$).

The snails which had been collected from the pond were inspected at this point for trematode infection. Snails found with cercariae, rediae, or sporocysts were discarded.

When a sufficient quantity of tissue had been collected (1-2 gms.), it was blotted, weighed on a torsion balance, and placed in a chilled Tenbroeck tissue grinder for preparation of homogenates. Tissue to be minced was placed on a chilled glass plate and minced free hand with two scalpels, one held in each hand. The tissue to be homogenized was ground with sufficient homogenizing solution to make a 25 per cent (w/v) homogenate.

For some assays this homogenate was used as the source of enzyme. For other assays the homogenate was centrifuged at 487 x g for 20 minutes at 5° C. and the supernatant fluid ("tissue extract") was used. In some cases the tissue extract was dialyzed for five hours against 500 ml. of cold 0.154 M KCl. The KCl solution was changed once at the end of two hours of dialysis. After dialysis the extract was transferred

to a centrifuge tube, diluted with an equal volume of homogenizing solution, and centrifuged at 1,658 x g for 10 minutes at 5° C. The supernatant fluid was decanted and saved for use in the assay. Caution was taken to keep the extract chilled at all times.

Analytical Procedures

The following procedures are general outlines of the method employed. Details of the individual assay conditions are given in context.

Oxygen consumption was measured in the Warburg apparatus by a standard method (Umbreit, et al., 1959). In all instances where anaerobic measurements were conducted, the reaction was carried out in the Warburg apparatus and the following procedure was employed. The manometers and flasks were placed in the waterbath and gassed for 7 minutes. Each sidearm vent and manometer stopcock was then closed and the contents of the sidearm tipped into the reaction chamber. An equilibration period of 3 minutes was allowed after tipping the contents of the sidearm. The initial manometer reading was recorded at the end of the equilibration period.

Anaerobic glycolysis was measured in an atmosphere of 95 per cent nitrogen and 5 per cent carbon dioxide. A

glycolytic reaction mixture patterned after that of LePage (1948), but omitting hexokinase and glucose, was used, and the enzyme preparation was placed in the sidearm of the reaction flask.

The manometric determinations of glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase employing cyanide followed the method of Cohen and Scott (1950). The method described by Kornberg (1950) for glucose-6-phosphate and 6-phosphogluconate dehydrogenase, which measures the rate of TPN reduction at 340 mμ, was also used.

Phosphoglucomutase activity was determined using glucose-1-phosphate as substrate by coupling the reaction to glucose-6-phosphate dehydrogenase and following the rate of TPN reduction at 340 mμ. The analysis is quite similar to the one for measuring phosphoglucoisomerase activity.

Phosphoglucoisomerase activity was determined by the method of Slein (1950). The conversion of fructose-6-phosphate to glucose-6-phosphate is coupled to glucose-6-phosphate dehydrogenase and TPN reduction is measured at 340 mμ.

Fructoaldolase activity was studied by chemical and spectrophotometric methods. In the chemical method a reaction mixture patterned after that of Taylor (1955) was employed. Cyanide was used to bind triose phosphate, and

inorganic phosphate was determined in aliquots of the reaction mixture before and after alkali hydrolysis. The spectrophotometric method of Warburg and Christian (1943) was also used to detect this enzyme.

Glyceraldehyde-3-phosphate dehydrogenase activity was estimated by following the rate of reduction of DPN at 340 mμ (Warburg and Christian, 1943). The reaction was initiated by the addition of excess glyceraldehyde-3-phosphate.

Glycerophosphate dehydrogenase was determined using a method suggested by Baranowski (1949). Activity was measured by following the oxidation of DPNH at 340 mμ in the presence of excess glyceraldehyde-3-phosphate.

3-Phosphoglyceric kinase activity was measured by the method of Axelrod and Baranowski (1953) in which hydroxylamine is used to bind acyl phosphate. The determination of acyl phosphate with hydroxylamine is based on studies reported by Lipmann and Tuttle (1945).

Lactic dehydrogenase activity was determined by two different methods. The first method is essentially that outlined by Kornberg (1955) which measures the oxidation of DPNH at 340 mμ in the presence of excess pyruvate. The second method was a colorimetric determination patterned

after the method of Ells (1959). This assay utilizes lactic acid as substrate and in this respect is similar to the assay procedure outlined by Neilands (1955).

The activity of pyruvic kinase was shown by a method patterned after that described by Bücher and Pfleiderer (1955). The pyruvate formed from phospho-enol-pyruvic acid and ADP is reduced by purified rabbit muscle lactic dehydrogenase* in the reaction mixture and the resulting oxidation of DPNH is followed at 340 mμ.

3-Phosphoglyceric acid mutase activity was demonstrated by the method of Sutherland, et al. (1949), which couples this enzyme to enolase, pyruvic kinase and lactic dehydrogenase. The activity was measured by following the oxidation of DPNH at 340 mμ. Purified rabbit muscle lactic dehydrogenase was added to the reaction mixture. The endogenous enolase and pyruvic kinase activities of the homogenate served to convert 2-phosphoglyceric acid to pyruvic acid in this system.

Enolase activity was determined by following the increase in absorption at 240 mμ which accompanies the conversion of 2-phosphoglyceric acid to phospho-enol-pyruvic acid (Warburg and Christian, 1941). The reaction was initiated

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*Sigma Chemical Co., Type II.

by the addition of excess 2-phosphoglyceric acid.

Inorganic phosphate was determined colorimetrically by the method of Fiske and Subbarow (1925). Pyruvic acid was determined by the method of Koepsell and Sharpe (1952).

At the end of every assay a suitable aliquot of the reaction mixture was treated with 10 per cent trichloroacetic acid and total protein in the resulting precipitate was determined by the method of Lowry, et al. (1951). This value for total protein was subsequently employed to calculate enzyme activity which is reported as a function of protein concentration and time.

All spectrophotometric measurements were made in a Beckman Model DU spectrophotometer, except the pyruvate kinase determination which was measured with a MacAlaster Bicknell Coenzometer.

Reagents

Glucose-6-phosphate, fructose-6-phosphate, fructose-1,6-diphosphate (barium salts), glucose-1-phosphate (dipotassium salt), glyceraldehyde-3-phosphate diethylacetal (monobarium salt), adenosine-5'-diphosphate (disodium salt) and 2,3-diphosphoglyceric acid (barium salt) were obtained from

Schwarz Laboratories.[1] 6-Phosphogluconate, 3-phosphoglycerate, 2-phosphoglycerate (barium salts), pyruvate (sodium salt), adenosine-5'triphosphate (disodium salt), and lactic dehydrogenase (type II) were obtained from Sigma Chemical Company.[2] Triphosphopyridine nucleotide and diphosphopyridine nucleotide (sodium salts) were obtained from Pabst Laboratories.[3] Lactic acid (lithium salt) was from Hartmann-Leddon Company.[4] 2,6-Dichlorophenolindophenol was from Eastman Organic Chemical Company.[5] Phenazine methosulfate was a gift from Dr. H. Alan Ells. Phosphopyruvic acid (Tricyclohexylamine salt) was obtained from California Corporation for Biochemical Research.[6]

Diphosphopyridine nucleotide was reduced following the method outlined by Beisenherz, et al. (1955). All barium salts, except glyceraldehyde-3-phosphate diethylacetal

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[1]Schwarz Laboratories, Inc., Mount Vernon, New York, USA.

[2]Sigma Chemical Company, St. Louis, Missouri, USA.

[3]Pabst Laboratories, Milwaukee, Wisconsin, USA.

[4]Hartmann-Leddon Company, Philadelphia, Pennsylvania, USA.

[5]Eastman Organic Chemical Company, Newark, New Jersey, USA.

[6]California Foundation for Biochemical Research, Los Angeles, California, USA.

were converted to the sodium salt before use with an equivalent amount of Na_2SO_4. Glyceraldehyde-3-phosphate diethylacetal barium salt was converted to glyceraldehyde-3-phosphate following a procedure suggested by the supplier. All substrate solutions were adjusted to pH 7.0 before they were introduced in the assay mixture. All other chemicals used in the study were commercial products of high purity.

Abbreviations

The following abbreviations are used, where applicable, throughout the paper: glucose-6-phosphate (G-6-P); glucose-1-phosphate (G-1-P); 6-phosphogluconic acid (6-PG); fructose-6-phosphate (F-6-P); fructose-1,6-diphosphate (FDP); glyceraldehyde-3-phosphate (G-3-P); 3-phosphoglyceric acid (3-PGA); 2,3-phosphoglyceric acid (2,3-PGA); 2-phosphoglyceric acid (2-PGA); phospho-enol-pyruvic acid (PE); adenosine-5'-triphosphate (ATP); adenosine-5'-diphosphate (ADP); triphosphopyridine nucleotide (TPN); triphosphopyridine nucleotide reduced (DPNH); iodoacetic acid (IAA); trichloroacetic acid (TCA); and inorganic orthophosphate (PI).

CHAPTER IV

RESULTS

Anaerobic Glycolysis

Measurements of the glycolysis of tissue extracts of P. halei and the effect of iodoacetic acid are presented in Figure 1. Since aquatic snails have been reported to produce volatile fatty acids as well as lactic acid (Mehlman and von Brand, 1951), total acid rather than lactic acid was measured. Fructose diphosphate stimulated and iodoacetic acid inhibited the production of acid. The observed gas uptake, in the absence of added substrate and in the presence of iodoacetic acid was unexpected. Gas uptake decreased rapidly after the first thirty minutes of measurements.

Control measurements using rat muscle extract did not show the peculiar gas uptake of the snail extract. The measurement with rat muscle agrees very well with results reported by LePage and Schneider (1948).

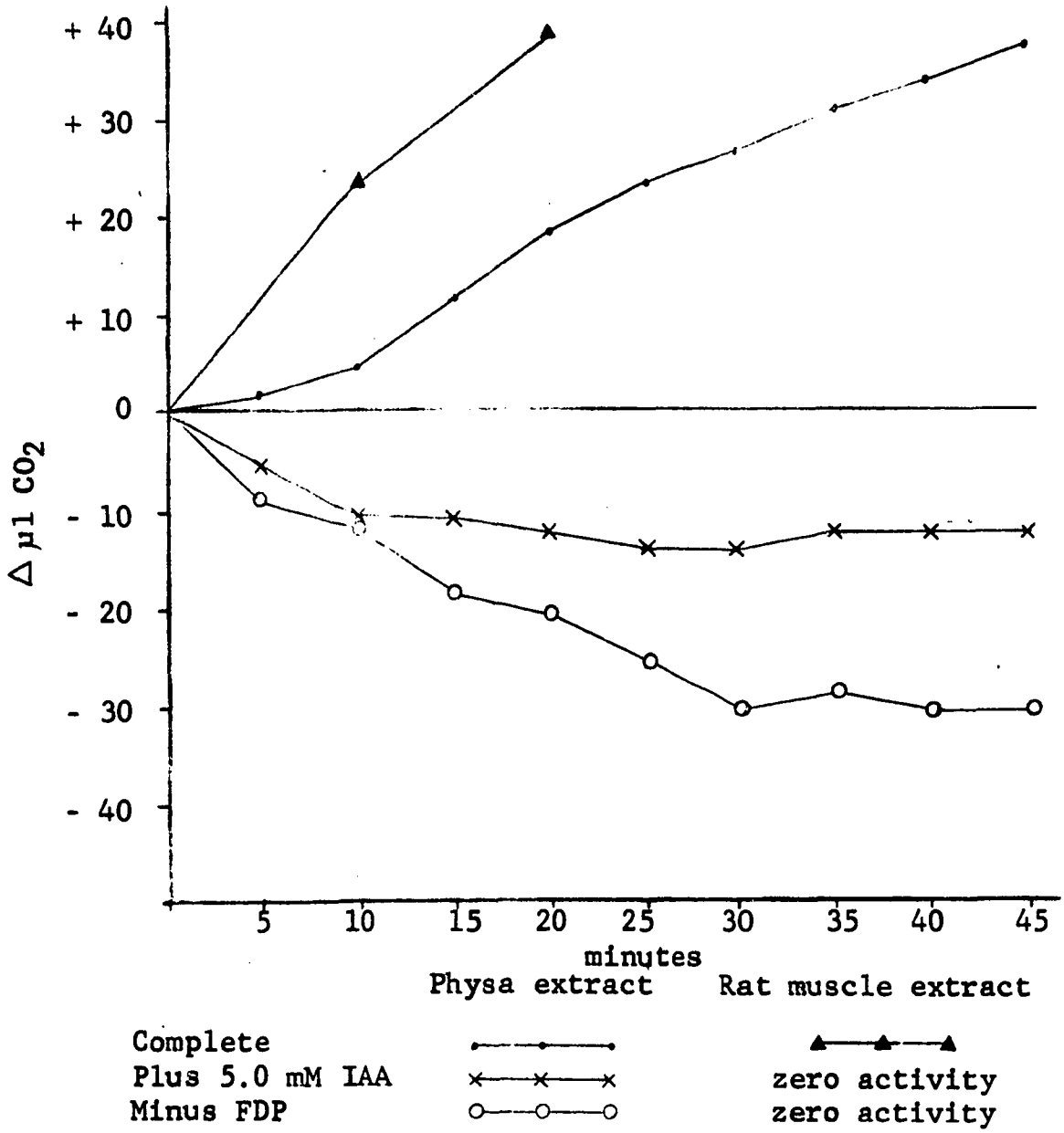


Fig. 1 Anaerobic Glycolysis

The complete system for P. halei and rat muscle contained: 10 µM glycylglycine, pH 7.4; 31 µM $KHCO_3$; 2 µM $MgCl_2$; 2 µM NaF; 2 µM Na pyruvate, pH 7.0; 8 µM nicotinamide; 0.6 µM DPN; 0.012 µM ADP; 20 µM FDP; 0.5 ml. tissue extract of 25% homogenate added from sidearm of flask after equilibration. Final volume, 2.0 ml. Gas phase, 95% N_2-5% CO_2. Temperature, 37°C. Reaction time, 30 minutes. Additions or omissions as indicated.

Glucose-6-Phosphate and 6-Phosphogluconic Dehydrogenase

Although these two enzymes are initial reactions of the hexose monophosphate shunt rather than steps of the glycolytic pathway, their association with one of the central glycolytic intermediates, G-6-P, makes measurements of them appropriate. The manometric measurements (Tables 1 and 2) were obtained by a procedure which ignored the cyanide-absorbing properties of potassium hydroxide (present in the center well of the reaction flasks). Control vessels were employed, however, and all readings were corrected to them. The data suggest the presence of both these steps in the tissue extract of P. halei. In both systems activity is stimulated by the addition of substrate. Phenazine methosulfate, a dye which will oxidize reduced pyridine nucleotides, and which is in turn oxidized by molecular oxygen (Dickens and McIlwain, 1938), also stimulates this activity. Further, the system with glucose-6-phosphate added has approximately twice the oxygen uptake of the system with 6-phosphogluconate. In a system where both dehydrogenase enzymes are present such a ratio of oxygen uptake would be expected.

Spectrophotometric evidence for the presence of these two enzymes is presented in Table 3. In both instances

activity was detected only after addition of the substrate. TPN served as the co-factor and DPN could not be substituted. The rate of activity of both enzymes was linear (Fig. 2) for the 6 minute period of measurement. This activity, based on the measurements with G-6-P as substrate, is in the same range as the activity reported for yeast autolyzate (Kornberg, 1950).

Measurements obtained with dialyzed tissue extract and 6-PG were comparable to those reported in Table 3. The addition of Mg^{++} was not necessary for activity and no increase in activity was observed when it was added to the system.

Phosphoglucomutase

This enzyme catalyzes the conversion of G-1-P to G-6-P. In view of the high G-6-P dehydrogenase activity in P. halei, evidence for the mutase step was sought by measuring TPN reduction in a mixture containing excess G-1-P. Table 4 gives the results of these measurements. Both Mg^{++} and cysteine were necessary for demonstration of activity. Neither of the co-factors alone nor both together in the system could stimulate activity in the absence of G-1-P. The rate of formation of reduced TPN is slower with G-1-P

TABLE 1

6-PHOSPHOGLUCONIC DEHYDROGENASE

System	µl O_2/mg. protein/hr.	
	Exp. 1	Exp. 2
Complete	77.2	47.3
Minus phenazine methosulfate	63.9	43.0
Minus 6-phosphogluconate	49.1	3.2

The complete system contained: 133 µM Na phosphate, pH 7.0; 1 µM $MgCl_2$; 8 µM nicotinamide; 2 µM KCN; 0.30 µM TPN; 10 µM IAA, pH 7.0; 0.4 mg phenazine methosulfate; 4 µM 6-PG, added from sidearm of flask after equilibration; 0.5 ml. tissue extract (762 µg. protein). Final volume, 2.0 ml. Gas phase, air. Temperature, 37°C. Reaction time, 30 minutes. Omissions as indicated.

TABLE 2

GLUCOSE-6-PHOSPHATE DEHYDROGENASE

System	µl O_2/mg. protein/hr.	
	Exp. 1	Exp. 2
Complete	135.6	120.8
Minus phenazine methosulfate	34.2	47.9
Minus glucose-6-phosphate	28.4	3.2

The complete system contained: 13.6µM Na phosphate, pH 7.0; 1 µM $MgCl_2$; 8 µM nicotinamide; 2 µM KCN; 0.30 µM TPN; 10 µM IAA, pH 7.0; 0.4 mg phenazine methosulfate; 4 µM G-6-P, added from sidearm of flask after equilibration; 0.5 ml. tissue extract (738 µg. protein). Final volume 2.0 ml. Gas phase, air. Temperature, 37°C. Reaction time, 30 minutes. Omissions as indicated.

TABLE 3

GLUCOSE-6-PHOSPHATE AND 6-PHOSPHOGLUCONIC DEHYDROGENASE

System	Change in $\frac{O.D.}{min.}$ (340 mμ)	Rate of formation of reduced TPN*
Complete		
with G-6-P substrate	0.033	9.13
with 6-PG substrate	0.010	2.76
Minus substrate	0.000	0.00
Either substrate minus TPN	0.000	0.00
Either substrate with DPN replacing TPN	0.000	0.00

*μM/min/mg. protein x 10^2

The complete system contained: 300 μM Na phosphate, pH 7.0; 4 μM substrate, added immediately before taking the zero reading; 0.30 μM TPN; 10 μM IAA, pH 7.0; 0.05 ml. tissue extract (61 μg. protein). Final volume 2.5 ml. Temperature, 25°C. Reaction time, 6 minutes. Omissions as indicated.

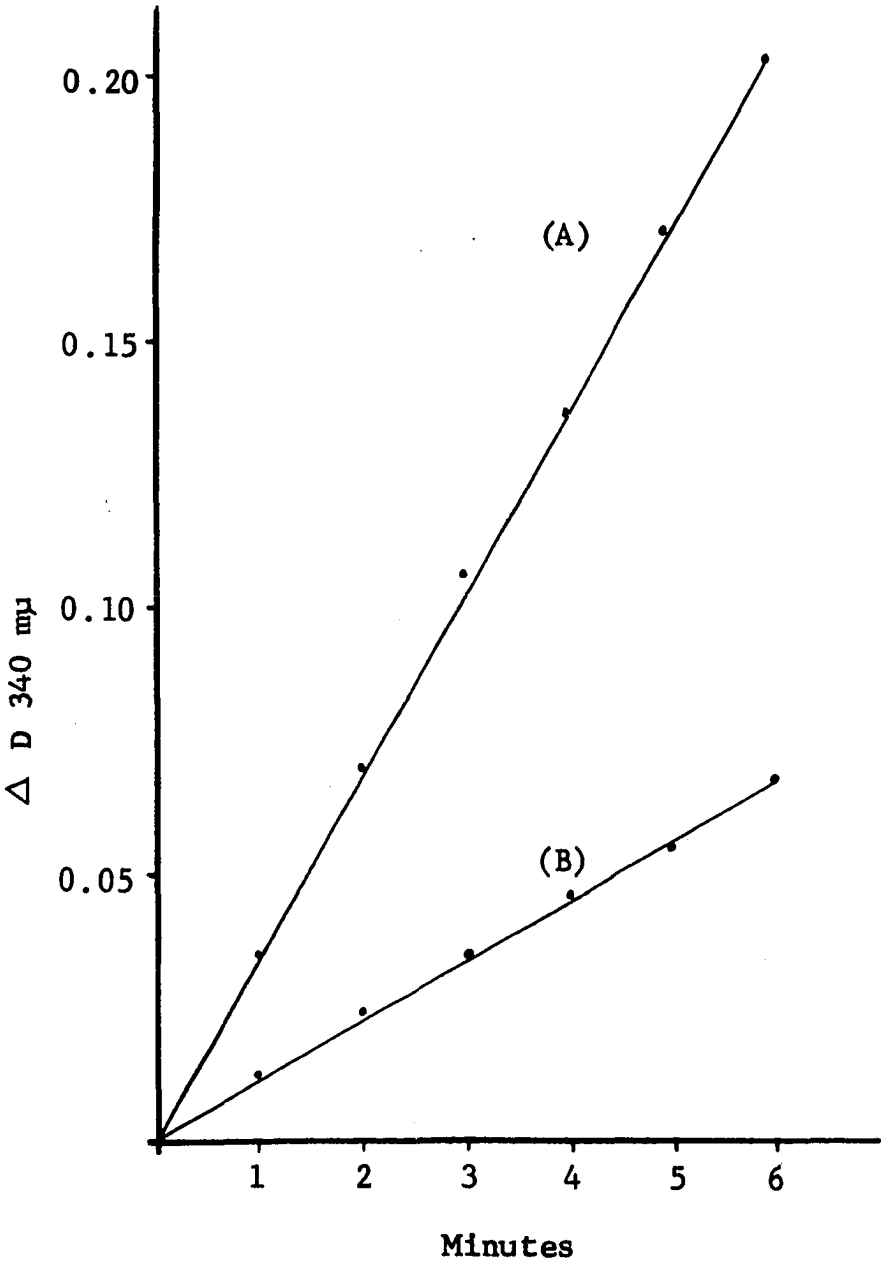


Fig. 2 Glucose-6-phosphate and 6-Phosphogluconate Dehydrogenase

Conditions given in Table 3.
Curve (A) is with G-6-P as substrate. Curve (B) is with 6-PG as substrate.

than with G-6-P. However this rate remains linear throughout the 7 minute period of measurement (Fig. 3).

Phosphoglucoisomerase

This enzyme mediates the inter-conversion of G-6-P and F-6-P. The ability of tissue extract of P. halei to isomerize F-6-P to G-6-P was demonstrated by coupling the step with G-6-P dehydrogenase and following the reduction of TPN. In this way, activity (Fig. 4) was determined in the presence and in the absence of F-6-P. With this mixture no added co-factor or chelating agent was necessary for activity and the reaction rate was linear for the 6 minute period of measurement. This rate is relatively high and approaches the rate observed for G-6-P dehydrogenase.

Aldolase

This enzyme catalyzes the splitting of fructose diphosphate into glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. Evidence for its presence in P. halei was sought by chemical and spectrophotometric methods. In studying the enzyme, tissue homogenates, tissue extracts, and dialyzed tissue extracts were used as the source of enzyme. The chemical method of assay was based on the measurement of the alkali-labile phosphate of the reaction

products (Meyerhof and Lohmann, 1943). The reaction was carried out in the presence of cyanide which binds the triose phosphates and, in so doing, prevents them from entering into the reverse reaction (Herbert, et al., 1940) or into additional degradation steps (Meyerhof, 1951; Taylor, 1955). The results of measurements carried out on tissue homogenates (Table 5) are quite similar to results obtained with crude and with purified preparations of muscle aldolase. Alkali-labile phosphate is present in the system with cyanide and absent in the system free of cyanide.

When the assay was repeated with tissue extract, the results did not agree with those obtained with the whole tissue homogenate. Much more alkali-labile phosphate formed with the tissue extract than with the homogenate and the amount formed without cyanide was approximately the same as it was with cyanide (Table 5). Although the equilibrium in muscle aldolase favors the formation of hexose diphosphate rather than triose phosphate (Herbert, et al., 1940) the possibility was considered of an aldolase in snail tissue which would favor formation of triose phosphates. The fact that with tissue homogenate there was no alkali-labile phosphate formation in the absence of cyanide suggested that the triose phosphate was being removed as rapidly as it was

TABLE 4

PHOSPHOGLUCOMUTASE

System	Change in $\frac{O.D.}{min.}$ (340 mμ)	Rate of formation of reduced TPN*
Complete	0.009	0.85
Minus Mg^{++}	0.000	0.00
Minus G-1-P	0.000	0.00
Minus cysteine	0.000	0.00

*μM/min/mg. protein x 10^2

The complete system contained: 2 μM Tris (hydroxymethyl) aminomethane, pH 7.4; 2 μM $MgCl_2$; 0.3 μM TPN; 20 μM G-1-P, added immediately before taking the zero reading; 20 μM cysteine, pH 7.4; 10 μM IAA, pH 7.0; 0.1 ml. tissue extract (181 μg protein). Final volume, 3.0 ml. Temperature, 25°C. Reaction time, 7 minutes. Omissions as indicated.

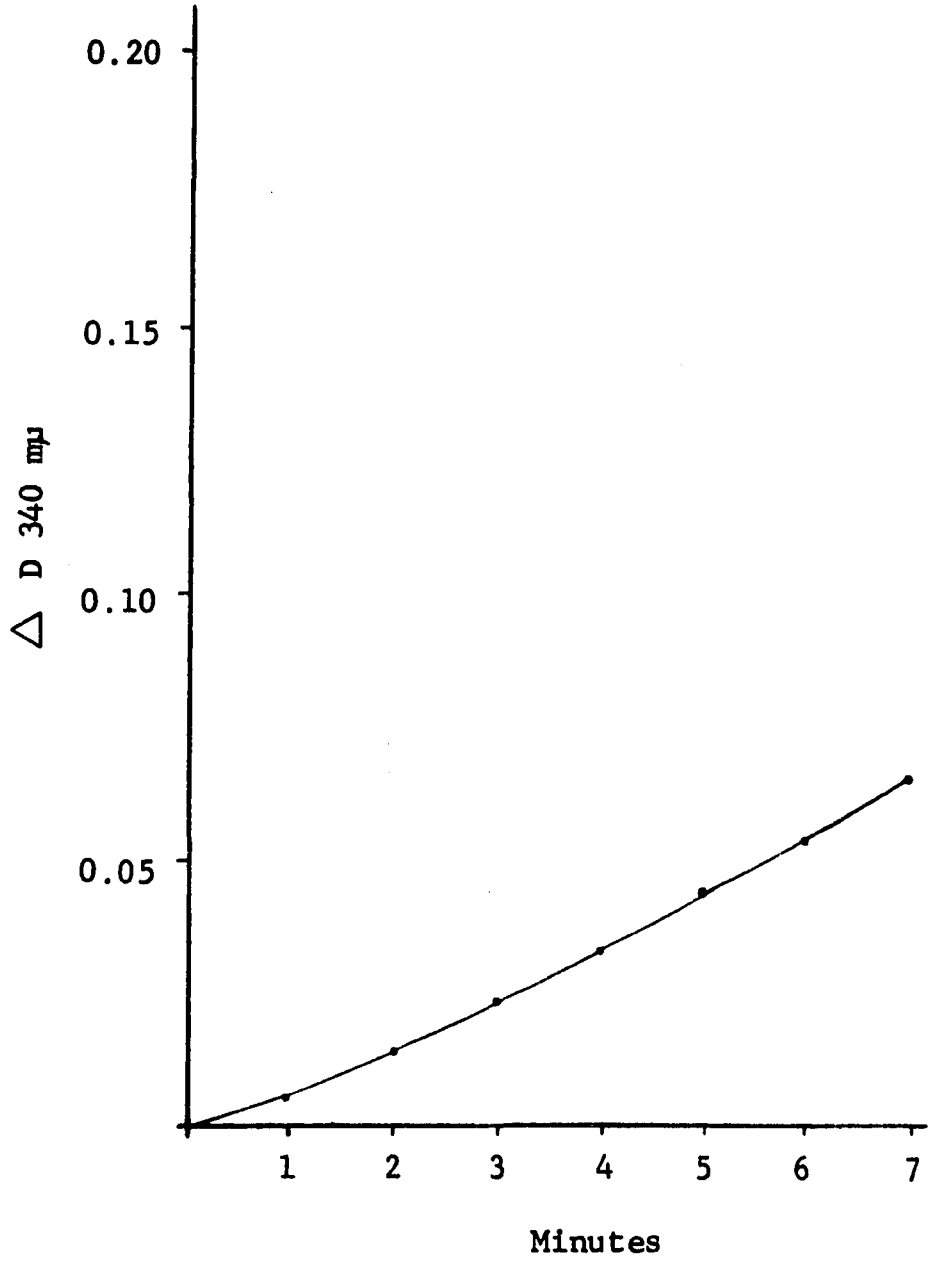


Fig. 3 Phosphoglucomutase

Conditions given in Table 4.

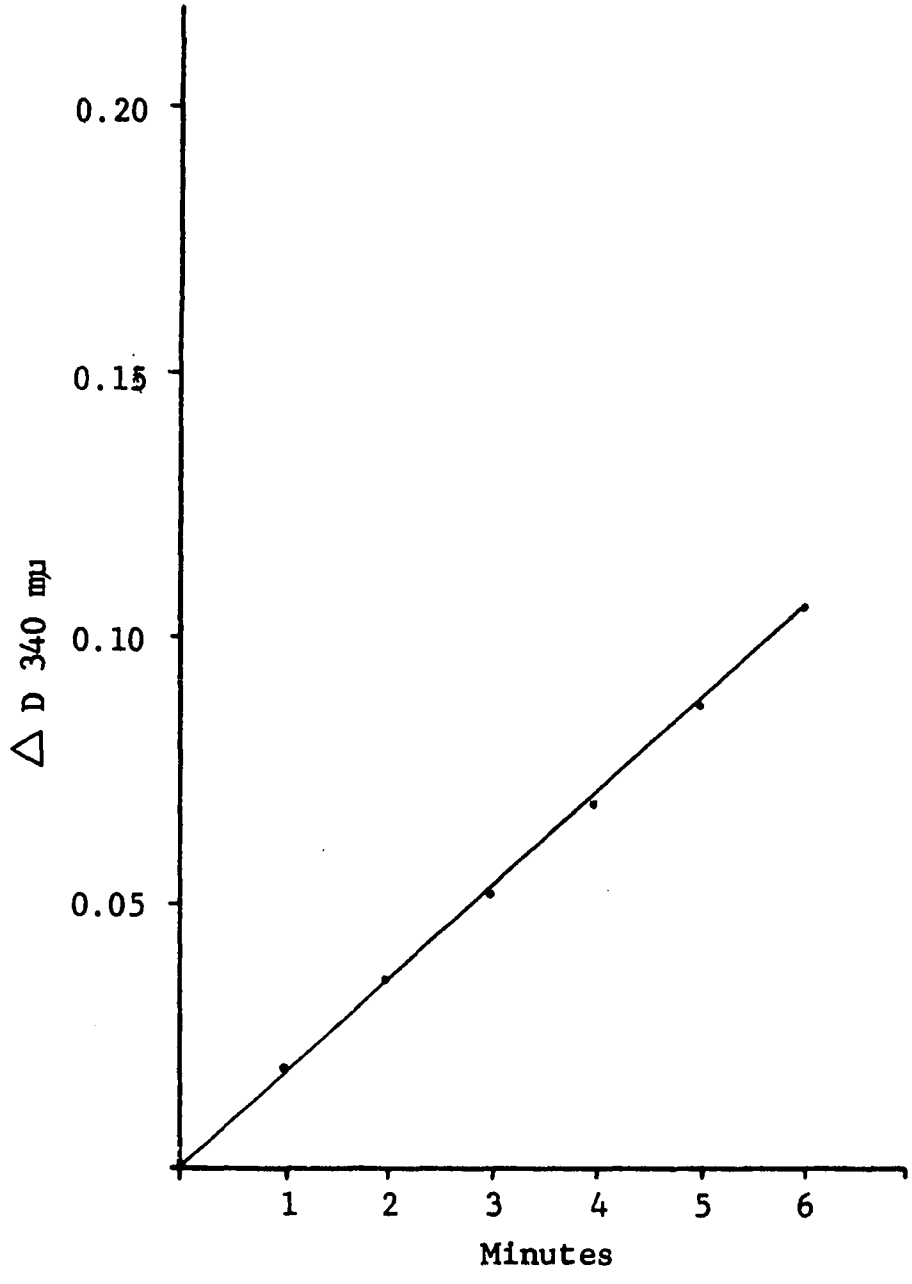


Fig. 4 Phosphoglucoisomerase

The complete system contained: 400 μM Na phosphate, pH 7.0; 10 μM F-6-P; 0.30 μM TPN; 0.05 ml. tissue extract (47 μg protein). Final volume, 3.0 ml. Temperature, 25°C. There was no change in O.D. in the absence of F-6-P.

formed. It seemed possible that removal of cellular debris also removed components necessary for the further metabolism of G-3-P. With this in mind the tissue extract mixture was fortified with substances which are known to facilitate activity of G-3-P dehydrogenase. These results are also included in Table 5. Neither DPN nor cysteine added to the medium alone or together had appreciable effect on the amount of alkali-labile phosphate formed.

Assays for aldolase were repeated with dialyzed tissue extract serving as the source of enzyme (Table 6). The experiments were designed to measure the action of aldolase in dialyzed tissue extract and also to determine the relationship of activity to concentration of enzyme. In every measurement with cyanide added to the reaction mixture, the total alkali-labile phosphate was far below the total alkali-labile phosphate without cyanide. The cyanide had a definite inhibitory effect on the dialyzed extract. This inhibition was related to the ratio of enzyme and cyanide in the mixture, since the alkali-labile phosphate in the determinations with cyanide is proportional to the concentration of enzyme added.

In view of the inhibitory effect of cyanide on dialyzed tissue extract it was also desirable to determine

TABLE 5

ALDOLASE

System	µg P_i/30 min./µg. protein	
	Homogenate	Tissue extract
Complete	0.000	0.432
Plus 175 µM KCN	0.085	0.446
Plus .60 µM DPN	-	0.438
Plus .04 µM cysteine	-	0.486
Plus .04 µM cysteine and .60 µM DPN	-	0.415

The complete homogenate system contained: 0.6 µM glycine, pH 9.6; 2 µM FDP; 0.5 ml. tissue homogenate (1.3 mg. protein). Final volume 3.0 ml. Temperature, 37°C. Reaction time, 30 minutes. Additions as indicated.

The complete tissue extract contained: 30 µM Tris (hydroxymethyl)aminomethane, pH 8.6; 20 µM FDP; 0.5 ml. tissue extract (700 µg. protein). Final volume 2.0 ml. Temperature, 37°C. Reaction time, 10 minutes. Additions as indicated.

activity by a spectrophotometric method patterned after that of Warburg and Christian (1943). In this method the enzyme is coupled to G-3-P dehydrogenase and the rate of reaction is measured by following the reduction of DPN. Since this dehydrogenase has been measured previously in P. halei (see G-3-P Dehydrogenase) and found to be very active in dialyzed extract, excess dehydrogenase was not added. The results for aldolase by this method are presented in Figure 5. In the presence of added FDP, reduced DPN is formed and the rate of formation is linear for the 6 minute period of measurement. When FDP was omitted from the mixture there was no measurable formation of reduced DPN.

Glycerophosphate Dehydrogenase and Triose Phosphate Isomerase

This dehydrogenase catalyzes the formation of glycerophosphate from dihydroxyacetone phosphate and the concomitant oxidation of DPNH. Measurements of this step in dialyzed tissue extract of P. halei (Fig. 6) were obtained by following the formation of oxidized DPN in the presence of excess G-3-P. Under the conditions of this assay, triose isomerase is also measured. Although the rate is low it remains linear for a relatively long period of time. The pH optimum for this system in the range 6.5 to 9.0 as determined in phosphate buffer

TABLE 6

ALDOLASE

System	Total Protein µg.	*Total alkali-labile P_i	µg P_i/30 min./ µg. protein
Complete	112	14.4	0.385
	224	28.0	0.375
	336	52.0	0.464
Plus 40 µM cyanide	112	0.0	0.000
	224	16.0	0.214
	336	22.4	0.200

*Figures represent total for 10 minute incubation period.

The complete system contained: 200 µM glycine, pH 9.0; 30 µM FDP; dialyzed tissue extract added to give µg. protein indicated. Final volume, 3.0 ml. Temperature, 25°C. Reaction time, 10 minutes. Additions as indicated.

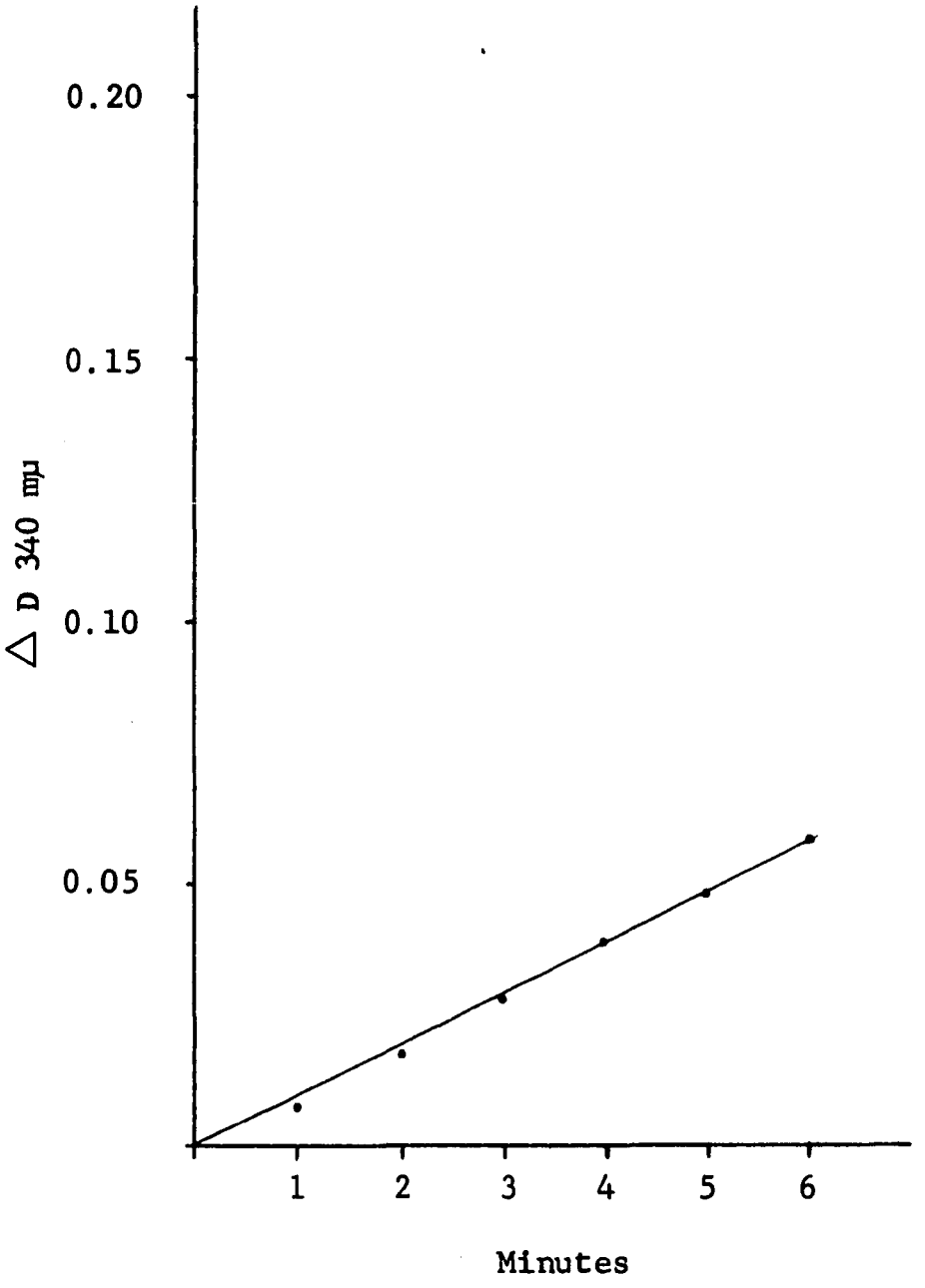


Fig. 5 Aldolase

The complete system contained: 60 μM pyrophosphate, pH 8.4; 8 μM cysteine; 0.6 μM DPN; 40 uM disodium arsenate; 3.0 μM FDP; 0.1 ml. dialyzed tissue extract (112 μg protein). Final volume, 3.0 ml. Temperature, 25°C. Reaction time, 6 minutes.

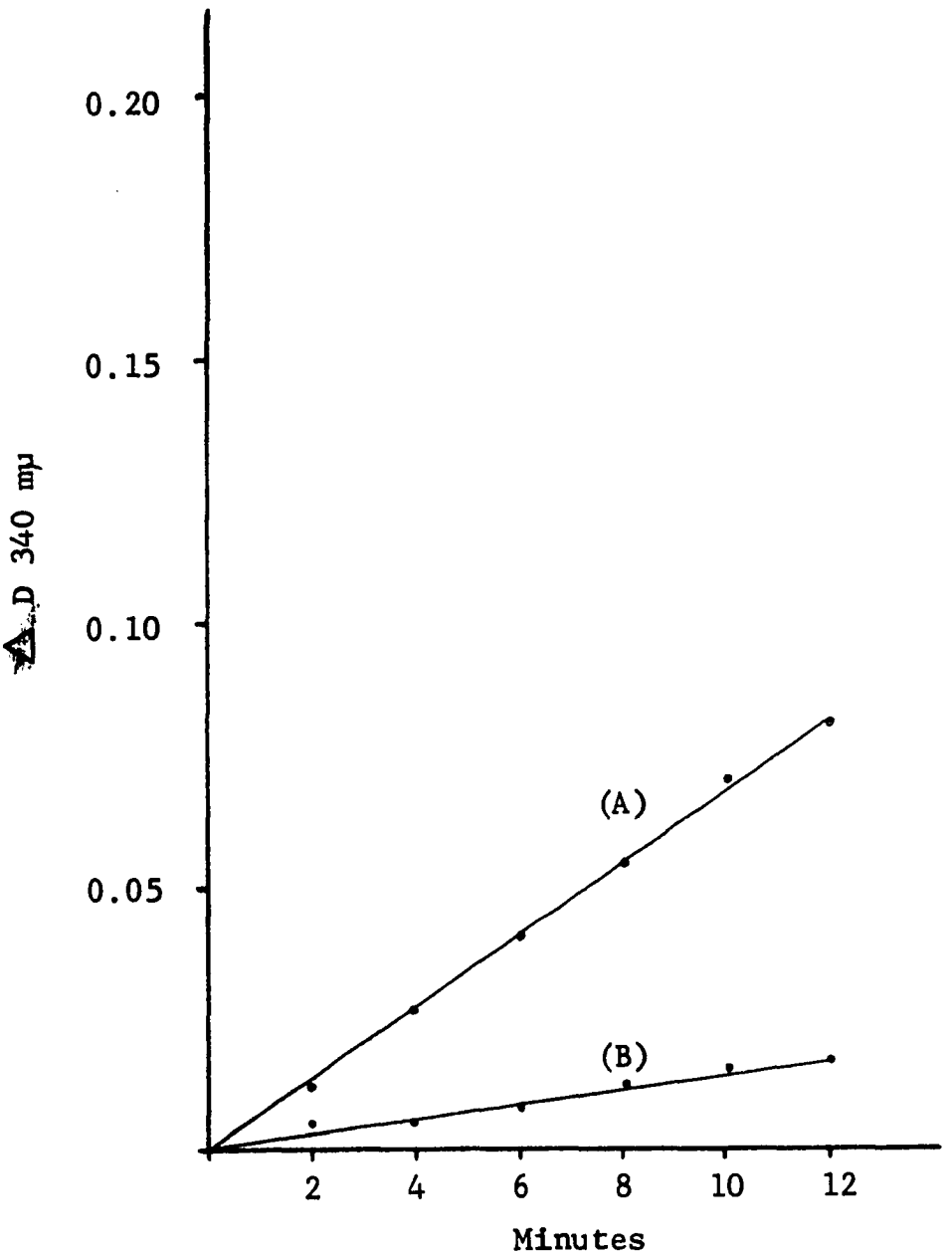


Fig. 6 Glycerophosphate Dehydrogenase and Triosephosphate Isomerase

The complete system, curve (A), contained: 40 μM Na phosphate, pH 8.0; 0.96 μM G-3-P; 1.5×10^{-3} μM DPNH; 0.1 ml. tissue extract (70 μg protein). Final volume, 2.5 ml. Temperature, 25°C. Curve (B) was measured when G-3-P was omitted from the system.

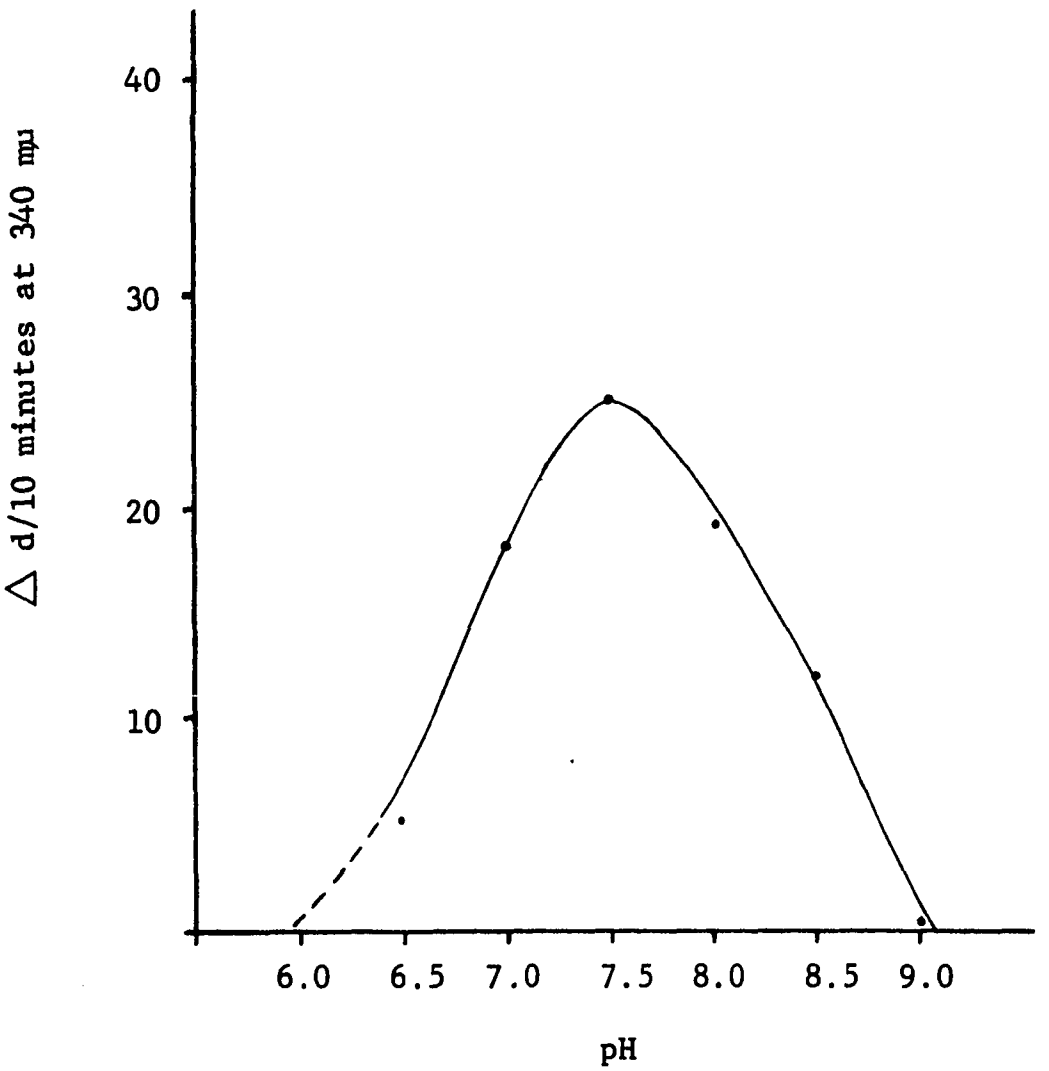


Fig. 7 pH Optimum for a-Glycerophosphate Dehydrogenase-Triose Isomerase System

Conditions as in figure 5 with pH as indicated.

was 7.5 (Fig. 7). This pH is recommended for maximum activity for the enzyme in rabbit muscle (Beisenherz, et al., 1953). Efforts to increase activity of the enzyme in P. halei with the use of triethanolamine buffer were not successful.

Glyceraldehyde-3-Phosphate Dehydrogenase

This enzyme mediates the reaction between G-3-P and 1,3-PGA. The classical inhibition of glycolysis by iodoacetic acid and certain other compounds is attributed to inhibition of G-3-P dehydrogenase and indicates sulphydryl groups to be the active catalytic site on the enzyme (Hellerman, 1937; Hellerman, et al., 1943).

Some of the characteristics of this enzyme in P. halei are shown in Table 7. In general the enzyme in P. halei is similar to the mammalian muscle enzyme. The negligible activity in the absence of arsenate or phosphate suggests that, as with the mammalian enzyme, arsenylation (or phosphorylation) is an obligatory accompaniment of oxidation of G-3-P. Decreased activity when phosphate replaces arsenate further suggests that the equilibrium relations of this reaction and the relative turnover rates of G-3-P dehydrogenase and phosphoglyceric kinase are similar in P. halei and in mammalian tissues (Cori, et al., 1948). The

requirement for cysteine, the inhibition by iodoacetic acid and the inactivity of TPN as a co-factor are other points of similarity between G-3-P dehydrogenase in P. halei and mammalian tissues. Activity, as measured spectrophotometrically, was detected only after addition of G-3-P. The rate of activity was high, compared with other oxidation steps measured in snail tissue, and remained linear for only a short period of time (Fig. 8). The failure of the system to retain the linearity was not due, however, to any inactivation of the enzyme since a constant rate of reaction was obtained with FDP as the substrate (Fig. 5).

3-Phosphoglyceric Kinase

This glycolytic enzyme catalyzes the conversion of 1,3-PGA to 3-PGA, with the accompanying phosphorylation of ADP to ATP. The reaction is reversible but the equilibrium favors 3-PGA formation (Colowick, 1951), and the acyl phosphate of 1,3-PGA is quite labile (Schlenk, 1951). Nevertheless, with hydroxylamine, which reacts with acyl phosphate under the proper conditions to form a hydroxamic acid, it is possible to measure the step by following the reverse reaction.

The data in Table 8 provide evidence for this step

TABLE 7

GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE

System	Change in $\frac{O.D.}{min.}$ (340 mμ)	Rate of formation of reduced DPN*
Complete	0.065	9.91
Minus G-3-P	0.000	0.00
Minus Arsenate	0.003	0.45
Minus Cysteine	0.000	0.00
0.3 μM TPN replacing DPN	0.000	0.00
Phosphate replacing Arsenate	0.022	3.94
Plus 10 μM IAA	0.000	0.00

*μM/min/mg. protein x 10^2

The complete system contained: 60 μM pyrophosphate, pH 8.4; 8 μM cysteine; 0.60 μM DPN; 40 μM disodium arsenate; 0.96 μM G-3-P; 0.1 ml. dialyzed tissue extract (112 μg protein). Final volume, 3.0 ml. Temperature, 25°C. Reaction time, 1½ minutes. Variations as indicated.

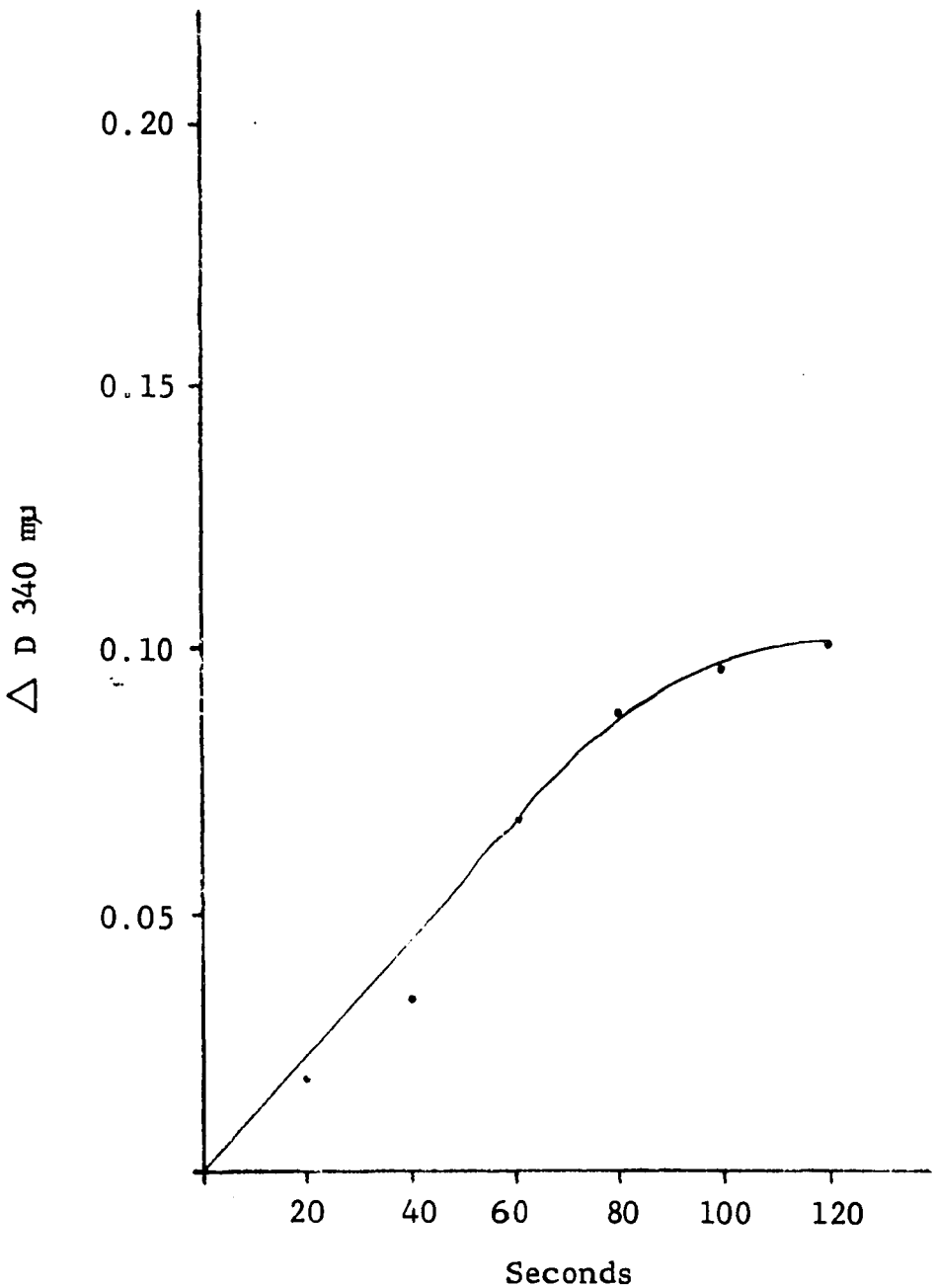


Fig. 8 Glyceraldehyde-3-Phosphate Dehydrogenase

Conditions given in Table 7.

in the tissue of P. halei. The measurements represent the increase of acyl phosphate over the zero time measurement. Magnesium stimulates the reaction rate. The activity of the complete system agrees very closely with the activity rate in cat muscle (Lipmann and Tuttle, 1945). Preliminary studies of the method were conducted with extracts of rat muscle and almost identical results were obtained.

Lactic Dehydrogenase

This enzyme catalyzes the reversible oxidation of lactic acid to pyruvic acid and, in vertebrate tissue, requires DPN as a co-factor. Under anaerobic conditions the oxidation of DPNH by this step balances the reduction of DPN by G-3-P dehydrogenase.

Evidence for the presence of this enzyme in dialyzed tissue extract was sought by following the oxidation of added DPNH in the presence of excess pyruvic acid. The results of these measurements are shown in Figure 9. Under the conditions employed in the assay, lactic dehydrogenase activity was very low. High concentrations of pyruvate (4 mM and higher) are known to have a marked inhibitory effect upon lactic dehydrogenase of vertebrate muscle (Kaplan, et al., 1960). The concentration of pyruvate used for the lactic

TABLE 8

3-PHOSPHOGLYCERIC KINASE

System	μM Acyl Phosphate	μM/hr/mg. protein
Complete	2.65	31.17
Minus ATP	0.79	9.29
Minus Mg^{++}	1.08	12.70
Minus 3-PGA	0.23	2.70

The complete system contained: 84 μM Tris(Hydroxymethyl) aminomethane, pH 7.4; 40 μM ATP; 12 μM $MgCl_2$; 40 μM 3-PGA; 200 μM Hydroxylamine, pH 7.0; 0.2 ml. tissue extract (340 μg. protein). Final volume, 3.0 ml. Temperature, 30°C. Reaction time, 15 minutes. Omissions as indicated.

dehydrogenase assay of P. halei was in the range reported to permit maximum activity with the vertebrate enzyme. That excess pyruvate was not the principal reason for the low lactic dehydrogenase activity is supported by the fact that DPNH oxidation was not observed upon addition of 3-PGA in the absence of a purified lactic dehydrogenase (Table 11). Pyruvate was formed upon addition of 3-PGA since oxidation of DPNH was observed in the presence of added lactic dehydrogenase. The initial concentration of pyruvate derived from added 3-PGA would not have been high. It seems, therefore, that inhibition from excess pyruvate would not explain the relatively low activity of lactic dehydrogenase in P. halei. Since pyruvate kinase activity is influenced by the type of buffer employed in the assay (Table 10), this could have an effect upon lactic dehydrogenase activity of Physa halei in the presence of added 3-PGA. Additional measurements were made with 3-PGA and with triethanolamine buffer instead of phosphate buffer, but this substitution did not result in an increase in DPNH oxidation. No increase in DPNH oxidation was observed when triethanolamine buffer was substituted for phosphate buffer in the presence of added pyruvate.

Further attempts to identify lactic dehydrogenase in snail tissue were made following the method of Neilands

(1955) which measures the reduction of DPN in the presence of excess lactic acid at pH 10 (the reverse of the system described above). No activity was detected by this method. This pH is an extreme condition, however, and, although mammalian enzyme can function in this range, it is conceivable that such alkalinity inactivates snail lactic dehydrogenase.

A colorimetric method for determining dehydrogenases at pH's near neutrality has recently been developed by Ells (1959). The method follows the conversion of lactic acid to pyruvic acid and has the characteristic of forcing the reaction toward pyruvic acid. The results presented in Figure 10 were obtained with this assay.

Using Ells' method, snail lactic dehydrogenase co-factor specificity was demonstrated to differ from that of mammalian muscle. In the latter, lactic dehydrogenase with TPN as co-factor has, at best, a small fraction of the activity it shows with DPN. The snail enzyme, on the other hand, is able, under the conditions of this assay, to utilize TPN as readily as DPN. To the author's knowledge, no previous report has been made of a lactic dehydrogenase which can effectively replace DPN with TPN.

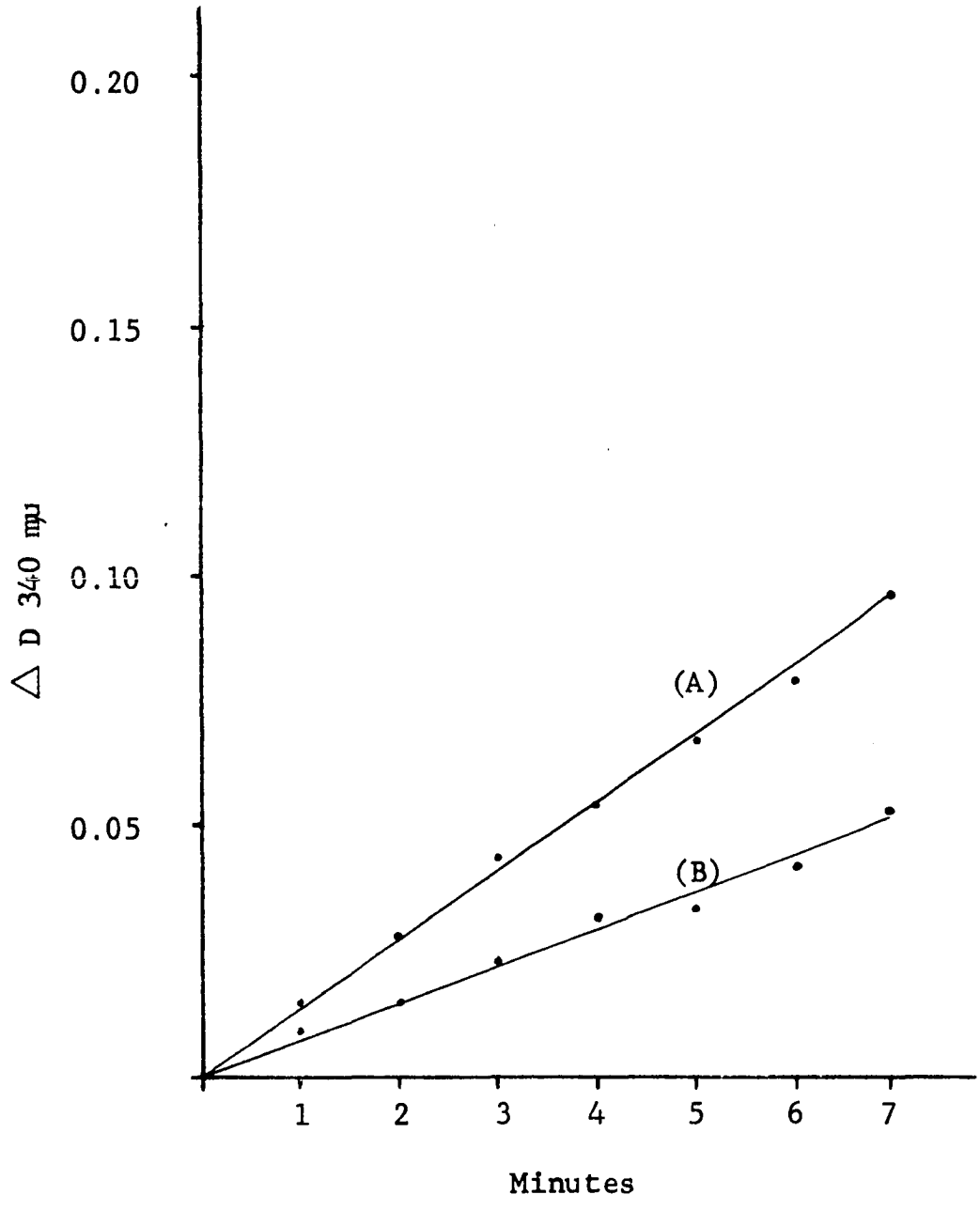


Fig. 9 Lactic Dehydrogenase

The complete system, curve (A), contained: 40 μM Na phosphate, pH 7.3; 6.7×10^{-3} μM DPNH; 10 μM Na pyruvate; 10 μM IAA; 0.2 ml. tissue extract (286 μg protein). Final volume, 3.0 ml. Temperature, 25°C. Curve (B) was measured when Na pyruvate was omitted from the system.

The Effect of Fluoride on Minced Tissue

Since fluoride has been reported to have little or no inhibitory effect on the minced tissue of Australorbis glabratus (Weinbach, 1953) it was desirable to test the effect of this ion on minced tissue of P. halei. The results of this study are presented in Table 9. The data agree with Weinbach's report (1953) that fluoride fails to inhibit pyruvate production in minced snail tissue. The inhibition of oxygen uptake is somewhat higher, however, in P. halei than in A. glabratus. This lack of fluoride inhibition made it desirable to obtain data on the individual enzymes of the terminal steps of glycolysis.

Pyruvic Kinase

This enzyme catalyzes the reaction between phosphopyruvic acid and ADP which yields pyruvate and ATP. In a system containing lactic dehydrogenase it is possible to study this reaction by following the oxidation of DPNH spectrophotometrically.

The approach described above was employed in demonstrating this kinase in P. halei. Initial attempts to demonstrate the enzyme in a mixture with sodium phosphate buffer, Mg^{++}, and ADP gave negative results. It seemed possible

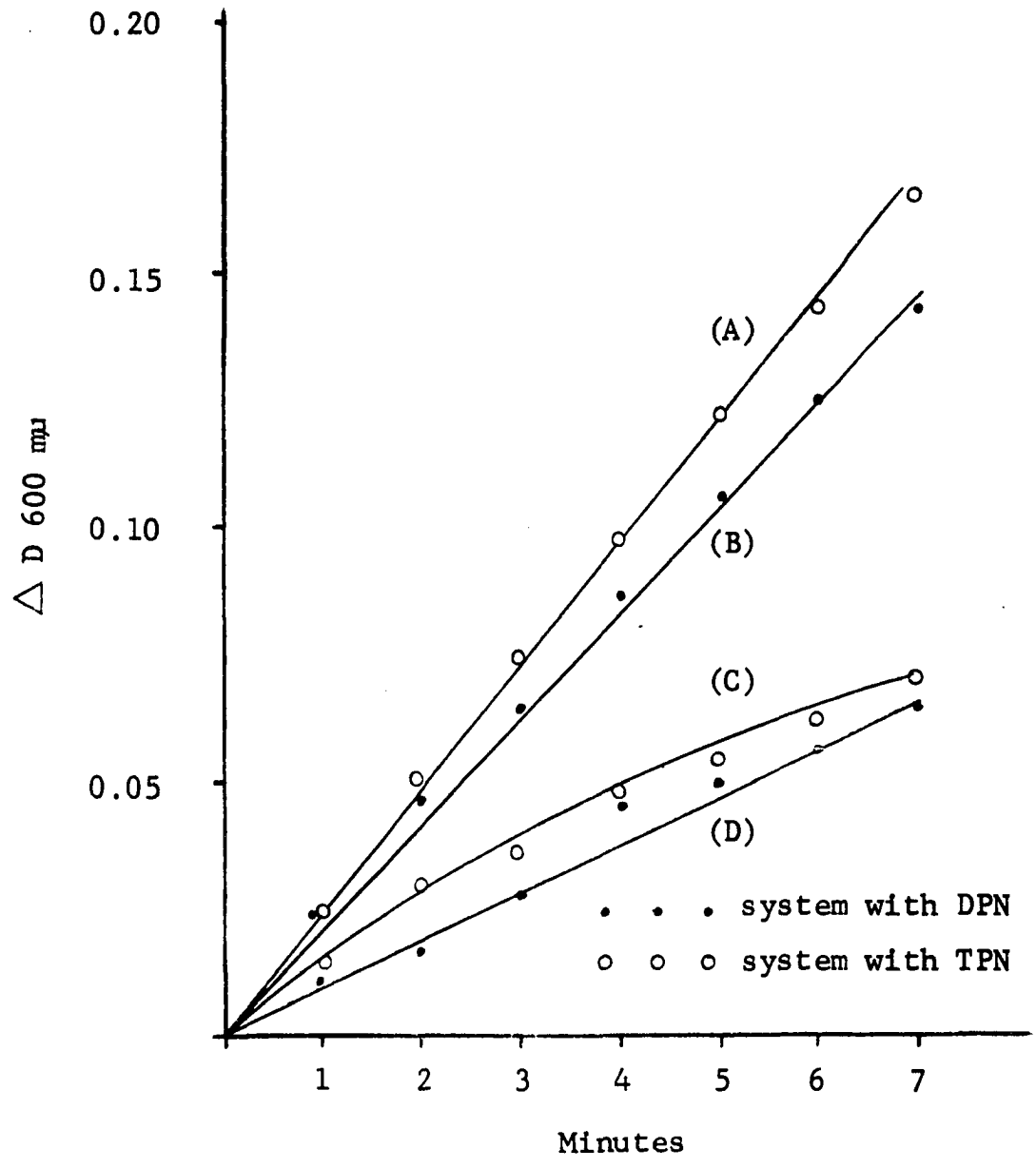


Fig. 10 Lactic Dehydrogenase

The complete system, curve (A) and (B), contained: 30 μM Na phosphate, pH 7.3; 600 x 10^{-3} μM DPN or 30 x 10^{-2} μM TPN; 20 μM lithium lactate; 10 μM IAA, 30 μM KCN, pH 7.3; 0.2 mg. phenazine methosulfate; 0.03 μM 2,6-dichlorophenolindophenol; 0.2 ml. tissue extract (234 μg protein). Final volume, 3.0 ml. Temperature, 25°C. Curves (C) and (D) were measured when lithium lactate was omitted from the system.

TABLE 9

EFFECT OF FLUORIDE ON OXYGEN CONSUMPTION
AND PYRUVATE PRODUCTION
OF MINCED PHYSA HALEI

Addition	µl O_2/hr./mg. tissue*	µg. pyruvate/hr./mg. tissue*
None (endogenous)	5.60	.148
30 µM Na fluoride	4.39	.168
Per cent inhibition	21.6	0.00

*Wet weight

Oxygen consumption was measured in a total volume of 3.0 ml. containing 50 µM Na phosphate, pH 7.4. Pyruvate was determined in a 2.0 ml. aliquot of the reaction mixture after addition of 1.2 ml. of 10% TCA and removal of precipitated protein. Each value is the mean of three determinations.

that the kinase was present but undetected due to the low rate of lactic dehydrogenase activity in the system. With this in mind, a highly purified, essentially kinase-free, lactic dehydrogenase was obtained and added to the assay mixture. The measurements with this system were also negative.

Mammalian tissue enzyme requires potassium and magnesium ions for activity and sodium ions are known to depress this activity by antagonizing the function of the potassium ion (Lardy, 1949). When sodium phosphate buffer was replaced with potassium phosphate the results shown in Table 10 were obtained. In this system activity was observed only when both Mg^{++} and ADP were present. The lack of activity with sodium phosphate buffer suggests that sodium antagonizes the P. halei enzyme in a manner similar to its antagonism to the mammalian tissue enzyme. Triethanolamine buffer has been reported (Beisenherz, et al., 1953) to increase activity of linked systems of this type and when it was substituted for potassium phosphate buffer the rate of activity was increased almost three times (Table 10). With triethanolamine buffer it was possible to test the effect of potassium upon the system and there is a very definite potassium requirement for maximum activity. Even in the absence of ADP in the triethanolamine buffered system there is formation of pyruvate.

This activity suggests the presence of a phosphatase in the system. Whether it is a specific or general phosphatase is not known.

Phosphoglyceric Mutase

This enzyme catalyzes the interconversion of 3-PGA and 2-PGA. The reaction has been studied in crude muscle extracts by following DPNH oxidation during lactic acid formation (Sutherland, et al., 1949). This approach was followed in demonstrating mutase activity in dialyzed extract of P. halei. The results of the study are presented in Table 11. Activity is reported as change in optical density per minute and the relative value of the figures was considered in evaluation of the data. The ability of 2,3-PGA to stimulate activity suggests the mechanism of reaction in Physa halei is similar to that in vertebrate tissue. Since the sequence would involve the enolase step, activity was determined in, and found to be inhibited by, the presence of fluoride.

Enolase

This enzyme catalyzes the conversion of 2-PGA to PE, and is characterized by its sensitivity to magnesium fluorophosphate, which is formed when fluoride is added to a system

TABLE 10

PYRUVIC KINASE

System	Change in $\frac{O.D.}{min.}$ (340 mμ)	
	With Phosphate Buffer	With Triethanolamine Buffer
Complete	0.066	0.160
Minus phosphopyruvate	0.005	-----
Minus ADP	0.008	0.037
Minus Mg^{++}	0.005	-----
Minus K^{+}	-----	0.078
Minus extract	0.003	-----

The complete system contained: 150 μM K^{+} phosphate, pH 7.5 or 15 μM triethanolamine, pH 7.5; 0.3 μM DPNH; 0.002 μM PE; 24 μM $MgCl_2$; 0.5 μM KCl; 0.2 ml. dialyzed tissue extract; 0.2 ml. lactic dehydrogenase (180,000 units). Final volume, 3.0 ml. Reaction time, 5 minutes except with the complete system in triethanolamine which was 2.5 minutes. Omissions as indicated.

TABLE 11

PHOSPHOGLYCERIC MUTASE

System	Change in $\frac{O.D.}{min.}$ (340 mμ)
Complete	0.016
Minus 3-PGA	0.003
Minus 2,3-PGA	0.011
Minus lactic dehydrogenase	0.000
Plus 17 μM Na Fluoride	0.003

The complete system contained: 100 μM triethanolamine, pH 7.5; .00135 μM DPNH; 0.4 μM ADP; 2.5 μM 3-PGA; 0.03 μM 2,3-PGA; 24 uM $MgCl_2$; 1.0 μM IAA; 0.2 ml. dialyzed tissue extract (160 μg. protein); 0.01 ml. purified lactic dehydrogenase (.5 mg/ml.). Final volume, 3.0 ml. Temperature, 25°C. Reaction time, 7 minutes. Omissions and additions as indicated.

containing Mg^{++} and inorganic phosphate. Since PE absorbs light at 240 mμ it is possible to follow its formation spectrophotometrically.

Evidence for this step in dialyzed tissue extract of P. halei was obtained by following the increase in optical density at 240 mμ with 2-PGA as substrate. Figure 11 shows the results of these measurements. Fluoride inhibits this system; in fact, the optical density of the mixture decreases at a steady rate in the presence of this ion.

Enzymes of Carbon Dioxide Fixation

The failure of fluoride to inhibit pyruvate formation in minced tissue of P. halei while the enolase of the snail is definitely inhibited by the ion suggests the presence of an alternate pathway for pyruvate synthesis. Two enzymes, oxalacetic carboxylase and TPN malic decarboxylase, both associated with carbon dioxide fixation, provide a route for pyruvic acid synthesis. Preliminary experiments have been carried out on oxalacetic carboxylase and TPN malic decarboxylase and also on a third enzyme, succinic decarboxylase. The last enzyme was included because of previous reports (Mehlman and von Brand, 1951) of anaerobic propionic acid formation in aquatic snails. Succinic decarboxylase is

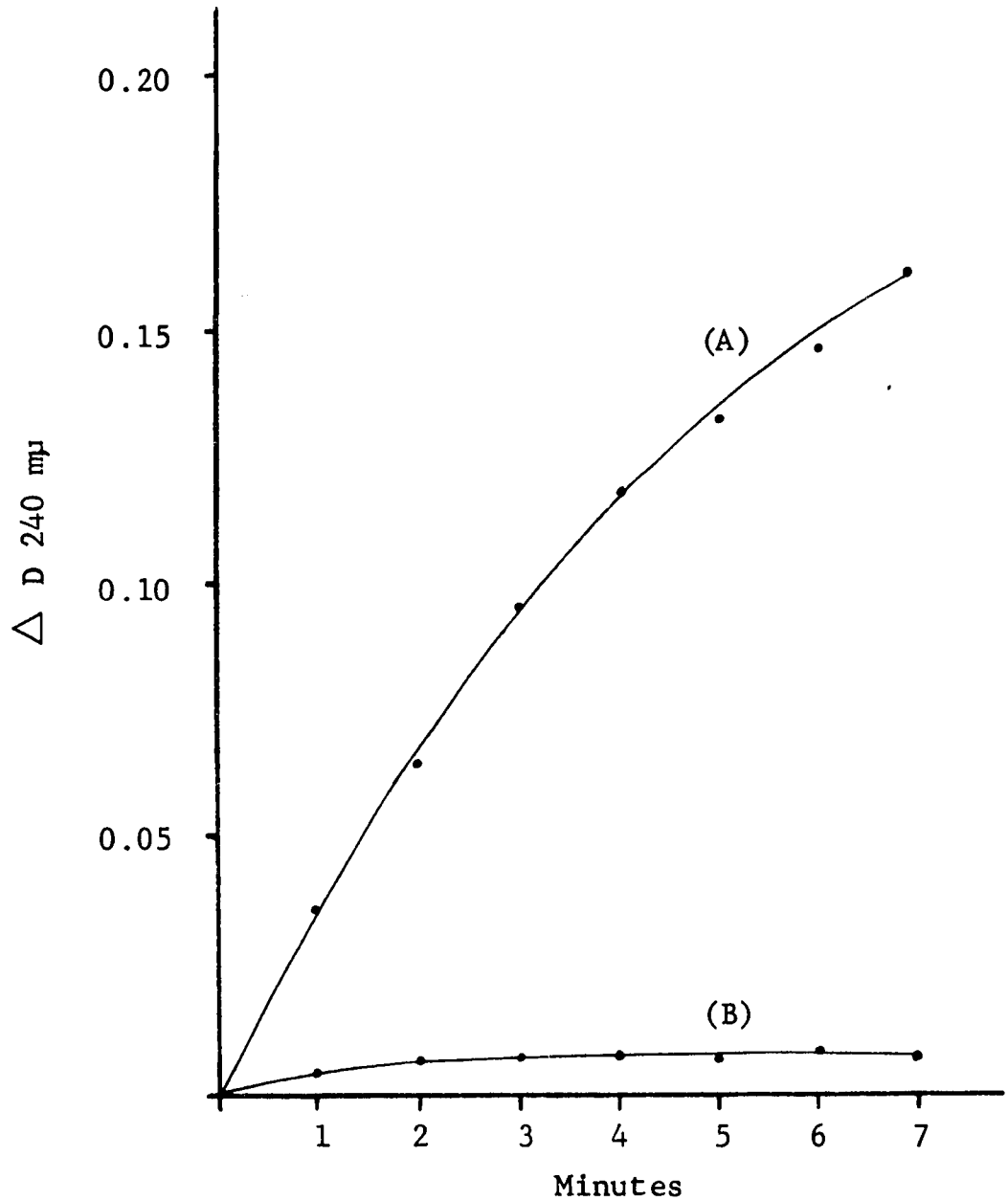


Fig. 11 Enolase

The complete system, curve (A), contained: 40 μM bicarbonate-carbonate buffer saturated with N_2-5%CO_2 gas to pH 7.6; 2.5 μM 2-PGA; 8.1 μM glycine; 24 μM $MgCl_2$; 0.2 ml. dialyzed tissue extract (125 μg protein). Final volume, 3.0 ml. Temperature, 25°C. Curve (B) was measured when 2-PGA was omitted from the system.

capable of catalyzing the fixation of carbon dioxide into propionic acid formation in aquatic snails. Succinic decarboxylase is capable of catalyzing the fixation of carbon dioxide into propionic acid to yield succinic acid. This reaction has recently been demonstrated in oyster tissue by Hammen and Wilber (1959). An attempt was made with dialyzed extract of P. halei to demonstrate this reaction by showing carbon dioxide evolution and propionate formation in a mixture containing excess succinic acid. Under the conditions employed, however, no activity was measured.

Oxalacetic carboxylase from pigeon liver catalyzes the fixation of carbon dioxide into pyruvic acid to give oxalacetic acid (Ochoa, et al., 1948). Jodrey and Wilber (1955) have demonstrated a high rate of activity for this enzyme in oyster tissue. Evidence for this enzyme in P. halei was sought by following carbon dioxide evolution in a mixture containing dialyzed tissue extract and excess oxalacetic acid. Under the conditions employed, the results of the assay were negative.

TPN malic decarboxylase was first described in a preparation from pigeon liver (Ochoa, et al., 1947). The reaction catalyzes the decarboxylation and oxidation of malic acid to pyruvic acid accompanied by the reduction of TPN to

TPNH. This reaction has also been demonstrated in muscle tissue of Ascaris lumbricoides (Saz and Hubbard, 1957).

This enzyme was tested for in P. halei by the procedure outlined by Ochoa, et al. (1948). The results of these spectrophotometric measurements are shown in Table 12. The formation of TPNH in the presence of malate and Mn^{++} suggests a TPN malic decarboxylase. Pyruvate was formed in concentrations far too high to be accounted for by the "malic" enzyme alone. No immediate explanation of this finding is apparent.

TABLE 12

TPN MALIC DECARBOXYLASE

System	Total μM TPNH Formed	Total μM pyruvate Formed
Complete	.034	3.27
Minus dl-malate	.004	1.69
Minus Mn^{++}	.002	0.00
Minus TPN	.003	2.00

The complete system contained: 75 μM glycylglycine, pH 7.2; 3 μM $MnCl_2$; 3.0μM dl-malate; 0.3 μM TPN; 0.2 ml. dialyzed tissue extract (151 μg. protein). Final volume 3.0 ml. Temperature 25°C. Reaction time 9 minutes. At the end of 9 minutes 1 ml. of 10% TCA was added to the mixture. The ppt. protein centrifuged and 1 ml. alq. of supernate taken, neutralized, diluted to 2.0 ml. and 0.5 ml. of this dilution assayed for pyruvate.

Pyruvate assay mixture contained: 40 μM Na phosphate, pH 7.4; 1.35×10^{-3} uM DPNH; 0.5 ml. neut. alq.; 0.1 ml. lactic dehydrogenase (.5 mg. protein/ml.). Final volume 3.0 ml. Temperature, 25°C. Reaction time, 10 minutes. Omissions as indicated.

CHAPTER V

DISCUSSION

The experimental data presented demonstrate the initial steps of the hexose monophosphate shunt and the major enzymes of the glycolytic pathway in P. halei. Most enzymes appear to be similar to comparable enzymes in vertebrate tissue. For a few enzymes, however, the characteristics are rather different. The reactions involved in glycolysis are summarized in Figure 12. The figure also includes the steps of the hexose monophosphate shunt that were measured in this investigation.

The unexpected gas uptake (reported as carbon dioxide) by tissue extract of P. halei under anaerobic conditions is quite high. The data in Table 1 suggest that exogenous substrate is not necessary for this gas consumption since there is no increase in uptake when FDP is present in the mixture. Freeman and Wilber (1948) demonstrated carbonic anhydrase activity in tissue extract of several marine mollusks and

suggested a possible role of carbonic anhydrase in the deposition of carbonate during shell formation. They state that hydration of carbon dioxide occurs in mantle tissue in the presence of both carbonate and bicarbonate. Recently Freeman (1960) has shown carbonic anhydrase activity (measured indirectly by following the effect of carbonic anhydrase inhibitors on shell growth) in the aquatic snail Physa heterostropha. It seems quite possible that the observed anaerobic gas intake of tissue extract of P. halei could be due to an active carbonic anhydrase. It is also reasonable to suppose that the carbon dioxide production upon addition of FDP is due to a high rate of acid production which "overshadows" the carbon dioxide uptake.

Measurements obtained with P. halei demonstrate both 6-PG and G-6-P dehydrogenase activities (reactions 2 and 3 in Figure 12). Activity of both steps is high relative to other enzymes (measured in this laboratory) which are concerned with hexose phosphate metabolism. The demonstration of these two dehydrogenases alone, however, is not conclusive proof for the existence of a complete shunt as it is known for the vertebrate tissue. The characteristics of the enzyme are apparently similar to those from vertebrate tissue, and the high activity points to the importance of

these enzymes in assimilation of hexose phosphates by these snails.

Both phosphoglucomutase and phosphoglucoisomerase (reactions 1 and 4, Figure 12) in P. halei have characteristics which are similar to these enzymes in vertebrate tissue. Under the conditions of assay the activity of the phosphoglucomutase step in snail tissue is rather low. With the mammalian enzyme, glucose-1,6-diphosphate has been shown (Leloir, et al., 1948) to activate the reaction. The addition of this co-factor was required, however, only with the purified enzyme. Furthermore, with the purified enzyme, cysteine was not required for activity. With the snail extract no effort was made to remove glucose diphosphate from the preparation nor was effort made to remove this substance from the G-1-P which was added as substrate to the reaction mixture. Whether the addition of glucose diphosphate to the mixture would have increased activity is not known. The response of the step to Mg^{++} and to cysteine make it reasonable to assume, however, that the mechanism of this reaction in P. halei is similar to that of the comparable reaction in vertebrate tissue.

The aldolase step, reaction 6, Figure 12, in P. halei seems definitely to be inhibited by cyanide. Aldolase

has been described as a metallo-protein in yeast (Warburg and Christian, 1942, 1943) in bacteria (Bard and Gunsalus, 1950) in fungi (Jagannathan and Singh, 1954) and in protozoa Baernstein, 1955). Warburg (1942, 1949) reported cyanide, pyrophosphate and cysteine inhibition of the yeast aldolase and the reversal of cyanide and cysteine inhibition upon addition of ferrous or cobaltous ion or zinc. With bacterial aldolase a,a'-dipyridyl, and o-phenanthroline are reported to inhibit and the effect can be reversed by ferrous or cobaltous ion but not by zinc ions (Bard and Gunsalus, 1950). The cell-free aldolase of bacteria, however, required a reducing agent such as cysteine for maximum activity. The aldolase of the fungus, Aspergillus niger, is reported to be stimulated by zinc ions and inhibited by the metal binding agents ethylenediaminetetracetic acid (EDTA), a,a'-dipyridyl, and pyrophosphate (Jagannathan and Singh, 1954). This enzyme was not inhibited by cysteine, and, in the crude preparation, pyrophosphate did not inhibit (Jagannathan and Singh, 1953). Baernstein (1955) found the aldolase of Trichomonas vaginalis to be a metallo-protein inhibited by the metal binding agent EDTA, but enhanced by cysteine. The inhibitory action of cyanide upon the P. halei aldolase suggests an enzyme associated with a heavy metal, and by assuming the enzyme to be a

metallo-protein which is not inhibited by cysteine or pyrophosphate one may explain this inhibition. The lack of inhibition in tissue extract and homogenate of snail tissue could well be due to the presence of relatively high concentrations of dialyzable compounds capable of reacting with the added cyanide in a manner which would tend to protect the aldolase enzyme. It is also possible that there was sufficient heavy metal in the non-dialyzed preparations to counteract the inhibitory effect of cyanide.

Lactic dehydrogenase, reaction 14, Figure 12, of P. halei differs considerably from the corresponding enzyme in vertebrate tissue. The low activity observed agrees with the report that lactic acid is a minor end product of carbohydrate metabolism in Physidae (Mehlman and von Brand, 1951). Lactic dehydrogenase in many invertebrates shows low activity with DPN or TPN, although in the presence of an artificial co-factor (3-acetyl pyridine diphosphopyridine nucleotide) a rapid conversion of pyruvate to lactate was observed (Kaplan, et al., 1960). Whether or not such activity in P. halei is due to low enzyme concentration has not yet been investigated. The ability of TPN to stimulate activity as readily as DPN distinguishes this enzyme from other lactic dehydrogenases which have been studied and suggests the possibility of an

efficient carbon dioxide fixation sequence such as the TPN malic decarboxylase-lactic dehydrogenase interrelation described by Ochoa (1952). In the presence of "malic" enzyme, malic acid and TPN react to give pyruvate, carbon dioxide, and TPNH. Pyruvate and TPNH are converted in the presence of lactic dehydrogenase to lactic acid and TPN. The TPN is then available to react with another molecule of malic acid and the cycle continues. The reverse reaction should also occur, in which carbon dioxide would be fixed into pyruvate to yield malic acid. In the oyster, Crassostrea virginica, however, the main pathway of carbon dioxide fixation has been shown to involve propionate. Pyruvate apparently does not participate in carbon dioxide fixation (Hammen and Wilber, 1959). Future work is planned to identify the products of the reaction and extend the data on co-factor specificity.

The classical steps for conversion of triose phosphate to pyruvate are present in P. halei and their characteristics appear to be quite similar to those of comparable steps in vertebrate tissue. The G-3-P dehydrogenase, reaction 9, Figure 12, is very active and the reaction rate of 3-PGA kinase, reaction 10, Figure 12, is comparable to that of the mammalian enzyme. The 3-PGA mutase, reaction 11,

Figure 12, possesses characteristics quite similar to those of the mammalian enzyme, although, with the method of assay employed, it was not possible to obtain values for activity rates which could be assigned to this enzyme alone. Pyruvate kinase, reaction 13, Figure 12, is quite active in the dialyzed tissue extract and requires both Mg^{++} and K^{+} for activity. The requirement for these two ions suggests that pyruvate kinase of P. halei more closely resembles the mammalian enzyme than the yeast enzyme since in yeast there is no requirement for K^{+} in the dialyzed extract (Muntz, 1947) whereas both ions are required for activity in the mammalian enzyme. The formation of pyruvate in the absence of ADP suggests the presence of a phosphatase capable of splitting inorganic phosphate from phosphopyruvate in triethanolamine buffer. Absence of activity in phosphate buffer could be due to phosphate inhibition of the enzyme. Several phosphatases are inhibited by inorganic phosphate; for example, intestinal phosphomonoesterase (Heppel, 1955), phosphomonoesterase of milk (Morton, 1955), and acyl (acetyl) phosphatase (Koshland, 1955).

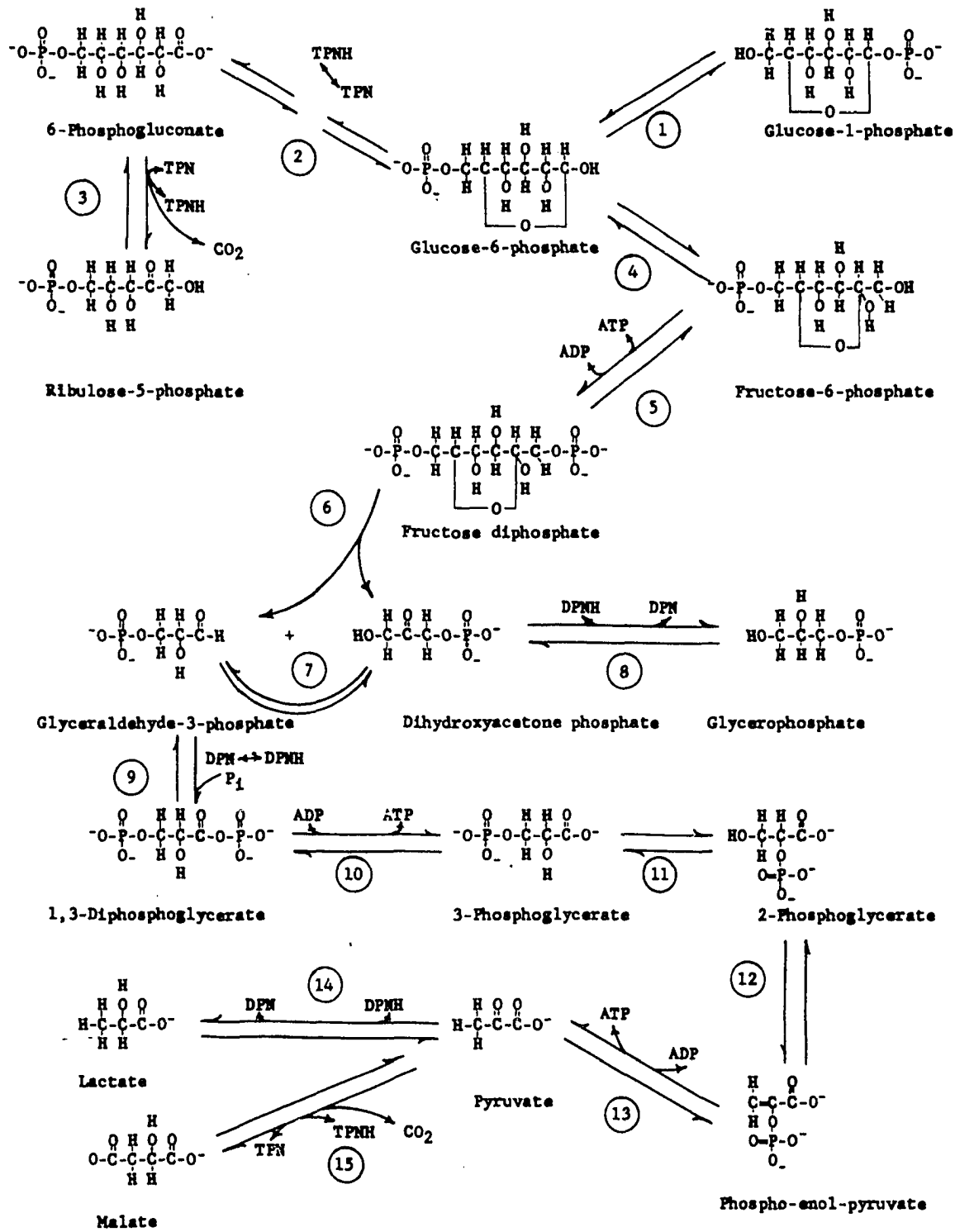
Although fluoride produced no measurable reduction in pyruvate formation in minced tissue, the ion has a very definite inhibitory action on the enolase step, reaction 12,

Figure 12, measured in tissue extract of P. halei. It is possible that most of the pyruvate formed in minced tissue is formed via some pathway other than the glycolytic scheme. The measurements of TPN malic decarboxylase activity, reaction 15, Figure 12, in P. halei indicate the formation of pyruvate from this source. This could well be a source of pyruvate in minced tissue. Weinbach (1956) has found that β-hydroxybutyric acid is vigorously oxidized by acetone powder of the albumen gland of Lymnaea stagnalis. It is possible that pyruvate is produced in quantity from some endogenous source of fatty acids.

The tissue preparations which served as the enzyme source for the measurements reported in this paper have permitted the demonstration and approximate measurements of activities of the glycolytic enzymes of P. halei. A study utilizing purified (or at least partially purified) enzymes would be desirable. This is particularly true of aldolase and lactic dehydrogenase, which apparently differ from the corresponding enzymes in vertebrate tissue. It is hoped that in the future it will be possible to obtain measurements on these steps with a more homogeneous tissue such as the hepatopancreas or albumen gland rather than the entire snail.

Fig. 12 Reaction scheme for the glycolytic pathway and initial steps of the hexose monophosphate shunt.

1. Phosphoglucomutase
2. G-6-P dehydrogenase
3. 6-PG dehydrogenase
4. Phosphoglucoisomerase
5. Phosphofructokinase
6. Aldolase
7. Triose isomerase
8. Glycerophosphate dehydrogenase
9. G-3-P dehydrogenase
10. 3-PGA kinase
11. Phosphoglyceric acid mutase
12. enolase
13. Pyruvate kinase
14. Lactic dehydrogenase
15. TPN malic decarboxylase


6-Phosphogluconate
TPNH
TPN
Glucose-1-phosphate
Glucose-6-phosphate
TPN
TPNH
CO2
Ribulose-5-phosphate
ATP
ADP
Fructose-6-phosphate
Fructose diphosphate
Glyceraldehyde-3-phosphate
Dihydroxyacetone phosphate
DPNH
DPN
Glycerophosphate
DPN ↔ DPNH
Pi
1,3-Diphosphoglycerate
ADP
ATP
3-Phosphoglycerate
2-Phosphoglycerate
Lactate
DPN
DPNH
Pyruvate
ATP
ADP
Phospho-enol-pyruvate
TPN
TPNH
CO2
Malate

CHAPTER VI

SUMMARY

1. Homogenates and extracts of the aquatic snail, Physa halei Lea, have been employed in studies of the initial steps of the hexose monophosphate shunt and glycolytic sequence.

2. Both glucose-6-phosphate and 6-phosphogluconate dehydrogenase have been demonstrated in P. halei. They resemble the mammalian enzyme in co-factor specificity.

3. Efforts to follow anaerobic acid formation by manometric methods have not produced satisfactory results. The measurements obtained suggest the presence of an active carbonic anhydrase which interferes with the measurement of carbon dioxide.

4. Evidence is presented for the presence of these glycolytic enzymes: phosphoglucomutase, phosphoglucoisomerase, aldolase, triosephosphate isomerase, a-glycerophosphate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase,

phosphoglyceric kinase, phosphoglyceric mutase, enolase, phosphopyruvate kinase, and lactic dehydrogenase.

5. Most of the enzymes of the glycolytic sequence have characteristics which are similar or identical to those of mammalian enzymes. Aldolase, lactic dehydrogenase, and pyruvate kinase in P. halei differ in some respects from corresponding mammalian enzymes.

6. Aldolase has characteristics which suggest that it is a metallo-protein and thus more closely resembles the enzyme found in microorganisms than that found in mammalian tissue.

7. Lactic dehydrogenase in P. halei, which has low activity, functions as well with TPN as with DPN. In this respect the enzyme is quite different from mammalian lactic dehydrogenase.

8. Fluoride did not inhibit pyruvate production in minced tissue of P. halei although the ion definitely inhibits enolase.

9. The presence of a "malic" enzyme was investigated, and, while malic acid was found to stimulate the formation of TPNH and pyruvate, pyruvate formation was not accompanied by an equal molar formation of reduced TPN. Pyruvate formation was observed in the absence of TPN. A

malate to pyruvate step could explain the accumulation of pyruvate observed in minced tissue when fluoride was added to the system.

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