ENZYMATIC PHOSPHATE TRANSFER IN PLANT SYSTEMS

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ABSTRACT

The experiments described in this thesis have established that the same phosphorylated intermediates of carbohydrate metabolism known to occur during the course of glycolysis in yeast and muscle tissue are involved in the carbohydrate metabolism of the pea seed. In the identification of these glycolytic intermediates use has been made of chromatographic and other techniques developed for the purpose.

Two enzymes of the glycolytic sequence not previously characterized in higher plants have been separated and studied. These are phosphofructokinase and hexokinase. Both of the enzymes are concerned with the direct phosphorylation of substrate with adenosine triphosphate (ATP) — a key compound in the energy metabolism of the cell.

Phosphofructokinase, which catalyzes the reaction

fructose-6-phosphate + ATP <⟶> fructose-1, 6-diphosphate + ADP

has been partially purified from pea seed meal and characterized with respect to its pH optimum, inhibitors, stability, substrate affinities, and substrate specificity. A method for the assay of the enzymatic activity has been developed in which aldolase is employed for the direct determination of fructose diphosphate formed.

Hexokinase, which catalyzes the reaction

hexose + ATP <⟶> hexose-6-phosphate + ADP

has, likewise, been partially purified and characterized with respect to several of its properties. Both enzymes have been found in a variety of higher plants, indicating the ubiquity of the glycolytic sequence.

Although phosphofructokinase is a soluble cytoplasmic constituent, hexokinase appears to occur both in an insoluble and in an soluble form, the distribution being dependent upon the tissue studied and the method of preparation used. The fact that the insoluble or mitochondrial fraction seems to contain the major fraction of the hexokinase activity is of interest in light of the fundamental role of the mitochondria in the generation of ATP via oxidative phosphorylation.

TABLE OF CONTENTS

GENERAL INTRODUCTION

With the demonstration by Harden and Young (1) that phosphate is essential to fermentation by cell-free extracts of yeast, a new and productive era of biochemical investigation was inaugurated. One of the most important biological concepts which emerged during the post Harden-Young period was that of the energy-rich phosphate bond and its dynamic function in the metabolism of the cell. Although a great number of investigators have turned their attention toward the study of energy-rich phosphate bonds, few have been concerned with the phosphorus metabolism of higher plants.

It is proposed to review briefly in this dissertation the historical development of our present knowledge of the glycolytic pathway in yeast and animal tissue, to correlate these findings with similar investigations of the carbohydrate metabolism in higher plants, and finally, to present new evidence concerning glycolysis in higher plants and to discuss original studies of two, hitherto uncharacterized enzymes involved in this metabolic pathway.

Harden and Young (2) were able to show that not only is phosphate essential for glycolytic activity in aged yeast extracts, but also a dializable heat stable cofactor, also from yeast, is required. This cofactor, diphosphopyridine nucleotide (DPN), was later isolated and identified by Warburg (3).

The identification of hexose diphosphate as an intermediate

in the fermentation of glucose by yeast extracts, and the isolation and identification (4) of hexose monophosphate (Robinson ester) were the first sign posts on the, as yet, relatively unknown pathway of carbohydrate metabolism.

Applying similar techniques to the study of muscle metabolism, Embden et al.(5) showed that fermentation of hexose by minced muscle preparations is dependent on phosphate. When he added fluoride to the reaction mixture to inhibit phosphatase activity, hexose diphosphate accumulated. He was then able to isolate and identify hexose diphosphate and to show that this product is identical with the hexose diphosphate isolated from yeast. It was, however, the brilliant work of Otto Meyerhof that led to the understanding of the reactions mediating the transformation of glycogen to lactic acid. Meyerhof (6) obtained a cell-free muscle extract, myozymase, which was able to convert starch, glycogen, and other polysaccharides to lactic acid. As with yeast, addition of phosphate to the medium yielded a threefold increase in the amount of lactic acid formed.

Freshly prepared myozymase was also able to ferment glucose, fructose, and mannose, but this ability was rapidly lost on aging. Meyerhof found that when both boiled muscle extract and cell-free yeast extract were added to the aged system, its activity was restored. The factor in yeast extract which restored activity to the muscle preparation was rapidly destroyed by heat. It was subsequently

shown that the activator in the yeast extract is the enzyme hexokinase, which mediates the initial phosphorylation of hexose. Boiled muscle extract was required to supply the two essential coenzymes — DPN and the source of energy-rich phosphate, adenosine triphosphate (ATP).

The isolation by Embden and Zimmerman (7) of adenylic acid in the course of their work on the isolation of hexose phosphates from muscle extracts led ultimately to the discovery by Lohmann (8) and by Fisk and SubbaRow (9) of ATP. Subsequently, Meyerhof and Lohmann (10) showed that glycolytic activity could be restored to autolyzed muscle extracts with purified ATP for which phosphate alone was ineffective. It was the monumental discovery by Meyerhof and Lohmann (11) that the hydrolysis of the terminal phosphates of ATP liberate 12,000 calories per mole per phosphate residue that directed attention to the study of energy transfer mechanisms in cellular metabolism.

Since yeast cells are able to derive all of their required metabolic energy by anaerobic fermentation of hexose, and since a part of this energy is evidently transferred as ATP, much early work centered on the study of the mechanism by which phosphate is esterified to the energy-rich form. During the study of the metabolism of phosphoglyceric acid, Parnas, Ostern, and Mann (12) were able to demonstrate the transfer of the phosphate group of phosphoenolpyruvic acid to adenosine diphosphate (ADP) with the formation of pyruvic acid and ATP. Soon after, Schaffner and Berl (13)

implicated an exothermic oxidation-reduction reaction involved in the esterification of inorganic phosphate. Needham and Pillai (14) demonstrated that the oxidation of triosephosphate by a DPN-linked enzyme resulted in a net esterification of inorganic phosphate. Warburg and Christian (15) crystalized the enzyme from yeast and showed that oxidation occurred only in the presence of inorganic phosphate. Recently, Racker and Krimsky (16) proposed a mechanism for the action of triosephosphate dehydrogenase involving glutathione as a prosthetic group of the enzyme.

The efficiency with which phosphate is esterified in the glycolytic process is quite high. Beginning with one mole of glucose-1-phosphate (G-1-P) derived from the phosphorolysis of starch, the formation of two moles of pyruvate yields three moles of energy-rich phosphate. If glucose is phosphorylated via hexokinase and then metabolized to two moles of pyruvate, a net synthesis of two moles of energy-rich phosphate is realized. The $-_{\Delta}F$ values for the two overall reactions are ca. 50,000 and 35,000 calories, and the resulting energy stored in the phosphate bonds is ca. 36,000 and 24,000 calories respectively. The $-_{\Delta}F$ involved in the transformation of glucose to pyruvate represents, however, only a small part of the 686,000 calories per mole potentially available when glucose is completely oxidized to CO_2 and water.

The observation by Kalckar (17) that the oxidation of various respiratory intermediates is coupled to phosphorylation in

kidney preparations and the subsequent elucidation of the mechanisms involved has made possible a greater understanding of many biochemical and physiological phenomena. It has now been clearly established that the oxidative metabolism of pyruvate to CO_2 and water represents the major source of energy-rich phosphate in the cell. Indeed, for each mole of pyruvate (1/2 mole of glucose) oxidized via the Krebs cycle, ca. fifteen moles of inorganic phosphate are esterified in the form of ATP.

Recently the studies of Millerd, et al. (18) and of Laties (19) in this laboratory have shown that in plants the mechanisms for the production of ATP by the oxidation of Krebs cycle intermediates are similar to the mechanisms in mammalian tissues. The systems capable of carrying out this complex series of reactions are localized in the mitochondria which, with suitable care, may be isolated intact.

As the individual enzymes of the glycolytic pathway were studied in detail by such investigators as the Coris, Warburg and Christian, Parnas, Ostern, Needham, and others, the role of phosphate in carbohydrate metabolism became clear. Figure 1 indicates the sequence of reactions and the enzymes involved in glycolysis as they are known to operate in yeast and in animal tissue.

The earliest demonstration of the existence of phosphorylating enzymes in higher plants was made by Bodnar (20) who found that inorganic phosphate disappeared during incubation of an aqueous

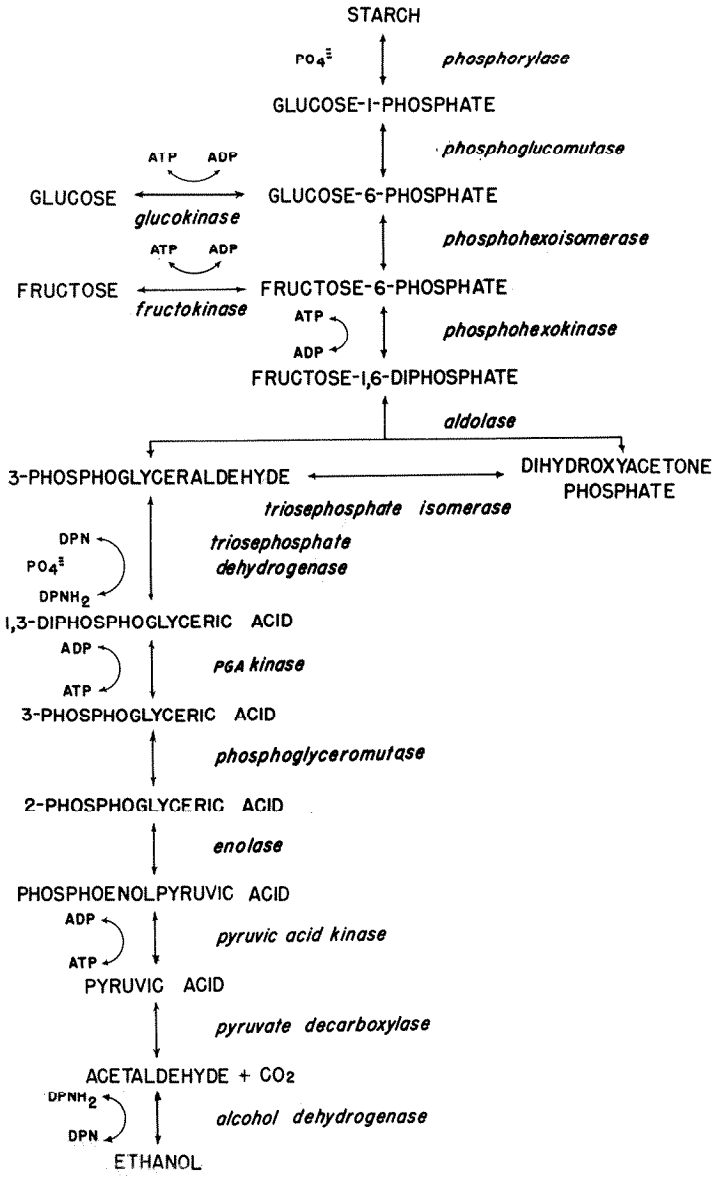


Figure 1.

Figure 1. Glycolytic pathway of carbohydrate metabolism.

suspension of pea seed meal with phosphate. Following Bodnar's lead, Tanko (21) demonstrated that 90°/o of the phosphate esterified may be recovered in the form of fructose-1, 6-diphosphate (FDP). The hexose monophosphates were also identified in the reaction mixture. Tanko was further able to demonstrate in this system a conversion of yeast Robison ester (6-phosphates of glucose, fructose, and mannose) to FDP, the first demonstration of a glycolytic enzyme in higher plants. This early work indicated then that the glycolytic pathway of plants might well resemble that in yeast and muscle. This was further indicated by the finding of James and James (22) that pyruvic acid accumulates in the fermentation of starch bycell-free extracts of barley. An increasing amount of interest then turned toward obtaining an understanding of the metabolic processes involved.

Hanes (23), using cell-free extracts of pea seed flour, was able to isolate and identify glucose-1-phosphate as the first product in the phosphorolysis of starch. Hanes further demonstrated the presence in the extract of phosphorylase, the enzyme mediating the reaction

$$\text{starch} + \text{phosphate} \longleftrightarrow \text{G-1-P}.$$

He also noted the conversion of the non-reducing G-1-P to the reducing glucose-6-phosphate (G-6-P) and fructose-6-phosphate (F-6-P), and thus presented evidence for the occurrence of phosphoglucomutase in plants.

Since Hanes' initial contribution on phosphorylase, a great deal of effort has been devoted to understanding the metabolism of the polysaccharides. Rather extensive reviews by Hassid (24), Stumpf(25), and Peat (26), outline the developments in this field.

With the mounting interest of biochemists in matters pertaining to plants, more detailed studies were reported on the individual enzymes in the glycolytic sequence. Phosphoglucomutase has been isolated and characterized from Jack bean seeds by Cardini (27). The dialyzed enzyme requires glucose-1, 6-diphosphate, Mg^{++}, and cysteine for activity as has been reported also for the yeast and muscle enzyme. Phosphohexoisomerase has been demonstrated in pea seed extracts by Somers and Cosby (28) who found a conversion of 67% of added F-6-P to G-6-P in 80 minutes. This enzyme has received little detailed attention from biochemists although its mechanism presents a unique problem.

Aldolase from pea seeds has been purified 90-fold and characterized by Stumpf (29), and its ubiquitous distribution in the plant kingdom has been demonstrated (30). No cofactors are required by the enzyme and its properties are quite similar to the enzyme of yeast and muscle. Although triosephosphate isomerase has not been isolated from plant tissue, its presence has been unambiguously demonstrated by Stumpf (31) who showed that one equivalent of FDP yields two equivalents of phosphoglyceric acid. This would only be possible if the dihydroxyacetone phosphate produced through the action of aldolase on FDP is converted to 3-phosphoglyceraldehyde.

Stumpf (31) showed that the DPN-linked triosephosphate dehydrogenase is to be found in extracts of acetone powder of pea seeds and that it requires either phosphate or arsenate for activity. However, when this study was extended to pea seedlings and green leaves (32) the enzyme could not be found. Axelrod and Bandurski (33) then showed that when TPN rather than DPN is used with spinach leaf preparations the reaction proceeded rapidly and did not require phosphate or arsenate for activity. More detailed studies of this enzyme have subsequently been made by Gibbs (34) and by Arnon (35). A very interesting problem in connection with this enzyme still remains unsolved. The requirement of the enzyme for arsenate or phosphate found by Stumpf in pea seeds was not found by Axelrod and Bandurski in spinach leaves. In the light of the results of Axelrod and Bandurski there is possibly an alternate mechanism to that proposed by Racker and Krimsky (16) involving neither glutathione as a coenzyme nor phosphorolysis or arsenolysis for activity. An understanding of the mode of action of the plant enzyme might be an important contribution to the whole matter.

Phosphoglyceric mutase and enolase were indirectly demonstrated to be present throughout the life cycle of the pea plant by Tewfik and Stumpf (32) who found that 3-phosphoglyceric acid is converted to pyruvic acid and that this conversion is inhibited by fluoride.

Phosphoenolpyruvic kinase has been studied by Stumpf (31)

who incubated phosphoenolpyruvic acid and AMP with extracts from pea seedlings and showed the formation of ATP in the reaction mixture.

The first demonstration of the occurrence of ATP in higher plants was by Albaum and Ogur (36) who isolated from oat seedlings an energy-rich nucleotide of low purity and low yield. The presence of large amounts of starch, meta-phosphate, phytic acid, and other contaminants in the oats prompted a search for a plant material more suitable for study. Mung bean seedlings proved to be an ideal tissue, since they have little polysaccharide and no phytic acid. Albaum et al. (37) were able to isolate an ATP-like substance in a fraction normally discarded in the Umbreit and LePage (38) technique for barium fractionation of phosphate esters. Only after treatment with mercury could identity of the plant material with ATP isolated from yeast and muscle tissue be established. The significance for this difference in preparative technique has not been investigated. Reference to Albaum's review (39) should be made for the details of the procedures.

Prior to the work of Albaum and co-workers, Bonner (40) had shown that FDP is formed upon the addition of glucose to cell-free extracts of Avena seedlings. The addition of ATP to this system increased the amount of FDP. Similar results were obtained with cell-free extracts of spinach leaves (41). It was assumed that endogenous ATP was involved in the initial synthesis of HDP without

added ATP, and further, that hexokinase was present to mediate the initial phosphorylation of the glucose. That this is indeed the case has now been definitely established.

The important finding of Millerd, et al. (18) that mitochondria isolated from mung bean seedlings are capable of carrying out oxidative phosphorylation has been instrumental in clarifying our knowledge of the respiratory processes operative in higher plants. It was possible to show by the use of P^{32} labeled phosphate and chromatographic techniques to be discussed in the next section of this thesis, that not only is there a net synthesis of ATP from AMP, but also that radioactive hexose phosphate is formed. This work provided the first direct evidence of the presence of hexokinase in higher plant tissue.

Although the literature on hexokinase in yeast and animal tissue is extensive (see reviews by Colowick (42) and by Slein, et al. (43)), investigations of this enzyme in higher plant materials are few. Griffiths (44), during a study of the mechanism of alloxan inhibition of a wide variety of sulfhydryl enzymes, notes the apparent presence of hexokinase in potato tuber. The experimental technique used, is not, however, definitive for the measurement of hexokinase activity in the presence of phosphorylase. Kotelnikova (45) also reported the occurrence of the enzyme in potato tuber, but her assay method measures the change in 7 minute labile phosphate, and is not sufficiently specific to establish her claim. Recently

Kotelnikova (46) repeated her original work employing the Nelson (47) method of assay for hexokinase and showed that the activity resides in an insoluble fraction. However, she did not identify this fraction as the mitochondrial fraction.

In order to understand more completely the carbohydrate metabolism of higher plants, investigations were undertaken in this laboratory to isolate and characterize the remaining uncharacterized enzymes of the glycolytic sequence. In the course of this work four enzymes involved in transphosphorylation were studied: phosphofructokinase, hexokinase, adenylate kinase (48), and phosphoglyceric kinase (49).

It has been clearly demonstrated by Axelrod and Bandurski (50) that a pathway in addition to that of the Embden-Meyerhof sequence for the metabolism of sugars is operative in higher plants. This oxidative sequence is shown in Figure 2. The importance of G-6-P, which can either be broken down to pyruvate via the glycolytic sequence yielding three moles of ATP per mole of G-6-P, or metabolized through the oxidative pathway resulting in the formation of pentose phosphate, heptose phosphate, and eventually, pyruvate, makes it of interest to study more closely the relative importance of these two modes of biosynthesis of pyruvate.

Yet another pathway for carbohydrates is indicated by the work of Tewfik and Stumpf (51) who reported the presence in plants of an oxidative system for the metabolism of HDP which does

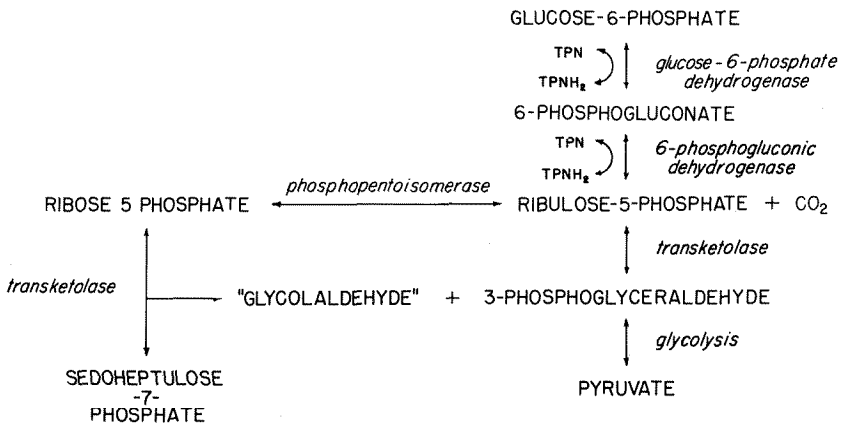


Figure 2.

Figure 2. Oxidative pathway of hexose phosphate metabolism.

not involve ATP, DPN, or TPN, but which seems to operate through flavine adeneine dinucleotide. An important problem confronting the plant biochemist is to determine the relative importance and interplay of the three metabolic systems.

Mention should be made of three recent comprehensive reviews of the literature: "Glycolytic Enzymes in Higher Plants" by Stumpf (25), "Metabolism of Phosphorylated Compounds in Plants" by Albaum (39), and "Phosphorus Assimilation in Plants" by Stumpf (52).

The investigations described in this thesis have demonstrated and characterized two enzymes intimately concerned with transphosphorylations involving ATP — phosphofructokinase and hexokinase. Evidence is also presented that the classical Embden-Meyerhof glycolytic pathway is operative in cell-free extracts of pea seeds.

PHOSPHATE UPTAKE BY PEA MEAL EXTRACTS

With the development by Bandurski and Axelrod (53) of a chromatographic method for the identification of the phosphate esters involved in glycolysis, it was possible to reexamine in greater detail the experiments of Tanko (21). By utilizing radioactive phosphate as a tracer the course of phosphate esterification could readily be followed, and this was done using a cell-free extract of pea seeds.

Materials and Methods

Preparation of the enzyme:

Dried pea seeds (Pisum sativum, var. Alaska) were ground in a Wiley Mill to pass a no. 40 mesh screen. The flour was extracted overnight at 2° with twice its weight of ice cold distilled water. The mixture was centrifuged for 30 minutes at 20,000 x g, the clear amber colored supernatant decanted, adjusted to pH 6.5, and used as the enzyme.

1.0 ml. of enzyme was incubated for two hours at 37° with 1.0 ml. of 0.16 M potassium phosphate buffer, pH 6.5, and 0.18 ml. of the radioactive phosphate solution described below. The reaction was then stopped with 10%/o TCA and fractionation of the barium salts of the phosphate esters carried out by the procedure outlined below.

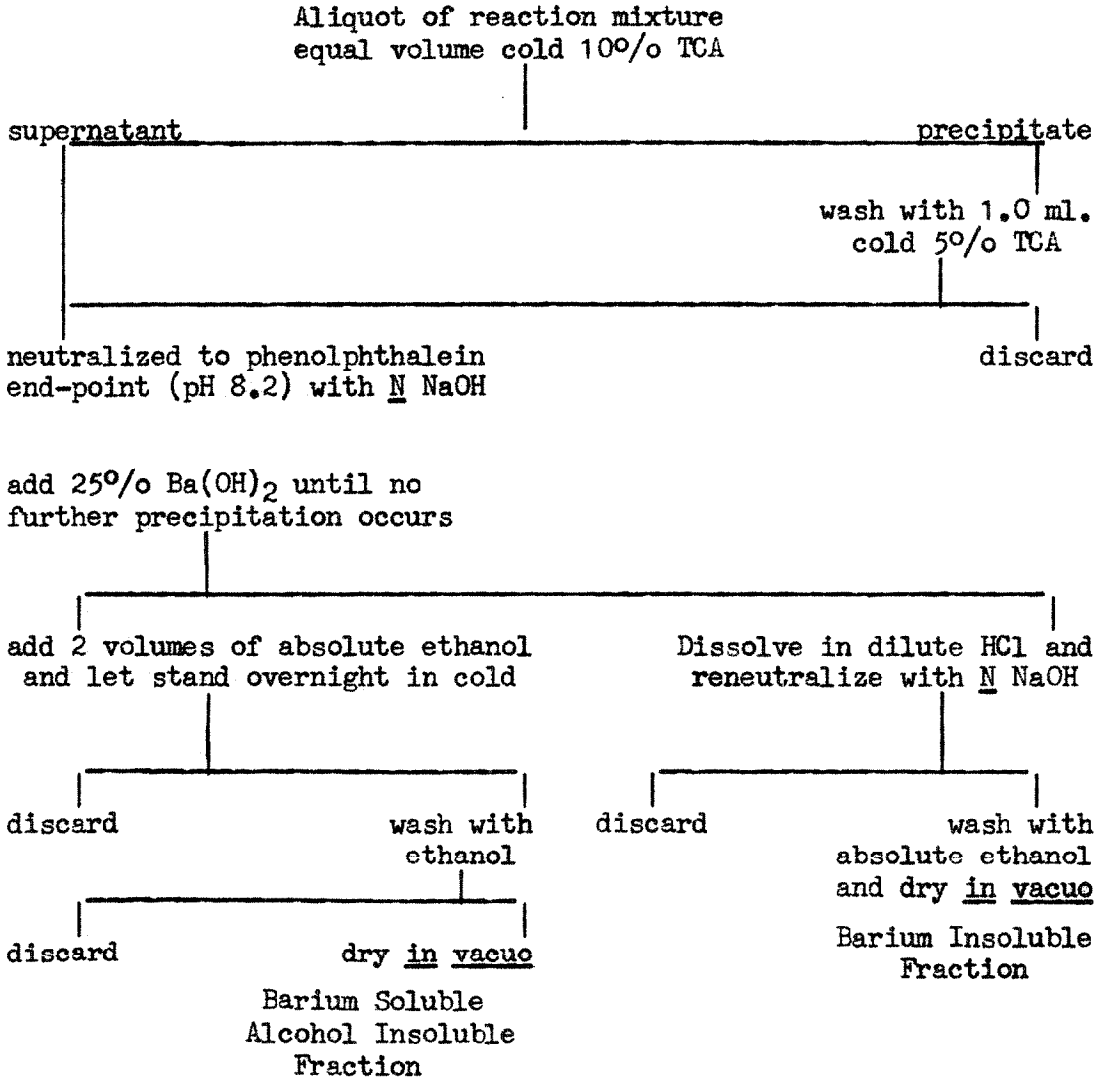
Radioactive phosphate:

P^{32} labeled radio-phosphate was obtained from the Oak Ridge

National Laboratory as phosphoric acid in a solution of dilute HCl. The stock solution had an activity of 4 millicuries per ml.

Isolation of the barium salts of phosphate esters:

The technique for fractionation of the barium salts is essentially that described by Umbreit et al. (38), and is outlined in the flow diagram below:



Chromatographic Technique:

The method used was exactly as described by Bandurski and Axelrod (51). This technique employs two dimensional ascending paper chromatography, developing first in an acid solvent (methanol 80, formic acid 15, water 5) followed by development in an alkaline solvent (methanol 60, ammonium hydroxide 10, water 30). The paper is then dried, sprayed with the Hanes-Isherwood (54) perchloric acid-ammonium molybdate reagent, and subjected to short ultraviolet radiation to detect by means of the characteristic blue color the organic phosphates as well as inorganic phosphate. In all cases authentic samples of glycolytic intermediates were co-chromatographed with the reaction products and identification made by superposition of the radio-autograph on the developed chromatograms. The barium salts were first deionized with Dowex no. 50 and were chromatographed as the free acids. The final activity of the applied spots was approximately 750,000 counts per minute per spot.

Results

Tracings of the radio-autographs are shown in Figures 3 and 4. The following radio-labeled compounds were identified in the reaction mixture: glucose-1-phosphate, fructose-1, 6-diphosphate, 3-phosphoglyceric acid, 2-phosphoglyceric acid, phosphoenolpyruvate, and ATP. It is not possible in this chromatographic procedure to resolve glucose-6-phosphate and fructose-6-phosphate. A spot was, however, observed on radio-autographswhich corresponded in size and

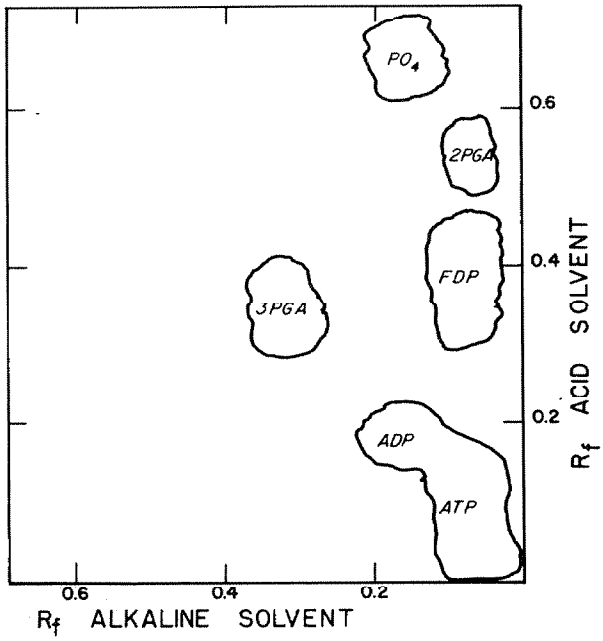


Figure 3.

Figure 3. Tracing of radio-autograph of two dimensional chromatogram. Barium Insoluble Fraction.

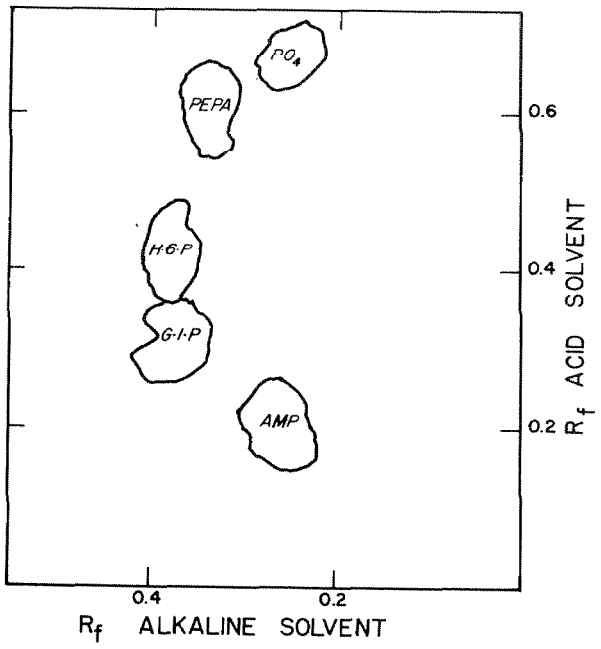


Figure 4.

Figure 4. Tracing of radio-autograph of two dimensional chromatogram. Barium Soluble, Alcohol Insoluble Fraction.

shape to that of a mixture of the authentic hexose phosphates.

From the fact that the compounds that became labeled during the course of the reaction were identical with the compounds known to be involved in glycolysis in yeast and muscle tissue, it seems likely that the Embden-Meyerhof pathway is operative in pea seed extracts. That ATP became radio-active is strong evidence not only for the presence of this important compound, but also for its participation in the metabolic reactions of pea seeds. Since it appeared that phosphofructokinase and hexokinase were active in this preparation, a study of these two enzymes was undertaken.

PHOSPHOFRUCTOKINASE IN HIGHER PLANTS*

Phosphofructokinase is the enzyme which catalyzes the reaction

fructose-6-phosphate + ATP <——> fructose-1, 6-diphosphate + ADP.

Its presence in tissues of higher plants had been indicated in several ways, although the enzyme had never been isolated and characterized.

Both Tanko (21) and Hanes (23) were able to isolate and identify FDP from reaction mixtures containing phosphate and soluble enzymes from pea seed meal. Bonner (40) was able to demonstrate the increased accumulation of FDP on the addition of ATP and glucose to Avena coleoptile brei; the amount accumulated was greater than the sum of that effected by glucose or ATP alone. A similar situation was observed with a preparation from spinach leaves (41).

Another line of evidence is from the work of Stumpf (29) who has isolated and characterized aldolase, the enzyme which cleaves one mole of FDP to form two moles of triose phosphate. It would seem likely, therefore, that the enzymes mediating the synthesis of FDP are present in higher plants.

The chromatographic evidence presented in the previous section of this thesis indicates that the Embden-Meyerhof glycolytic pathway is operative in pea seed extracts. If this is true, then

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* The studies reported in this section have now appeared in published form: Axelrod, B., Saltman, P., Bandurski, R.S., Baker, R.S., J. Biol. Chem., 197, 89 (1952).

phosphofructokinase, one of the enzymes directly implicated in this scheme, should be present.

The experiments presented in this section will show that phosphofructokinase is, indeed, present in all of the several plant tissues investigated. The enzyme from pea seed meal has been partially purified, and characterized, with respect to several properties.

Experimental

Assay for phosphohexokinase activity:

Enzyme activity was measured by determining the amount of FDP formed from F-6-P and ATP under the conditions described below.

To a solution containing 0.25 ml. of 0.1 M F-6-P, 0.25 ml. of 0.04 M ATP, 0.20 ml. of 0.15 M THAM-HCl buffer, pH 8.5, and 0.05 ml. of 0.2 M $MgCl_2$, was added 0.25 ml. of enzyme solution. The ATP and F-6-P solutions were prepared from commercial products, freed from Ba^{++} with K_2SO_4, and adjusted to pH 8.5. The F-6-P employed throughout these experiments was chromatographically homogeneous except for the presence of a trace of inorganic phosphate. The ATP was, however, only 70% pure; the necessary correction was applied in computing concentrations.

After incubation for 30 minutes at 37° the reaction mixture was treated with 1.0 ml. of 5% trichloroacetic acid. A time zero control was obtained by introducing the trichloroacetic

acid prior to the addition of enzyme. After removal of the protein by centrifugation, 1.0 ml. of the supernatant was neutralized with 0.5 ml. of 0.3 N NaOH and then treated with 0.5 ml. 0.15 M THAM-HCl buffer, pH 8.5, and 0.5 ml. of 0.25 M NaCN, pH 8.5 (freshly prepared). This mixture was incubated with 0.1 ml. of aldolase at 37° for 10 minutes, following which two aliquots of 0.5 ml. were withdrawn. In one aliquot the amount of inorganic phosphate was determined directly by the method of Allen (55); in the other, inorganic phosphate was determined after 15 minutes hydrolysis with 0.5 ml. of 2 N NaOH at room temperature, followed by neutralization with 1.0 ml. of 1.0 N HCl. 2 moles of alkali-labile phosphate are liberated per mole of FDP present. This determination is essentially similar to that described by Herbert et al. (56).

Aldolase obtained from rabbit muscle at the first precipitation stage by the method of Taylor et al. (57) was suitable for our purpose. An aqueous solution of this precipitate (containing 0.51 mg. of protein nitrogen per ml.) was stored in 0.25 ml. portions at -20° until needed. Aldolase handled in this way was still satisfactorily active after 9 months of storage. Before use in the assay, it was diluted 1:10 with water.

The unit of phosphohexokinase activity was arbitrarily defined as that quantity of enzyme which produces 10γ of alkali-labile phosphate per reaction tube in 30 minutes. Figure 5 shows the relationship between enzyme units and FDP formed with increasing amounts of a partially purified enzyme preparation described below.

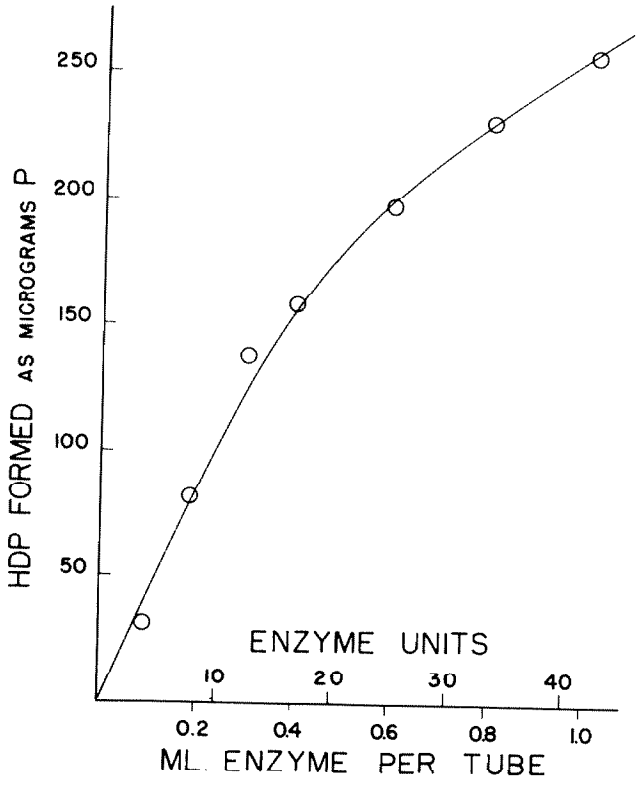

Figure 5.

Figure 5. Standard assay curve for phosphohexokinase. The substrate contained 0.25 ml. of 0.1 M F-6-P, 0.25 ml. of 0.04 M ATP, 0.20 ml. of 0.15 M THAM-HCl buffer, pH 8.5, and 0.05 ml. of 0.2 M $MgCl_2$. The enzyme was contained in 0.25 ml. of H_2O. Total reaction volume, 1.0 ml. Reaction time, 30 minutes; temperature 37°.

Preparation of enzyme from pea meal:

Phosphohexokinase was prepared as follows: Pea seed meal (40 mesh), Alaska variety, was suspended in 2 to 4 times its weight of cold (2°) water for 30 minutes and agitated occasionally. This mixture was centrifuged (16,000 x g) for 20 minutes. The supernatant was poured off and adjusted to pH 7.5 with 2 N NaOH and made $28^{\circ}/o$ saturated with respect to ammonium sulfate by the addition of the proper volume of saturated ammonium sulfate solution, pH 7.5. After standing 30 minutes, the mixture was centrifuged 20 minutes at 16,000 x g and the supernatant made $38^{\circ}/o$ saturated with the required volume of saturated ammonium sulfate solution. The precipitate collected after 30 minutes standing and 20 minutes centrifugation at 16,000 x g was dissolved in a volume of water equal to approximately one-third of the original volume of extract and dialyzed with gentle agitation against three changes of 40 volumes of freshly prepared 10^{-4} M cysteine. All preparative procedures were carried out at 2°. The above fractionation resulted in a 6-fold increase in specific activity, as based on units of activity per biuret density value determined as follows: X ml. of protein and (2 - X) ml. of water are added to 8.0 ml. of biuret reagent prepared according to Gornall et al. (58). After 20 minutes the optical density is determined in an Evelyn colorimeter with a 540 mμ filter.

pH Optimum:

The plot of activity of phosphohexokinase as a function of pH, Figure 6, shows a sharp maximum at about pH 6 and another maximum, approximately 15°/o lower, at pH 9. Acetate buffer was used in the pH range 4 to 6, while THAM was used above pH 6.0. This double peak has been repeatedly verified and is probably not caused by the change in buffers, since identical activities were obtained with acetate and with THAM buffers at pH 6.0 and 6.5.

The more alkaline region was chosen for the standard assay procedure because the relative broadness of the pH optimum made precise pH control unnecessary. The more alkaline pH also tended to suppress interference by any contaminating phosphatase, since the optimum of this enzyme is at pH 6.0 or lower. The unusually wide range over which phosphohexokinase shows appreciable activity, pH 5 to 11, is noteworthy.

Temperature optimum and energy of activation:

The temperature optimum of the phosphohexokinase reaction was 38° as determined by 30 minutes incubation of the enzyme in the standard reaction mixture at a series of different temperatures (Figure 7). A calculation of the energy of activation based on the low temperature portion of the plot of log activity *versus* reciprocal absolute temperature yields a value of 3700 calories per mole (Figure 8).

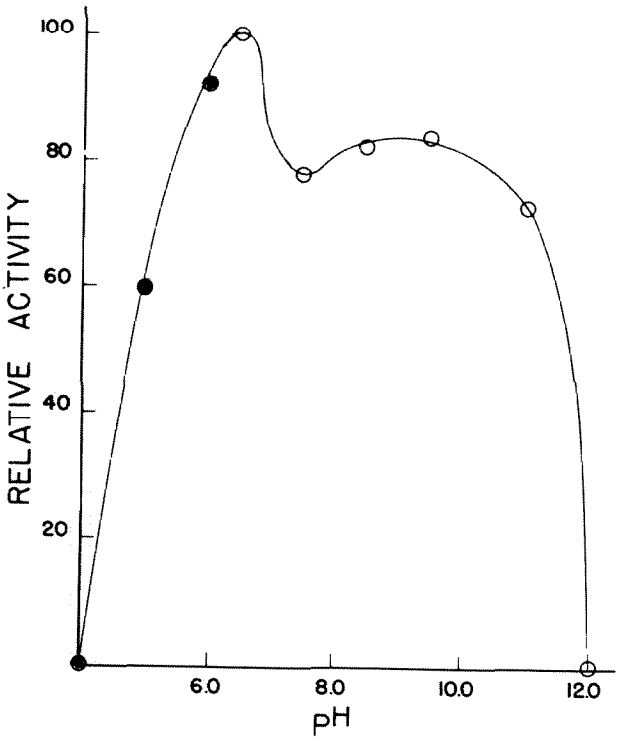

Figure 6.

Figure 6. Effect of pH of reaction mixture on phosphohexokinase activity. Conditions as in the standard assay except that the composition of the buffer was varied: ● ,0.15 M acetate buffer; ○, 0.15M THAM-HCl buffer.

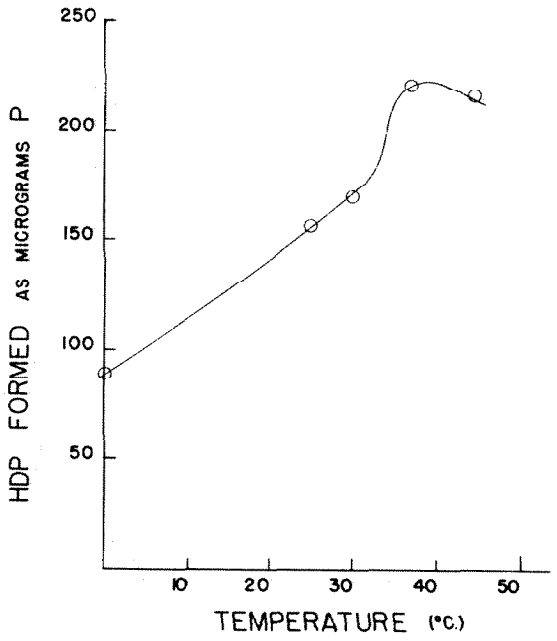

Figure 7.

Figure 7. Effect of temperature on phosphohexokinase activity. Conditions as in the standard assay except that the temperature was varied.

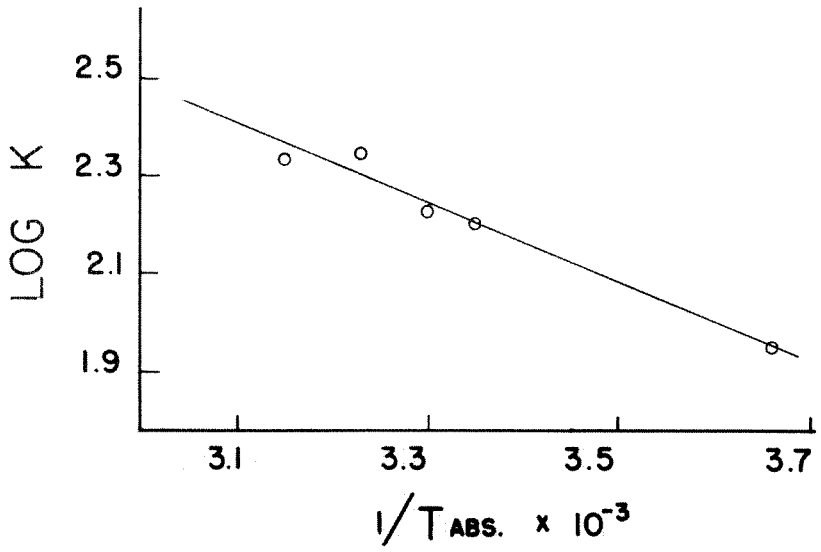

Figure 8.

Figure 8. Graphical determination of energy of activation of FDP synthesis by phosphohexokinase. Slope, -815 deg.$^{-1}$ Energy of activation, 3700 calories per mole.

Temperature stability:

Portions of a purified preparation of phosphohexokinase were allowed to stand at three different temperatures for varying lengths of time and the activity was determined at 37° by the standard assay procedure. The enzyme was stable at -5° for as long as 2 weeks, but did lose over 50°/o of its activity in 20 hours at 2°, and more than 90°/o in 6 hours at 25°.

Figure 9 shows the effect of exposing the enzyme for 2 minutes to various temperatures above 25°, followed by assay at 37°.

Metal requirements:

The presence of Mg^{++} was essential to phosphohexokinase activity. Figure 10 illustrates the influence of Mg^{++} concentrations on the rate of the reaction. The optimum Mg^{++} concentration was about 0.01 M, and above this concentration inhibition occurred. $CuSO_4$ and $ZnSO_4$ at the same concentration were completely ineffective in replacing Mg^{++}, but $MnCl_2$ was about half as effective at 0.01 M. As seen in Table I, Cu^{++} was actually a potent inhibitor. Since $MgSO_4$ and $MgCl_2$ were interchangeable, the effects were due only to the cation.

Inhibitors:

Of a number of substances tested for their inhibitory effect on phosphohexokinase the following were ineffective: NaF (0.01 M), KCN (pH 8.5, 0.03 M), cysteine (pH 8.5, 0.025 M),

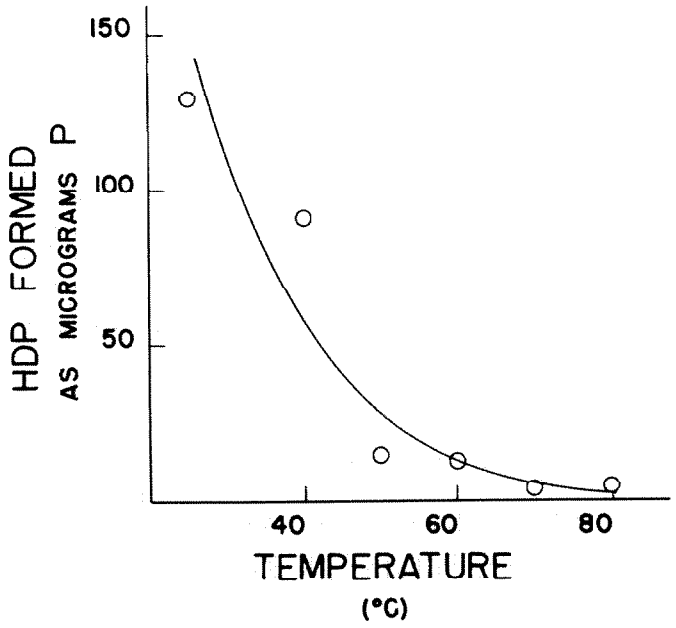

Figure 9.

Figure 9. Stability of phosphohexokinase as a function of temperature. The enzyme was held for 2 minutes at the temperature indicated and then assayed under standard assay conditions.

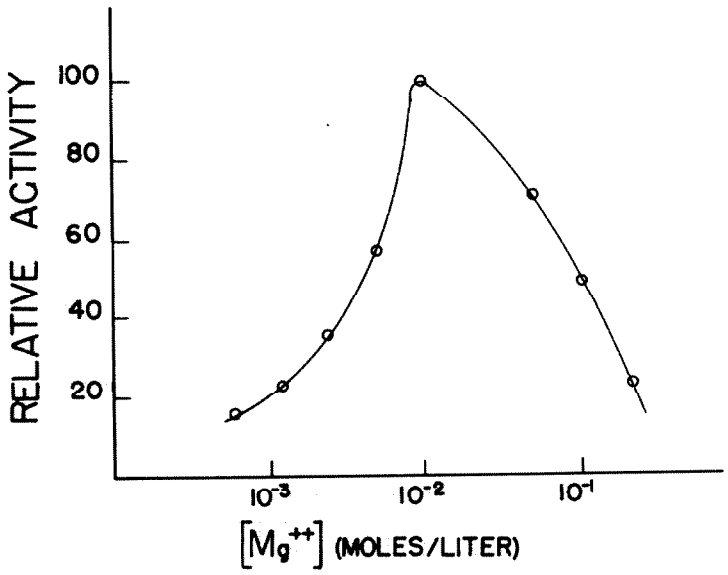

Figure 10

Figure 10. Effect of Mg^{++} concentration on phosphohexokinase activity. Conditions as in the standard assay except for Mg^{++} concentration.

TABLE I

INHIBITORS OF PHOSPHOHEXOKINASE

Substance	Concentration	Inhibition
	M	per cent
$CuSO_4$	0.01	100
Iodoacetate	0.005	51
$HgCl_2$	0.005	96
$K_4P_2O_7$	0.005	73

Standard assay conditions; 13 units of enzyme.

fructose (0.04 M), glucose (0.04 M), sucrose (0.04 M), NaN_3 (0.005 M), $Be(NO_3)_2$ (0.005 M). Those substances effective as inhibitors are listed in Table I. In all cases in which inhibition occurred, it was demonstrated that the effect was not caused by interference with the aldolase reaction in the assay.

Effect of substrate concentration:

Affinities of phosphohexokinase for its substrates as expressed by the Michaelis-Menten constants, K_s, were determined as follows: Reaction mixtures of 2 ml. containing ATP (0.0059 M), $MgCl_2$ (0.0059 M), enzyme, and concentrations of F-6-P varying from 0.005 to 0.05 M were incubated for 20 minutes at 37°. The resulting data were analyzed by the Lineweaver-Burk method (59). The K_s for F-6-P, as determined graphically, is 7.1×10^{-3} M (Figure 11).

The corresponding constant for ATP was determined in a like manner, except that the F-6-P concentration was fixed at 0.025 M and the ATP concentration varied from 0.001 to 0.016 M. The K_s, as calculated from Figure 12, is 2.4×10^{-3} M.

In the determination of the two constants, two assumptions have been made, neither of which is entirely valid: (a) that the substrates are chemically pure, and (b) that the reaction velocities as determined are proportional to initial rates. The values which have been obtained are therefore only indicative of the order of magnitude of the Michaelis-Menten constants.

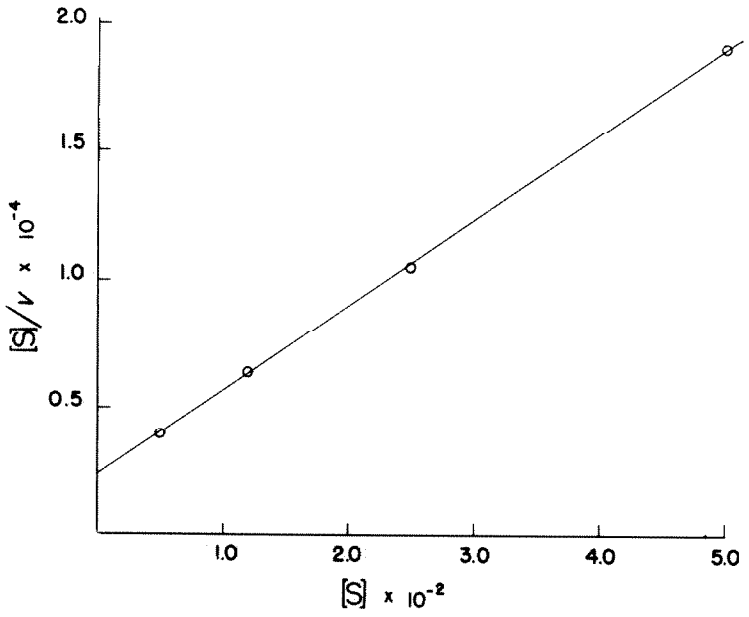

Figure 11.

Figure 11. Graphical determination of Michaelis-Menten constant for F-6-P in phosphohexokinase reaction. Conditions of activity determinations as in Figure 1 except that the concentration of F-6-P was varied and the reaction time was 20 minutes.

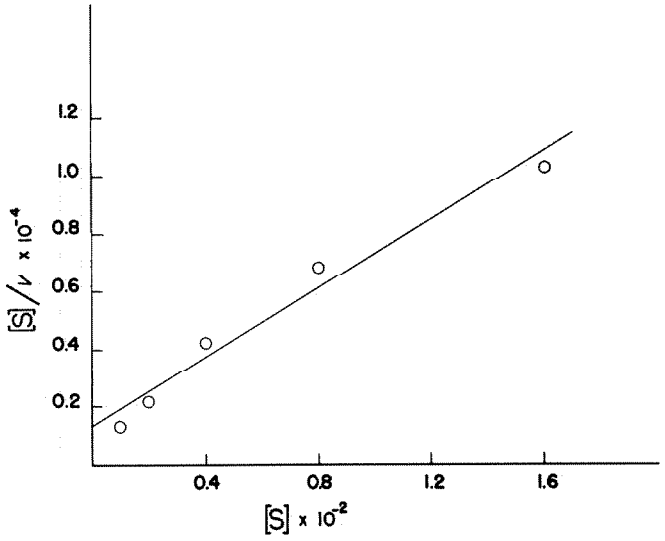

Figure 12.

Figure 12. Graphical determination of Michaelis-Menten constant for ATP in the phosphohexokinase reaction. Conditions of activity determinations as in Figure 1 except that the concentration of ATP was varied and the reaction time was 20 minutes.

Substrate specificity:

Phosphocreatine, phosphoenolpyruvate, cocarboxylase, pyrophosphate, and p-nitrophenylphosphate were all unable to replace ATP. Adenosinediphosphate proved to be half as effective as ATP under the standard assay conditions. The apparent activity of ADP is due to the presence of myokinase in the enzyme preparation (48).

Glucose-6-phosphate is able to function as well as F-6-P, but, owing to the possible contamination of the enzyme with phosphohexose isomerase, no conclusion is warranted in regard to the ability of the enzyme to utilize glucose-6-phosphate directly. Glucose-1-phosphate, glucose, and fructose are unable to replace F-6-P.

Occurrence of phosphohexokinase in higher plants:

Some information on the occurrence of phosphohexokinase in higher plant materials in addition to pea meal is summarized in Table II. Unless otherwise indicated, the enzyme preparations were obtained by grinding the plant material to 20 to 40 mesh size in a micro Wiley mill, extracting the meal with 2 times its weight of water for 1 hour, and centrifuging. The supernatant was finally dialyzed for 4 hours against 10^{-4} M cysteine before assaying by the usual procedure. All preparative operations were performed at 2°. The orange flavedo was extracted with its own weight of 0.2 M sodium acetate. After 1 hour the supernatant obtained by centrif-

TABLE II

PHOSPHOHEXOKINASE IN SOME PLANT TISSUES

Plant	Tissue used	Units per gm. fresh weight	Units per biuret density
Avena sativa (oats, var. Victory)	Seed	13.3	6.0
Citrus (orange, var. navel)	Flavedo	0.0	0.0
Helianthus annuus (sunflower)	Fruit	6.7	2.4
Lycopersicum esculentum (tomato)	Seed	3.3	0.8
Pinus ponderosa (ponderosa pine)	Pollen	187.3	44.3
Pisum sativum (pea, var. Alaska)	Leaf	20.1	52.0
	Seed	260.0	74.5
Raphanus sativus (radish)	Seed	50.0	8.1
Soya hispida (soy bean)	Seed	73.3	6.4
Vicia faba (Windsor broad bean)	Seed	96.7	18.1
Zea mays (corn)	Seed	53.3	41.7

ugation was dialyzed 11 hours against 10^{-4} M cysteine. 3 gm. of pine pollen were made into a paste by grinding with 20 ml. of water and a few drops of toluene. The paste was then dialyzed overnight against 10^{-4} M cysteine and used directly. In all cases the enzyme preparations were adjusted to pH 8.5 prior to assay.

Discussion

Comparison of phosphohexokinase from pea seed and that isolated from yeast (60) and muscle (61) must be limited to the few available points of common information. In all cases Mg^{++} was required as a cofactor. The heat lability of the pea enzyme was far greater than that of the enzyme of rabbit muscle studied by Racker (61). The muscle enzyme lost no activity after 4 minutes at 56°, while 2 minutes at 40° resulted in the loss of about 50% of the original activity for the pea enzyme.

The inhibition of phosphohexokinase by $CuSO_4$, iodoacetate, and $HgCl_2$ would indicate the presence in the enzyme of an essential sulfhydryl group. In this connection a rapid loss of activity took place if the enzyme was dialyzed against distilled water, owing presumably to oxidation of the sulfhydryl group. Dialysis was therefore carried out against 10^{-4} M cysteine. The inhibition by pyrophosphate is probably caused by a binding of Mg^{++}.

The assay for activity by direct measurement of FDP formed during the reaction has the advantage over previous

manometric and spectrophotometric techniques in greater specificity, and offers greater adaptability to studies of pH optimum, of inhibitors, of substrate specificity, and of temperature optimum.

HEXOKINASE IN HIGHER PLANTS*

Hexokinase is the enzyme which catalyzes the initial phosphorylation of free hexose by the following reaction:

hexose + ATP <—> hexose-6-phosphate + ADP.

The historical aspects of hexokinase have been discussed in some detail in the first section of this thesis. Although this enzyme has been studied extensively in muscle and yeast, little definitive work had been done with it in tissues of higher plants. With the finding of Millerd et al. (18) that mitochondria are capable of phosphorylating hexose during the course of oxidative phosphorylation, it became of interest to examine this enzyme in more detail.

It will be shown in this section that hexokinase occurs in the plant in a soluble form, as well as associated with the mitochondria. Several characteristics of the enzyme have been investigated and will be discussed.

Materials and Methods

Assay for Hexokinase activity:

Two methods were used for the measurement of hexokinase activity. The first involves precipitation and removal of the hexose phosphates formed from hexose and ATP, followed by determination of the residual free hexose. This is the modification

*The studies reported in this section have now appeared in published form: Saltman, P., J. Biol. Chem. 200, 145 (1953).

of the procedure of Nelson (47), described by Brown*, in which after 10 minutes incubation of the reaction mixture the reaction is stopped by the addition of $Ba(OH)_2$ and $ZnSO_4$. Residual glucose is measured by the method of Somogyi (62). A control without ATP is also carried out, and the activity is expressed on the basis of the net difference in free glucose over the control.

Because an active ATPase is associated with the mitochondrial hexokinase, KF must be added to suppress its activity. The reaction mixture of 1.5 ml. contains ATP (0.01 M), glucose (0.0033 M), $MgCl_2$ (0.01 M), Tris buffer (0.06 M), pH 8.0, KF (0.01 M), and enzyme.

A second method for assay is the manometric technique of Colowick and Kalckar (63). The reaction is carried out in 0.02 M bicarbonate buffer at pH 7.5. For each mole of phosphate transferred from ATP to the acceptor, 1 acid equivalent is generated, and, hence 1 mole of CO_2 is liberated. This method was used for the investigation of acceptors other than glucose.

Preparation of enzyme:

Wheat germ was found to be a satisfactory source of both the insoluble and soluble form of hexokinase**.

The insoluble or mitochondrial fraction was prepared as

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* Personal communication.

** A wheat germ suitable for this was obtained from General Mills, Inc.

follows: 15 gm. of wheat germ and 60 ml. of cold 0.5 M mannitol were homogenized with sand in a cold mortar. The mixture was centrifuged at 500 x g for 10 minutes to remove starch and cell debris. The supernatant fluid was removed and recentrifuged at 18,000 x g for 15 minutes, and the supernatant fluid from this centrifugation was removed by suction. The residue was resuspended in 20 ml. of 0.5 M mannitol and recentrifuged at 18,000 x g for 10 minutes, the supernatant fluid was again removed, and the residue suspended in 5.0 ml. of cold distilled water. This suspension was used as the enzyme. It may be stored at 2° for 10 days with the loss of about 10°/o of the original activity.

The soluble fraction was obtained by extracting the wheat germ with 4 times its weight of cold water for 30 minutes at 2° with agitation. The mixture was centrifuged at 18,000 x g for 20 minutes, and the cloudy supernatant fluid was decanted, adjusted to pH 5.5 with N acetic acid, and immediately centrifuged. The precipitate was discarded and the clear supernatant fluid was brought to pH 7.0 with N KOH. A saturated solution of ammonium sulfate, pH 7.0, was added to bring the supernatant solution to 55°/o saturation with respect to ammonium sulfate and the resulting precipitate discarded. Additional saturated ammonium sulfate was added to bring the enzyme solution to 65°/o saturation, and the precipitate was collected by centrifugation, dissolved in distilled water, and dialyzed overnight against distilled water.

By this procedure a 5-fold increase in the specific activity was obtained.

Results

Distribution of Hexokinase in tissues of higher plants:

That hexokinase is widely distributed in higher plants is evident from the data of Table III. In Table III data are also provided on the partition of enzyme between insoluble and soluble forms, a partition which varies from tissue to tissue. In each case the material was prepared as described above for wheat germ unless otherwise indicated. The supernatant fluid and the washings of the particulate residue were combined and assayed as the soluble fraction; the particles were suspended in water and considered as the insoluble fraction. Leaf tissue was homogenized with sand and 0.1 M phosphate buffer in 0.4 M mannitol. Seeds were ground to no. 40 mesh in a Wiley mill before homogenization.

The proportion of soluble hexokinase appears to depend markedly on the method of preparation. Thus, when potato tubers were ground with sand in the cold according to the procedure found to be optimum for the preparation of intact plant mitochondria (18), all hexokinase activity was associated with the particles. When, by contrast, the tubers were homogenized in a Waring blendor for 3 minutes, over two-thirds of the enzyme appeared in the soluble form.

In the case of wheat germ, the proportion of soluble to particle-bound hexokinase was found to depend on the treatment

TABLE III

DISTRIBUTION OF HEXOKINASE IN SOLUBLE AND INSOLUBLE FRACTION OF SEVERAL TISSUES

Tissue	Insoluble units* per fraction	Per cent activity	Soluble units per fraction	Per cent activity
Wheat germ (commercial)	46.8	33	93.6	67
Wheat germ (dissected)	100.8	68	46.8	32
Potato	16.2	100	0.0	0
Potato (blendor)	11.7	30	27.9	70
Pea seed (var. Alaska)	6.1	30	13.8	70
Mung bean seed	16.4	28	42.0	72
Mung bean hypocotyl	14.0	19	61.0	81
Avena seed	3.5	19	14.7	81
Spinach leaf	1.3	28	3.4	72

* 1 unit = 1 mole of glucose phosphorylated per 10 minutes in 1.5 ml. of standard reaction mixture.

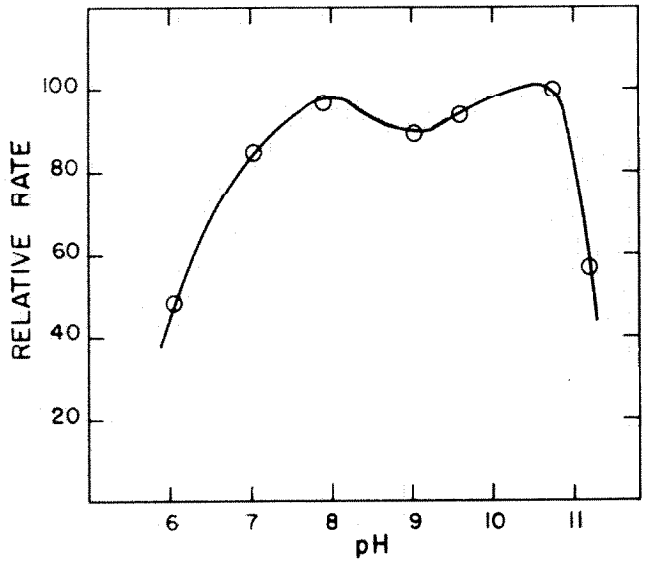

Figure 13.

Figure 13. Effect of pH of reaction mixture on hexokinase activity. The reaction mixture of 1.5 ml. contained ATP (0.01 M), glucose (0.003 M), $MgCl_2$ (0.01 M), KF (0.01 M), enzyme, and Tris buffer (0.06 M) at the various pH indicated. Reaction time 10 minutes; temperature, 37°.

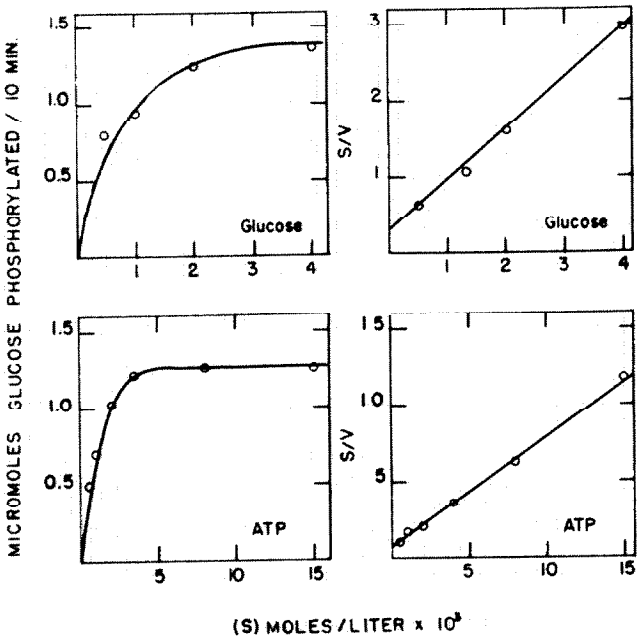

Figure 14.

Figure 14. Effect of glucose and ATP concentrations on hexokinase activity. Assay conditions as for Figure 1. Right-hand section shows Lineweaver-Burk plots from which the K_m values were calculated.

Metal requirements:

Magnesium ions are essential to activity of plant hexokinase. Figure 15 shows the relationship of reaction rate to the concentration of Mg^{++}. At a concentration of ATP of 0.01 M, the optimum concentration of Mg^{++} was found to be 0.01 M. This is in agreement with the observation of Hers (66) for fructokinase from liver in which a 1:1 relationship of Mg^{++} to ATP was found to be optimum. Since $MgSO_4$ and $MgCl_2$ are interchangeable, the activation is due only to the cation.

$MnCl_2$ was found to activate plant hexokinase 80% as effectively as $MgCl_2$ at 0.01 M. $CoCl_2$ was completely ineffective, while $CuSO_4$, $ZnCl_2$, and $HgCl_2$ were potent inhibitors as will be shown below. K^+, Na^+, and NH^+_4 showed very little effect either as activators or inhibitors.

Temperature optimum and energy of activation:

The temperature optimum for the insoluble hexokinase was found to be 37°, as determined by a 10 minute incubation of the enzyme in the standard reaction mixture at a series of different temperatures (Figure 16). A calculation of the energy of activation based on the low temperature portion of the plot of log activity versus reciprocal of absolute temperature yields a value of 11,670 calories per mole (Figure 17).

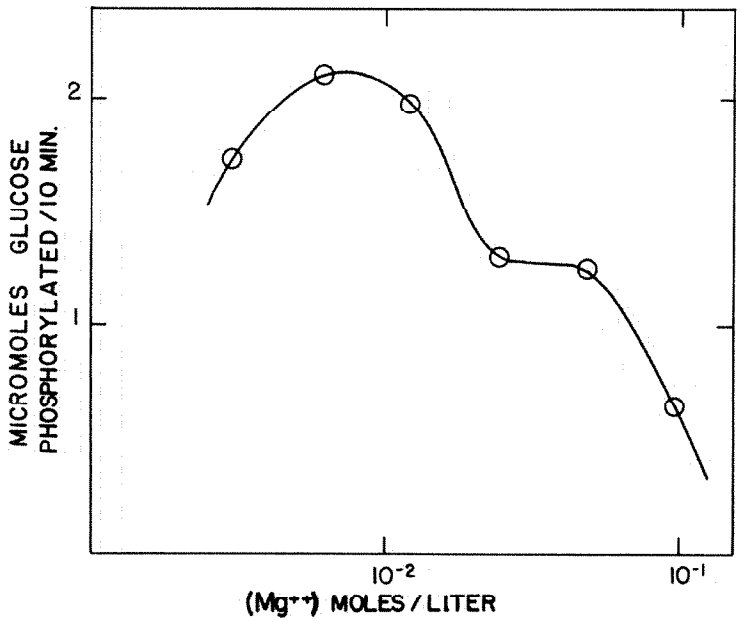

Figure 15.

Figure 15. Effect of Mg^{++} concentration on hexokinase activity. Conditions as in standard assay except for Mg^{++} concentrations.

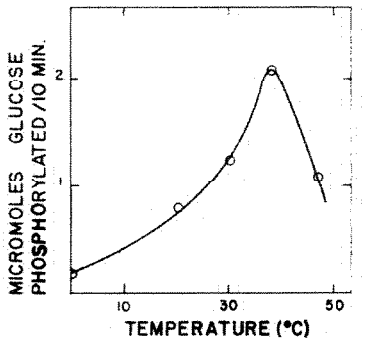

Figure 16.

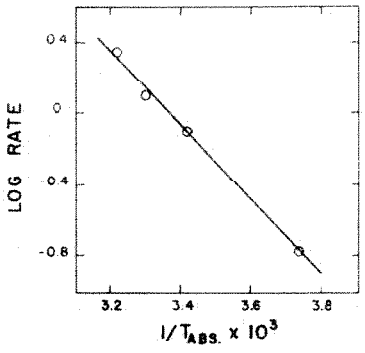

Figure 17.

Figure 16. Effect of temperature on hexokinase activity. Conditions as in standard assay except that temperature was varied.

Figure 17. Graphical determination of energy of activation of G-6-P synthesis by hexokinase. Energy of activation, 11,670 calories per mole.

Substrate specificity:

The insoluble plant hexokinase is capable of phosphorylating glucose, fructose, mannose, and glucosamine. The relative rates of phosphorylation with a concentration of acceptor of 0.0033 M in the standard reaction mixture are respectively 1.00, 0.62, 0.68, and 0.52.

The following substrates were completely ineffective as acceptors: galactose, ribose, arabinose, ribulose, adenosine, glyceraldehyde, dihydroxyacetone, mannitol, and glucose-1-phosphate. The ability of sucrose to act as an acceptor could not be ascertained because of the presence of invertase in the particulate preparation.

Inosine triphosphate is 35% as effective as ATP as a phosphate donor in the reaction. Adenosinediphosphate and adenosine monophosphate show a low activity which may be ascribed to the presence of adenylate kinase on the particles. Potassium pyrophosphate was ineffective.

Inhibitors:

A number of substances were tested for their inhibitory effect on hexokinase, and the following were found ineffective: KH_2AsO_4 (0.005 M), NaF (0.05 M), NaN_3 (0.005 M), sodium pyrophosphate (0.005 M), iodoacetic acid (0.005 M), iodoacetamide (0.001 M), and maleic hydrazide (0.005 M). The plant growth substances indoleacetic acid (0.005 M) and 2, 4-dichlorophenoxyacetic acid (0.005 M) were also without influence on hexokinase activity in vitro.

Data concerning the effective inhibitors are summarized in Table IV.

It appears that substances which influence —SH groups exert only a small effect on plant hexokinase. This indicates that the insoluble hexokinase of higher plants is not markedly dependent upon functional —SH groups for activity. In this respect plant hexokinase resembles the hexokinase of yeast (Slein et al. (43)). Zn^{++} was found to be a non-competitive inhibitor.

Discussion

The characteristics of the hexokinase of higher plants more closely approximate those of yeast hexokinase than those of the mammalian enzyme. This parallelism consists in lack of dependence on functional —SH groups for activity, acceptor specificity, and in energies of activation.

Soluble hexokinase was examined with respect to its $K_{(glucose)}$, specificity of acceptors, and its behavior toward inhibitors. The data presented in Table V indicate the striking similarity between the insoluble and soluble form.

Although in many tissues a considerable fraction of the hexokinase activity appears in the soluble form, the experiments with potato and wheat germ (see Table III) suggest that the major part of the activity may be associated in vivo with the mitochondria and that it is only inability to prepare the enzyme in a sufficiently

TABLE IV

INHIBITORS OF PARTICULATE HEXOKINASE IN STANDARD ASSAY

Substance	Concentration	Inhibition
	M	per cent
Dinitrophenol	5×10^{-2}	62
Dinitrophenol	5×10^{-3}	37
Dinitrophenol	5×10^{-4}	23
$HgCl_2$	10^{-3}	32
$CuSO_4$	10^{-3}	19
$ZnCl_2$	5×10^{-3}	70
p-Chloromercuribenzoic acid	10^{-4}	24
p-Chloromercuribenzoic acid	10^{-5}	18
Alloxan	5×10^{-3}	15
Sodium tripolyphosphate	5×10^{-3}	8
Glucose-6-phosphate	5×10^{-3}	17

TABLE V

COMPARISON OF PARTICULATE AND SOLUBLE HEXOKINASE FROM WHEAT GERM

Inhibitors	Concentration	Particulate	Soluble
		$K_{(glucose)} = 4.4 \times 10^{-4}$ M	$K_{(glucose)} = 4.6 \times 10^{-4}$ M
		Acceptor specificity, glucose, mannose, fructose, glucosamine	
		per cent inhibition	per cent inhibition
Alloxan	0.005	15	0
p-Chloromercuribenzoic acid	0.0001	24	31
Iodoacetamide	0.001	0	0
$CuSO_4$	0.001	19	20
$ZnCl_2$	0.005	70	53

delicate fashion that results in its solubilization.

That hexokinase should be present in the tissues of several higher plants both in an insoluble and in a soluble form is an interesting facet of the carbohydrate metabolism. As has been shown with mung bean, not only does esterification of phosphate in the energy-rich bond of ATP take place on the plant mitochondria, but the mitochondria can also use this ATP for the synthetic reaction mediated by hexokinase. It has also been demonstrated (Bonner and Millerd (67)) that the endogenous hexokinase of the mitochondria of mung bean hypocotyl sustains the maximum rate of oxidative phosphorylation in the presence of limiting amounts of adenylate with glucose as the acceptor. Addition of wheat germ hexokinase or yeast hexokinase showed no stimulatory effect on either the rate of oxidation or phosphate esterification.

ARGININE KINASE

In experiments designed to determine the specificity of phosphate acceptors for mitochondrial hexokinase by the manometric technique of Colowick and Kalckar (63), an apparent transphosphorylation occurred in the presence of arginine, but not with any other of several amino acids tested. At the same time, Bonner and Millerd (68) in investigating oxidative phosphorylation by plant mitochondria found that arginine appeared to be able to replace hexose as a final phosphate acceptor although arginine was only about half as effective as was glucose. These two independent experiments pointed to the possible existence in the plant of an arginine kinase, catalyzing the reaction

arginine + ATP <—> arginine phosphate + ADP.

The above reaction plays a dominant role in the muscular metabolism of invertebrates. The isolation and the identification of arginine phosphate in crab muscle by Meyerhof and Lohmann (69) was followed by the elucidation by Eggleton (70) of the function of this phosphagen during muscular contraction in Mytilus. It was found that during the interval of contraction, the level of arginine phosphate falls rapidly, but the phosphagen is rapidly resynthesized during the recovery phase. Thus, arginine phosphate serves, as does creatine phosphate in the vertebrates, as a reservoir of energy-rich phosphate which can be quickly mobilized as required for muscular activity.

Although the mechanism of the formation of energy-rich phosphate compounds in higher plants is now relatively well understood, it has not been clear whether the plant possesses a system by which this energy may be held in reserve. It was of interest to examine the arginine kinase of higher plants in more detail in the light of the hypothesis that arginine phosphate might contribute to such a reserve.

Preparation of enzyme:

The procedures found optimal by Bonner and Millerd (67) for isolation of intact mitochondria were followed (see page 42 of this thesis) with some modifications in the preparation of this enzyme. For the manometric experiments and in the experiments involving the determination of inorganic phosphate, 0.5 M mannitol (no added phosphate) was used as the extraction medium for the mitochondria. The particulate fraction of Neurospora crassa was prepared from a wild type strain according to the procedure of Haskins et al. (71).

Soluble enzymes were obtained during the preparation of the particulate fractions by combining the supernatant of the first high speed centrifugation with the supernatant from the washings of the particulate residue. Soluble preparations from acetone powders were obtained by extraction of the powder with eight times its weight of cold distilled water, and collecting the supernatant after centrifugation at 18,000 x g for ten minutes.

Methods of Assay for Activity

Manometric technique of Colowick and Kalckar:

The reaction was carried out as described for hexokinase in the previous section. The concentrations of the reagents in the final reaction mixture were: ATP, 0.01 M; bicarbonate, 0.02 M; arginine, 0.1 M; NaF, 0.01 M; 0.5 ml. enzyme in total volume of 1.5 ml. A control with ATP but without arginine was included for each enzyme preparation.

Mitochondrial and soluble enzymes from the following sources were assayed by this method: wheat germ, mung bean hypocotyl, Avena coleoptile, corn hypocotyl and mesocotyl, kidney bean hypocotyl, lupine cotyledons, and Neurospora crassa. In the experiment in which a net evolution of gas was observed, the reaction mixtures were assayed either for the formation of arginine phosphate or for the disappearance of free arginine (see following) with negative results. It seemed possible, therefore, that the gas exchange observed might not be the result of transphosphorylation, but rather, of a direct action of the enzyme system on the free arginine. To test this hypothesis experiments were set up in which a control mixture containing arginine but no ATP, and enzyme was incubated. In these experiments there was no detectable net evolution of gas due to the interaction of ATP and arginine. The net gas exchange noted in the first series of experiments could be accounted for by the evolution of gas in the flask containing arginine and enzyme but not ATP.

The evolution of gas by crude plant enzyme mixtures in the presence of arginine might be accounted for by the coupled action of two enzymes known to occur in higher plants -- arginase and urease.

$$\text{arginine} \xleftrightarrow{\text{arginase}} \text{ornithine} + \text{urea}$$

$$\text{urea} \xleftrightarrow{\text{urease}} 2\,NH_3 + CO_2$$

$$\text{arginine} \longleftrightarrow \text{ornithine} + 2\,NH_3 + CO_2 .$$

Thus, the presence of arginine kinase in plant tissues could not be demonstrated by the manometric techniques of Colowick and Kalckar.

Oxidative phosphorylation with limited adenylate:

It has been shown by Bonner and Millerd (67) that mitochondria isolated from higher plants are capable of oxidizing Krebs' cycle acids with the concomitant esterification of inorganic to energy-rich phosphate. The oxidation fails to proceed in the absence of suitable phosphate acceptor, in this case AMP, unless an uncoupling agent such as dinitrophenol is introduced. Thus, in the presence of limiting amounts of AMP, the oxidation will take place until all of the adenylate is converted to ATP. If hexokinase and glucose are present, ATP as it is formed during the oxidation is utilized to phosphorylate the glucose thereby regenerating and providing a continuous source of phosphate acceptor.

If arginine kinase were a component enzyme of the mito-

chondria and if arginine were present to act as the terminal phosphate acceptor, the oxidation of succinate or α -keto glutarate would lead to a net esterification of inorganic phosphate. This approach to the study of arginine kinase was therefore undertaken.

The reaction mixtures were prepared as described by Bonner and Millerd (68), except that the final concentration of arginine was 0.1 M rather than 0.01 M. The reaction mixture contains enzyme, phosphate 0.01 M, NaF 0.01 M, $MgCl_2$ 0.001 M, AMP 10^{-4} M, succinate or α -keto glutarate 0.01 M, and glucose or arginine 0.1 M, in a final volume of 3.0 ml.

As soon as the enzyme was added to the reaction mixture a 1.0 ml. aliquot was withdrawn into 1.0 ml. of ice cold 10% TCA, the remaining reaction mixture incubated 30 minutes at 30° on a Dubnoff Metabolic shaker, and another 1.0 ml. aliquot withdrawn into TCA. The precipitate was removed by centrifugation, and an 0.5 ml. aliquot of the supernatant diluted to 3.5 ml. with water. 1.0 ml. of the diluted TCA supernatant was then assayed for inorganic phosphate by the method of Lowry and Lopez (72). A reaction mixture utilizing glucose as the phosphate acceptor was included as a check on the effectiveness of oxidative phosphorylation in the particular preparation. Results of three experiments are given in Table VI.

As can be seen in Table VI, when succinate was used as the substrate and arginine as the acceptor a slight increase in the amount of phosphate esterified was observed, but no increase was noted with

TABLE VI

ARGININE AS PHOSPHATE ACCEPTOR FOR PLANT MITOCHONDRIA*

Substrate	Phosphate Acceptor	μ moles P esterified per ml. reaction mixture per 30 min.
Succinate 0.02 M	none	0.22
Succinate 0.02 M	glucose 0.01 M	1.86
Succinate 0.02 M	arginine 0.01 M	0.35
Succinate 0.02 M	none	0.18
Succinate 0.02 M	glucose 0.01 M	1.94
Succinate 0.02 M	arginine 0.01 M	0.39
α -ketoglutarate 0.02 M	none	0.39
α -ketoglutarate 0.02 M	glucose 0.01 M	0.94
α -ketoglutarate 0.02 M	arginine 0.01 M	0.25

* The reaction mixture contains enzyme, mannitol 0.3M, phosphate 0.01 M, $MgCl_2$ 0.001 M, NaF 0.01 M, AMP 0.0001 M; final volume, 3.0 ml.

α - keto-glutarate as substrate. In the experiments of Bonner and Millerd (68) arginine was able to act as the phosphate acceptor with one-half the effectiveness of glucose; in the experiments above the arginine was only, at most, one-tenth as effective.

Phosphate transfer from arginine phosphate to ADP:

It has been shown by Sorenyi et al. (73) that the equilibrium for crystalline arginine kinase from lobster favors the formation of ATP and arginine rather than the synthesis of arginine phosphate and ADP. An experiment was designed to take advantage of this equilibrium in the determination of arginine kinase. Two coupled reactions were used in this assay system:

$$\text{arginine phosphate} + \text{ADP} \xleftrightarrow{\text{arginine kinase}} \text{arginine} + \text{ATP}$$

$$\text{ATP} + \text{glucose} \xleftrightarrow{\text{hexokinase}} \text{ADP} + \text{glucose-6-phosphate}$$

$$\text{arginine phosphate} + \text{glucose} \longleftrightarrow \text{arginine} + \text{glucose-6-phosphate}.$$

Arginine phosphate was incubated with limiting amounts of ADP in the presence of mung bean mitochondria, and the ATP formed was to be trapped with yeast hexokinase and glucose. The concentration of free arginine was determined at the end of this incubation period.

Arginine phosphate was prepared from lobster muscle as described by Lohmann (74). The flexor muscles of twelve lobster were excised after the living animals had been cooled to 2°. The tissue was immediately frozen in liquid air, ground in a Waring

blendor with 5°/o TCA, filtered, and the supernatant neutralized and precipitated with $BaCl_2$. The mixture was then centrifuged, and the clear supernatant made 60°/o with respect to ethanol. The precipitate formed was collected and reprecipitated as the barium soluble alcohol insoluble salt. The material was isolated after the second barium precipitation and assayed, on the basis of phosphate and arginine, as 60°/o arginine phosphate.

The reaction mixture, total volume 1.5 ml., contained 0.5 ml. enzyme, 0.4 ml. arginine phosphate (2 mg. 60°/o pure barium salt per ml.), 0.05 ml. yeast hexokinase, and was 0.01 M with respect to $MgCl_2$; 0.01 M, KF; 0.06 M Tris (pH 8), 0.0001 M ATP, 0.1 M glucose. A control reaction was run concurrently without the hexokinase and glucose. After incubation for 15 minutes at 30° the reaction was stopped with 1.5 ml. ice cold 5°/o TCA, the precipitate removed by centrifugation, and 1.0 ml. aliquots assayed for arginine by the method of Dubnoff (75). It had been previously demonstrated that arginine phosphate does not react under the conditions of this assay.

No significant difference could be detected between the amounts of arginine liberated in the presence and absence of ATP.

Determination of activity by disappearance of free arginine:

In preliminary experiments it was found that arginine phosphate could be completely removed from a reaction mixture, without the inclusion of free arginine, by precipitation as the barium salt in 60°/o ethanol. An assay for arginine kinase activity

similar to that of Nelson (47) for hexokinase activity was therefore devised. This was carried out as follows: A reaction mixture of 1.5 ml. 0.01 M $MgCl_2$, 0.01 M NaF, 0.06 M Tris (pH 8.0), 0.01 M arginine, 0.01 M ATP, and containing 0.5 ml. of enzyme, was incubated at 37°. At various time intervals aliquots were withdrawn into ice cold 5°/o TCA and the precipitate removed by centrifugation. An aliquot of the supernatant was made 60°/o (volume °/o) with respect to ethanol, barium chloride added, and the insoluble barium salts removed by centrifugation. Arginine was determined in the alcohol supernatants by the method of Dubnoff (75). Controls without ATP were also carried out. Any formation of arginine phosphate will be reflected in a decrease in free arginine which will be greater in the presence of ATP than in the control. In the several experiments attempted with enzymes from mung bean, spinach acetone powder, Avena, and lupine, no significant difference was observed.

Measurement of change in labile phosphate:

The method of Lohmann (74) for the measurement of arginine kinase activity takes advantage of the extreme lability of the guanidino phosphate of arginine phosphate. One minute hydrolysis at 100° in 0.1 M HCl is sufficient to hydrolyze arginine phosphate completely, while only 3°/o of the ATP is hydrolyzed. A reaction mixture as described in the previous method was incubated at 37° for various time intervals; the reaction was stopped with an equal volume of ice cold 5°/o TCA, and the precipitate removed by

centrifugation. An aliquot of the TCA supernatant was withdrawn, hydrolyzed at 100° for one minute, and inorganic phosphate determined by the method of Lowry and Lopez (72). A control was taken at zero time.

No net increase of hydrolyzable phosphate could be observed for enzymes from Avena, mung bean, and spinach acetone powder.

Chromatographic identification of arginine phosphate formed:

The elegant methods for the chromatography of phosphate ester developed by Bandurski and Axelrod (53) offered yet another technique for the determination of arginine kinase activity. Since phosphorylated compounds can be detected in amounts as small as 5 μ gm., the isolation and characterization of the products of the reaction of ATP and arginine in the presence of enzymes from various tissues was undertaken. If arginine phosphate were formed in a reaction mixture, then co-chromatography of the reaction products with authentic arginine phosphate would indicate the existence of the enzyme.

In collaboration with Dr. Akio Fujiwara, the phosphorylated products of a reaction mixture of ATP and arginine incubated with a particulate mung bean enzyme preparation were isolated by fractionation of the barium salts as described on page 16 of this thesis. The barium soluble, alcohol insoluble fraction was then chromatographed by the technique of Bandurski and Axelrod (53), as described in section 2 of this thesis, using unidimensional development in the acid

solvent. The chromatograms were sprayed with the Hanes-Isherwood reagent to detect phosphorylated compounds as well as with ninhydrin to detect α -amino acids.

In one experiment, Dr. Fujiwara located a spot which gave a positive reaction both for arginine and for phosphate. Several attempts to repeat this experiment using Avena as well as mung bean particles were unsuccessful. It was found, however, that if the barium soluble, alcohol insoluble precipitate was not reprecipitated several times a ninhydrin spot appeared which moved with the same Rf as arginine phosphate, but gave a negative test for phosphate. In chromatograms of the three times reprecipitated material, no spots could be detected other than those due to adenylates and inorganic phosphate. Since free arginine under the conditions of the acid development moves with the same Rf as does arginine phosphate, it seems likely that arginine was carried down with the initial barium precipitate in sufficient quantity to give a positive amino acid test.

Conclusions

By the several criteria outlined above it appears unlikely that arginine kinase is found in higher plants. It must be emphasized, however, that negative experiments are not always conclusive. It is possible, that arginine kinase may be present in higher plants, but the inability to obtain the proper conditions for its isolation and characterization prevent its demonstration at this time.

CONCLUSION

The experiments described in this thesis have provided evidence that in higher plants, as in yeast, bacteria, and muscle, glycolysis is a principal pathway for the metabolism of carbohydrates. At the inception of this research in 1950, not only was the participation of glycolysis much in doubt, but also the integrated mechanisms of oxidative phosphorylation and of the energy-rich phosphate esters had yet to be implicated in the respiration and metabolism of higher plants.

With the discovery by Axelrod and Bandurski (50) of an oxidative pathway for the metabolism of carbohydrates in higher plants a new problem arises: What are the relative contributions of each of these biochemical sequences in the breakdown of hexose by the living plant? It may be possible to gain insight into this relationship by infiltrating leaves with radio-labeled C^{14} glucose. C_1^* labeled glucose would be applied to one group of leaves, and uniformly labeled C^* glucose to a second similar group. After a period of incubation, the tissue would be homogenized in cold TCA and the organic acids extracted into ether. Non-labeled pyruvic acid would then be added as a carrier and crystallized to constant activity.

For uniformly labeled glucose, the fractional recovery in pyruvate of the total counts applied as glucose will establish the minimum fraction of the glucose metabolized through pyruvate. For C_1^* labeled glucose the situation is different. Any C_1^* glucose

metabolized via the oxidative pathway will lose its label in the initial reaction. The fractional recovery of the counts in pyruvate will, therefore, be lower than in the case with the oxidative pathway. If all of the hexose metabolism proceeds through the oxidative pathway, the recovery of C_1^* glucose will be 0%; if all of the metabolism proceeds through glycolysis, the recovery in pyruvate will equal that found for uniformly labeled glucose. Thus, the recovery experimentally found will establish directly the contributions of each of these pathways.

The combined contributions of several workers in this and other laboratories have clarified our knowledge of the basic processes operative in plant respiration. Thus, ATP has been identified and characterized, glycolysis and the oxidative "shunt" have been studied in some detail, and the multi-enzyme system mediating oxidative phosphorylation isolated and subjected to study.

The studies of the individual enzymes phosphofructokinase and hexokinase have shown that there is a striking similarity in the characteristics of these enzymes as isolated from different tissues. That hexokinase from higher plants should be so similar in all of its properties to the enzyme isolated from yeast is an interesting facet of biochemical evolution.

As our understanding of the biochemistry of living organisms is extended, certain basic patterns of anabolism and catabolism emerge. Superimposed upon the fundamental pathways of synthesis and breakdown

are the biochemical embellishments characteristic of the particular organism or group of organisms. The fruitful search for these enzymic pathways will doubtless continue to promote greater understanding of the physiological phenomena dependent upon them.

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APPENDIX

The energy of activation as given for phosphofructokinase (pages 26 and 28) and for hexokinase (pages 49 and 51) were obtained as follows:

The relationship between the rate of a chemical reaction and the temperature at which the reaction occurs is given by the equation

$$(1) \qquad \frac{d \ln k}{dT} = \frac{E}{RT^2}$$

where

k - velocity constant of reaction

T - absolute temperature

R - gas constant

E - energy of activation.

Integrating equation 1, one obtains

$$(2) \qquad \log k = \frac{-E}{2.303\ R} \left(\frac{1}{T} + C\right).$$

Therefore, if log k is plotted against the reciprocal of the absolute temperature, the slope of the resulting "best" straight line is equal to $-E/4.606$.

Another method is to determine the rate of reaction at two temperatures and calculate E from the relationship

$$(3) \qquad \frac{k_2}{k_1} = e^{\frac{E}{R}\frac{T_2 - T_1}{T_2 T_1}} .$$

Data for Phosphofructokinase

	Temperature (°K)	$1/T \times 10^3$	k	log k
a	273.6	3.66	88.0	1.94
b	298.6	3.35	156.3	2.19
c	303.5	3.30	168.2	2.23
d	310.5	3.23	220.5	2.34
e	317.9	3.15	218.5	2.34

Slope of best straight line from Figure 8 = -815 deg.$^{-1}$

$$E = 815 \times 4.606$$

$$= 3{,}740 \text{ cal./mole.}$$

Values for E from equation 3 for all possible pairs of temperatures are given below:

T_1	T_2	E
a	b	3730
a	c	3820
a	d	4350
a	e	3740
b	c	3480
b	d	5350
b	e	3500
c	d	2730
c	e	3180
d	e	0

Average - 3,390 cal./mole.

Data for hexokinase

	Temperature (°K)	$1/T \times 10^3$	k	log k
a	273.6	3.66	0.17	$\bar{1}.228$
b	293.8	3.41	0.79	$\bar{1}.895$
c	303.6	3.30	1.26	0.100
d	311.1	3.22	2.22	0.342
e	320.3	3.12	1.10	0.042

Slope of best straight line from Figure 17 = -2,550 deg.$^{-1}$

$$E = 11{,}600 \text{ cal./mole.}$$

Values for E from equation 3 for all possible pairs of temperatures are given below:

T_1	T_2	E
a	b	12,600
a	c	11,400
a	d	12,000
b	c	9,720
b	d	11,300
c	d	14,400

Average - 11,900 cal./mole.

Estimation of K_m

Michaels and Menten (Biochem. Z., 49, 333 (1913)) have shown that the dissociation constant, K_m, for the enzyme-substrate complex can be expressed by the equation

(4) $$v = \frac{V(S)}{K_m + (S)}$$

where

v - velocity of reaction

V - maximum velocity of reaction

K_m - dissociation constant

(S) - substrate concentration.

Lineweaver and Burke (59) found that it was more convenient to express equation 4 in the linear form

(5) $$\frac{(S)}{v} = \frac{(S)}{V} + \frac{K_m}{V} \quad .$$

Thus, if (S)/v is plotted against (S) the slope of the resulting straight line is 1/V and the ordinate intercept is K_m/V.

Data for Phosphofructokinase

Fructose-6-phosphate (pages 34 and 35)

Slope = 3.4×10^{-3}

Intercept = 2.4×10^{-5}

$K_m = 7.1 \times 10^{-3}$ M.

ATP (pages 34 and 36)

Slope = 5.4×10^{-3}

Intercept = 1.3×10^{-5}

$K_m = 2.4 \times 10^{-3}$

Data for Hexokinase

Glucose

Slope = 0.66

Intercept = 2.9×10^{-4}

$K_m = 4.4 \times 10^{-4}$

ATP

Slope = 6.9×10^{-7}

Intercept = 6.0×10^{-4}

$K_m = 8.7 \times 10^{-4}$