UNIVERSITY OF MANITOBA

PURIFICATION AND KINETICS OF ACID PHOSPHATASE IN PLANT TISSUES

A THESIS SUBMITTED TO
THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE

by

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September, 1961

ACKNOWLEDGEMENTS

The author is greatly indebted to Dr. E. R. Waygood for his constant guidance and encouragement during the course of investigation.

Thanks are also due to Drs. B. D. Sanwal and D. T. T. Wang for their valuable advice and interest in the investigation and preparation of the manuscript.

The assistance and encouragement of Miss Manju Kapoor throughout the studies and preparation of the manuscript are sincerely appreciated.

Norland variety potatoes have been generously donated by Mr. K. Fegol, Grain Research Laboratory, Winnipeg.

This investigation was supported by grants-in-aid from The University of Manitoba and the National Research Council of Canada.

ABSTRACT

A phosphatase was isolated from potato tubers and 100 fold purification was achieved with DEAE cellulose column chromatography.

The phosphatase has the typical properties of a phosphomonoesterase II with pH optimum at 5.5. All the phosphomonoesters tested were hydrolysed to various extents. The Michaelis-Menten constant calculated with phenyl phosphate as substrate was 11.1 x 10^{-4} M. The potato phosphatase can be heated up to 60 minutes at 50°C without loss of activity. A shorter period of heat treatment appears to activate the enzyme.

The potato phosphatase was activated by oxatate < azide < formate< L-tartarate at a concentration of 1 x 10^{-4} M.

Of the metal ions tested Mn^{++} and Fe^{++} were slightly stimulatory to the phosphatase activity, whereas $Hg^{++} > Cu^{++} > Mg^{++} > Zn^{++}$ were inhibitory (conc. 1 x 10^{-4} M).

Arsenate > fluoride (1 x 10^{-4} M) also inhibited the phosphatase activity. There was complete inhibition with molybdate 10^{-4} M.

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CHAPTER I

INTRODUCTION

Among all the essential elements phosphorus plays a unique role in living tissues. Phosphates form natural buffers and control H-ion concentration in body fluids, and protoplasm of the living organisms. Phospholipids play a major part in transport of ions across cellular membranes. Phosphate bonds link together the nucleotides which form RNA and DNA, thereby playing a significant role in growth and heredity. Such phenomena as bioluminescence and bioelectricity are dependent on the phosphates. Phosphorylated compounds are the basis of almost all metabolic pathways. All this is possible because of the "kinetic stability of a thermodynamically labile phosphate bond" (Lipmann). Energy binding, transference and release are centred on it through the mediation of a host of enzymes (kinases, phosphorylases, phosphatases, pyrophosphatases and pyrophosphorylases) and a large amount of literature has already accumulated on phosphate metabolism.

In general, the literature deals chiefly with the pathways of phosphate metabolism in animal tissues and microorganisms (yeast, bacteria and fungi) and the information on phosphate metabolism in higher plant tissues is meagre. Much less is known about the enzymes in plant tissues, with respect to their suitability as source material, their substrate specificity and kinetics.

Phosphatases, as an example, are a group of enzymes universally present in living organisms and have been demonstrated in many plant tissues. The investigation reported herein was undertaken with the purpose purifying and elucidating the properties, kinetics and specificity of the acid phosphatase from potatoes in order to determine the extent to which it compares with the phosphatases from other sources. Furthermore, in order to facilitate studies in fields of carbohydrate and nucleotide metabolism being conducted in this laboratory, an understanding of the acid phosphatases is imperative. With this in view the purification of an acid phosphatase from potatoes was undertaken.

One of the steps in purification was the use of ion exchange chromatography. Several substituted celluloses, especially DEAE cellulose (Sober and Peterson, 1956) have been introduced in the recent years for the purification and separation of various proteins. A large number of enzymes have been purified to an extent which was not possible by the conventional methods with the aid of other ion exchange beds. Again, most of the literature refers to the application of chromatographic techniques to enzymes from animal or microbial sources. It was with this point in mind that the purification of the phosphatase was undertaken from potatoes by the use of ion exchange chromatography with DEAE cellulose as the ion exchanger.

CHAPTER II

REVIEW OF LITERATURE

This survey of literature is mainly concerned with the phosphomonoesterases. A brief reference is made to the history of phosphate metabolism and the various types of reactions involved therein. The phosphatases are, likewise, considered before dealing with the phosphomonoesterases in a greater detail. One of the purification procedures followed in the present investigation has been ion exchange chromatography. Since ion exchange chromatography is a major advancement, a short note on its history and various aspects is included separately, with special reference to the chromatography of proteins. This review is, therefore, divided into two sections, one dealing with phosphatases and the other concerning chromatography.

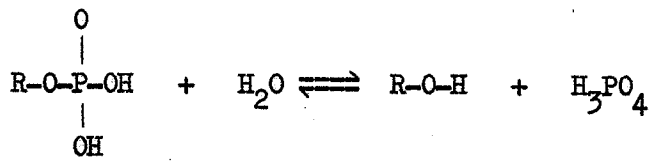
HISTORICAL PHOSPHATASES

Sujuki, Yoshimura and Takaishi (1907) described a phosphatase from rice bran which splits off a phosphate group from phytin. McCollum and Hart (1908) demonstrated a similar system in animal tissue. Following this Harden and Young (1909-10) reported the production by yeast juice of inorganic phosphate and sugar from a hexose phosphate. Von Euler and Funke (1912) and Grosser and Husler (1912) noted the presence of a phosphatase in intestinal epithelium, bone, kidney, spleen and pancreas. Robison (1922) discovered that a phosphatase produced a precipitate of tricalcium phosphate in the presence of a soluble calcium

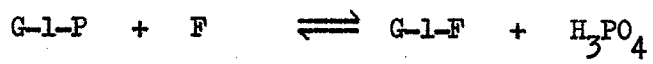
salt and hexose phosphate. This reaction formed the basis of the theory of bone calcification advanced by Robison and Soames in 1924. Consequently the phosphatases became progressively more important as the studies on ossification of bone and carbohydrate metabolism disclosed new areas of phosphatase activity. Various types of enzymes involved in phosphate metabolism have since been discovered (phosphorylases, phosphatases, transphosphorylases, etc.).

The following are the reactions typical of the above mentioned enzymes:

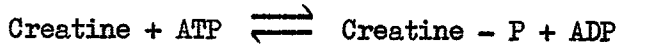
1. Phosphatases: Hydrolysis of phosphate esters



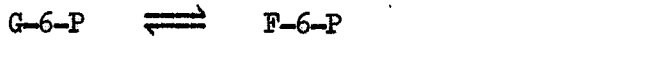
2. Phosphorylases:



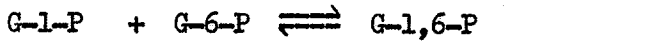
3. Phosphokinases: Transphosphorylation using ATP



4. Isomerases (phosphohexoisomerases)

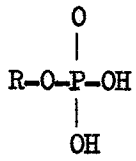


5. Mutases:

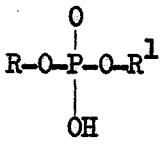


Phosphatases selectively hydrolyze the phosphate bonds of a wide variety of compounds. These bonds include the phosphates,

pyrophosphates, polyphosphates and metaphosphates. Among the phosphates, the monoesters -



and diesters -



are hydrolyzed but not the triesters. This holds true for pyrophosphoric and polyphosphoric esters as well. The nature of R radical may also have an effect on the specificity of the enzyme. Roche (1950) classified phosphatases on the basis of their substrate specificity, (Table I).

Akamatsu (1923) demonstrated the presence of phosphatases showing the same specificity but with different pH optima. These enzymes were called isodynamic phosphatases and were classified on the basis of their pH optima. According to Roche (1950) four types of phosphomonoesterases, three of pyrophosphatases and three of phosphodiesterases have been identified.

Phosphomonoesterases are widely distributed in living tissues. They liberate the phosphate from a variety of substrates according to the formula:

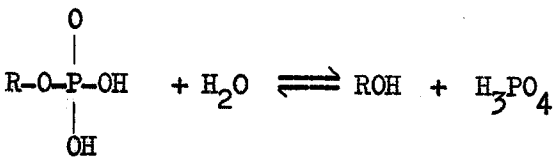


TABLE I. Substrate Specificity and Chief Sources of the Phosphatases.*

Enzyme Type	Substrates	Chief Sources
A. Phosphatases specific for one bond		
Phosphomonoesterases	Orthophosphoric monoesters	Bone, intestinal mucosa, kidney, seeds fungi, molds, yeasts
Phosphodiesterases	Orthophosphoric diesters	Liver, kidney, yeasts snake venoms
Pyrophosphatases	Phosphoric acid and its esters	Intestinal mucosa, kidney, yeasts, fungi molds
Phosphoamidases	Orthophosphoric amides	Kidney, cereals
Phosphoacylases	Orthophosphoric anhydrides of organic acids	Muscle, liver
B. Phosphatases specific for one or several substrates		
Adenosinetriphosphatases	Adenosinetriphosphate	
1. Adenylpyrophosphatase		Muscle, intestinal mucosa, potato
2. Apyrase		Muscle, potato
Phytase	Phytic acid	Cereals, seeds
Polyphosphatase	Triphosphoric acid and higher homologues	Intestinal mucosa, molds, yeasts
Cholinephosphatase	Cholineglycerophosphate	Intestinal mucosa, snake venoms
Hexosediphosphatase	1,6-Fructosediphosphate	Liver, kidney, yeasts
Polynucleotidases	Nucleic acids and polynucleotides	Intestinal mucosa, pancreas, liver
5'nucleotidase	5'-nucleotides	Testis, snake venoms
Metaphosphatases	Metaphosphoric acid	Kidney, molds, yeasts

* (After Roche, 1950)

TABLE II. Classification of Isodynamic Phosphomonoesterases*

Type	Optimal pH	Chief Sources	Chief characteristics in cell or tissue extracts
I	8.6-9.4	Bone, kidney, intestinal mucosa, mammary gland	Activation by Mg^{++}; inhibition by -SH; more on β- than on α-glycerophosphate; optimal stability at pH - 7.5 - 8.5.
II	5.0 - 5.5	Liver, seeds fungi, prostate	No activation of Mg^{++}; inhibition by F; more active on β- than on α-glycerophosphate; optimal stability at pH - 5.0-6.0.
III	3.4-4.2	Liver, top yeasts	Inhibition by Mg^{++}; more active on β- than on α-glycerophosphate; optimal stability at pH - 4.5-5.5.
IV	5.0-6.0	Red blood cells, bottom yeasts	Activation by Mg^{++}; more active on α-than on β-glycerophosphate; optimal stability at pH - 6.5-7.5.

* (After Roche, 1950)

The structure of R-radical may have limited influence on the hydrolysis. The phosphomonoesterases occur usually in association with pyrophosphatases. Their separation could be achieved by selective adsorption, thermal inactivation and maintainance at optimal pH. In general phosphomonoesterases of higher plants belong to type 11 (Roche 1950).

SOURCES

Seeds (Joyce and Grisolia 1960; Turner and Turner 1960; Newmark and Wenger 1960) and potatoes (Roche 1950) were found to have a high phosphatase activity. Roberts (1956 a,b; 1957) has extracted acid phosphatases from wheat leaves. Ito, Kondo and Watanabe (1955) used sweet potato as an enzyme source. A phosphatase was extracted from citrus fruit (Axelrod 1947).

SPECIFICITY

The phosphomonoesterases are generally nonspecific. They catalyze the hydrolysis of a number of phosphomonoesters (hexose phosphates, nucleotides and polynucleotides) at different rates (Newmark and Wenger 1960). Ito et al (1955) have presented data to show that the hydrolysis of β-glycerophosphate, ATP and inorganic pyrophosphate by a purified preparation from sweet potato, is catalyzed by a single enzyme. Axelrod (1947) and Newmark and

Wenger (1960) demonstrated a pyrophosphatase activity with their purified preparations (cf. Roche 1950). Lee and Eiler (1951) obtained a preparation from potato tubers which splits only one phosphate group from ATP on incubation below 7°C. At higher temperatures a second phosphate group splits off, but at a slower rate compared to the first. Whether this is due to an apyrase (Krishnan 1949) or a phosphomonoesterase is not certain. A substrate specific 3'-nucleotidase has been found in barley leaves (Shuster and Kaplan 1953). Chakravorty, Chakrabortty and Burma (1959) purified an acidic C-1 diphosphatase from spinach leaves which was active on Fructose diphosphate and ribulose diphosphate. Its activity toward G-6-P was only 4% of that toward the diphosphates.

ACTIVATORS

One of the characteristics by which phosphomonoesterase 11 is distinguished from type 1 is its insensitivity to Mg^{++} and other divalent ions, sulphhydryl groups, amino acids and alkaline cyanides. Joyce and Grisolia (1960) detected a slight stimulation in the activity of one of the chromatographic fractions of acid phosphatase by $Mg^{++} < Mn^{++}$. Newmark and Wenger (1960) reported that a. EDTA and Triton X-100 have a protective action on the activity of the enzyme; b. slight activation by Mg^{++} and Mn^{++}, $Mg^{++} < Mn^{++}$; and EDTA augments the activity of Mg^{++}.

In 1958 Roberts studied the effects of several ions on wheat leaf phosphatases .. From his data it appears that there is a mild activation with Mg^{++} and Fe^{++}.

INHIBITORS

Fluorides and molybdate are strong inhibitors of phosphomonoesterase 11 activity. Oxalates, phosphates and pyrophosphates are usually strong inhibitors. Metallic ions like Hg^{++}, Fe^{++}, Cu^{++} were reported to inhibit the activity (Joyce and Grisolia 1960; Newmark and Wenger 1960). Roberts (1960) demonstrated inhibition by fluoride, molybdate and arsenate. Naganna et al (1955) reported a complete inhibition by copper of a phosphatase from potato extracts.

THERMAL STABILITY

Phosphomonoesterases are remarkably heat stable. They withstand prolonged periods of heating at relatively high temperatures with only a slight loss of activity. Thermal inactivation of other proteins is used as a step in purification of the phosphatases (Newmark and Wenger 1960). Roberts (1957) studied the effect of heat treatment on acid phosphatase from wheat leaf juice. His results are summarized in Table III. Newmark and Wenger (1960) studied the thermal stability of phosphatases as a function of pH. At the optimum pH the enzyme retained over 90%

TABLE III. Effect of temperature treatment on acid phosphatase from wheat leaf juice.*

Temperature	Time of treatment Min.	Percentage initial activity retained after treatment
50°C	30	30 - 90
	60	10 - 83
60°C	30	15 - 52
	60	17 - 39

*(After Roberts, 1957)

of its activity for two hours at 37°C or 1 hour at 50°C but less than 50% only, after heating for half an hour at 60°C.

The data available so far on the phosphatases are meagre. The individuality of several phosphatases, their structure, the factors influencing their specificity and isodynamics are still obscure. While the significance of phosphates is known in the general metabolism, the role of phosphatases (with the exception of alkaline phosphatases from bone) in the general metabolism remains unknown.

HISTORICAL CHROMATOGRAPHY

Chromatography is widely used for the purification and separation of both organic and inorganic substances. Yet there is no general agreement as to the origin of chromatography. Early

observations on the adsorption of ions by soils, decolouration of solutions and on the filtration of petroleum revealed that filtration of a solution through absorptive media retarded the migration of solutes but this did not provide a resolution of the mixture of solutes. Tswett (1906) described a chromatographic method of separating leaf pigments on an inulin column. This method consisted mainly of adsorbing a coloured substance on a column and washing the column with a pure solvent, until a proper separation was achieved. The column was then cut, with or without extrusion, into sections and each section eluted appropriately.

Reichstein (1938) introduced the liquid or flowing chromatogram. The column was washed successively with different solvents, each of a higher eluting power, and each fraction was collected separately.

Tiselius (1941), introduced the techniques of frontal analysis and displacement development. In the frontal analysis a solution of substances is continuously forced through a column and their concentration is measured on leaving. In the displacement development the substances to be separated are first adsorbed on the column and then they are displaced by passing through the column the solution of a substance which gets adsorbed more strongly.

Martin and Synge (1941) made use of the partition coefficient of solutes to separate them from solvent A into solvent B, A and

B being immiscible.

Consden, Gordon and Martin (1944) showed that filter paper sheets and strips could also be used as a support for the stationary phase in partition chromatography. Further refinements added were, (a) impregnation of the paper with a variety of sorpants; (b) and the development of chromatogram by radial, ascending, descending or two dimensional (employing the same solvent or two different solvents) techniques.

ELECTROCHROMATOGRAPHY

This technique makes use of the differential migration of solutes in an electric field. Strain (1939) described a combination of chromatography and electrophoresis using two electrodes inserted in a column (alumina, glasswool, asbestos fibre or glass powder etc.).

Martin and Synge (1945) used a silica gel for the separation of peptides. Other types of gels (agar and starch) are employed for the separation of proteins. Wieland and Fischer(1948) were the first to separate amino acids and peptides on a paper strip moistened with a buffer.

Haugaard and Kroner (1948) used simultaneous development with a solvent and electrophoretic separation. This process is known as continuous electrochromatography or electrophoretic separation.

ION EXCHANGE CHROMATOGRAPHY

The phenomenon of ion exchange was first noticed and systematically studied in soil by Way (1850). But with one exception, however, minerals are of no practical value. Compounds resembling zeolite were successfully produced by Gans in 1907.

In 1934 Crosfield, Jos and Sons Ltd., (Brit. 455, 374) produced sulfonated coal with ion exchange properties. Adams and Holmes (1935) showed that the products of condensation of polyhydric phenols with formaldehyde could be charged with cations including H^+ - ions and these cations could then exchange with those in a solution. Following this logic they produced anion exchanging resins from polyamines. In the succeeding years synthetic resins were improved considerably in structure, stability variety of exchange groups (sulfonic, carboxyl, hydroxyl groups) and the total capacity of exchange groups. D'Alelio in 1940 announced the preparation of a cross-linked polystyrene resin.

In 1949 Cassidy conceived and executed the idea that it should be possible to produce polymers having groups capable of reversibly exchanging electrons with molecules or ions of the surrounding solution. These redox resins have been used for oxidations and reductions.

Guthrie (1952) described methods whereby both cation and anion exchange groups could be introduced into cellulose in the form of cotton. Of the several substituted celluloses, Diethy-

laminoethyl cellulose (DEAE cellulose) has been widely used in the purification of enzymes (Peterson and Sober, 1956).

Column chromatography of proteins

Chromatography is based on the differential migration of solutes between two phases. One of the phases is fixed and nonmobile and the other mobile. The migration is effected by the flow of the mobile phase while the differentiation is determined by the selective and reversible distribution of the solutes between the two phases. The latter may be solid-liquid, solid-gas, liquid-liquid or liquid-gas. Each phase may be composed of one or more constituents. The fixed phase may be a sheet or a column or one of the possible permutations and combinations of the two. The method of handling the mobile, soluble phase is referred to as the development of a chromatogram. A chromatogram includes the whole experiment together with the graphic representation of the results. The quantity of the liquid phase held within the column is referred to as the dead volume. Column chromatography alone will be dealt with here, in which the fixed phase is a solid ion exchanger.

Ion exchangers are insoluble, high polymer skeletons capable of reversibly exchanging large number of ionizable groups. The labile groups, depending on their charge, form salts with anions (i.e. positively charged hence anion exchangers) or cations (cation

exchangers). The kinetics of ion exchange of proteins is a complex process and the analyses of the chromatographic separation of proteins are few. Chromatography has been the subject of the monographs of Calman and Kressman (1957), Lederer and Lederer (1957), Lederer (1959), Cassidy (1951) and Osborn (1955).

The value of ion exchange chromatography, as a technique of resolving complex protein mixtures, lies to a large extent, in its flexibility. The nature of the phases, the method of development and the physical characteristics are all capable of several variations, (Table IV).

Development

Chromatography is started with a homogeneous ion exchanger in equilibrium with a buffer of a particular ionic strength at a specific pH. This buffer is the starting buffer. For the development of such a column there are three possibilities:

(1) To develop the chromatogram with the same buffer with which the column is at equilibrium. This proceedure is known as the starting buffer development. Hirs (1953) used this technique in resolving two different samples of crystalline chymotrypsinogen on Amberlite XE-64. The method has been applied only to a few proteins (see Moore and Stein, 1956; Boardman and

TABLE IV. Variables in Protein Chromatography*

Variable	Alternatives
Solid phase:	Anion exchangers resin or Cation exchangers cellulose
Techniques of development:	Starting-buffer (constant agent) Gradient (continuous change) Step-wise (different agents)
Liquid phase:	Anion or cation buffers pH and ionic strength Organic solvents, polymers (carrier or displacing agents)
Dimension factors:	Ratio of column (diameter/length) Load (substance applied/amount of adsorbent) Flow rate Scale

* (After Boman 1961)

Partridge, 1955). It is very sensitive to changes in pH and ionic strength and causes large dilutions. Boman and Westlund (1956) used Dowex-2 for the purification of horse radish peroxidase.

(2) Gradient development:- The composition of the developing agent is gradually changed from that of the starting buffer to a solution of higher ionic strength resulting in a continuous alteration of conditions effecting the equilibrium. To this end a buffer of higher eluting power is mixed, in gradually increasing amounts, with the starting buffer in a mixing chamber, before it is allowed to flow through the column. Lakshmanan and Lieberman (1953) and Drake (1955) have reviewed the theoretical and the practical aspects of this problem.

(3) Stepwise development:- This consists of development with a buffer other than the one used for equilibration of the column by means of changes in the ionic strength or pH. Boman and Westlund (1956) studied the behaviour of serum albumen on Dowex-2 by this method.

For the collection of the effluent, several types of fraction collectors are available. This is essentially a turntable carrying tubes around its periphery.

It changes the position of the receiving tubes by a slight rotation at a specific interval. The movement of the fraction collector is controlled by a specific time interval, volume/weight of the effluent or by drop counting.

The choice of the liquid phase is based on (a) the nature of the ion exchanger and the effects of effluent on the ion exchanger; (b) the sensitivity of the protein to be chromatographed, and the interaction between the solute-solvent; (c) the interference or otherwise of the eluant with the analytical methods to be employed subsequently and (d) the eluting power of the phase (Cassidy 1957; Wheaton and Bauman 1951; Boman and Westlund 1956).

Dimensional factors

The choice of the dimentional factors can only be made by trial and error and varies with the mode of development employed. Hirs (1953), with starting buffer technique, used a ratio of 1:60. In general, especially with stepwise development, a ratio of 1:20 can be used. On account of the sensitivity of the proteins (an exception being ribonuclease which can be chromatographed at room temperature) flow rate should be as high as possible without the separation being effected. In the preliminary

experiments a minimum load should be used on the column for a better resolution of the proteins. Subsequently the load should be varied to achieve optimum resolution.

CHAPTER III

MATERIALS AND METHODS

ENZYME PREPARATION

Potatoes (var. Norland*) were used as a source of acid phosphatase. Purification was carried out according to the method of Newmark and Wenger (1960) up to step IV, with the following modifications:

(a) KCN was included in the extracting buffer (conc. 1×10^{-2} M) to prevent browning reaction brought about by the activity of polyphenol oxidase;

(b) EDTA was omitted from the initial extracting mixture. Fraction III (Newmark and Wenger, 1960) was dialysed overnight against forty volumes of acetate buffer 0.02 M, pH 5.5. The turbidity developed was removed by centrifugation. The clear supernatant solution was stored at -10°. All centrifugations were carried out in a refrigerated Servall centrifuge at 0°C and 20,000 x g for 10 minutes, and all other operations were conducted in a cold room at temperatures below 5°C.

SUBSTRATES

Adenosine triphosphate, Adenosine diphosphate, Adenosine

monophosphate, Ribose-5-phosphate, Glucose-6-phosphate, Glucose-1-phosphate, Fructose-6-phosphate, Fructose-1,6-diphosphate, Galactose-6-phosphate, 3,D(-) phosphoglycerate, 1,3-Propanediol phosphate, p-Nitrophenyl phosphate were used to study the acid phosphatase activity. The pH of the solutions (0.02M) was adjusted to 7.0. Disodium phenyl phosphate 0.1 M, pH 5.5, was used for the study of kinetics.

PROTEIN DETERMINATIONS

Protein content of the enzyme samples, in the initial preparations, was measured by microkjeldahl estimation of total nitrogen. In the latter stages, in the absence of coloured phenolic compounds, spectrophotometric determinations ($E_{280/260}$) were made.

PHOSPHATE DETERMINATIONS

Inorganic phosphates liberated were determined by an adaptation of the Fiske-Subbarao method (Waygood, 1948) by measuring the optical densities of phosphomolybdate complex reduced by ascorbic acid at 650 mμ in a Coleman universal spectrophotometer Model 14.

ASSAY

A rapid spectrophotometric assay based on an increase in optical density, as phenol was liberated from phenyl phosphate,

was used (Figure 1). This method was suggested by Brawerman and Charagaff, (1954). The assay system for the acid phosphatase contained: Sodium acetate buffer 0.1 M, pH 5.5, 2.5 ml., disodium phenyl phosphate 0.1 M, pH 5.2, 0.01-0.02 ml enzyme 0.05 ml in a total volume of 3.0 ml. The increase in the optical density at 270 mμ was followed in silica cuvettes of 10 mm light path using either a Hilger or a Zeiss spectrophotometers.

The assay system for other substrates consisted of 1-4 μm. of the substrate, Sodium acetate buffer 0.1 M, pH 5.5, 1.0 ml and enzyme 0.2-0.4 ml in a total volume of 2.0 ml. The reaction mixture was incubated for 10 minutes at room temperature following which inorganic phosphate was determined.

ENZYME UNITS

A unit of enzyme activity is defined as the amount needed to cause an increase of 0.01 in optical density at 270 mμ or release of 1.0 μg of inorganic phosphate in 10 minutes at room temperature.

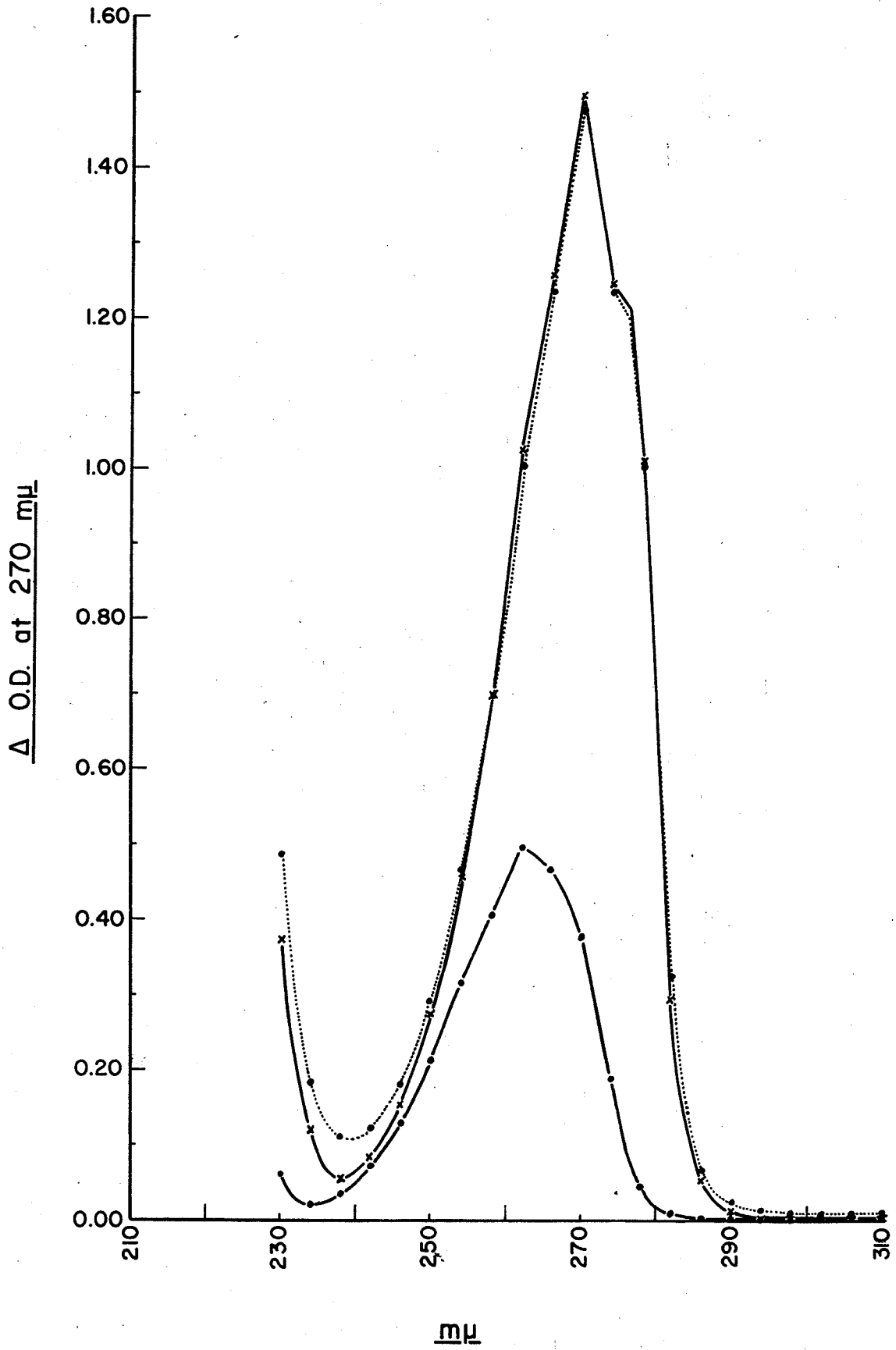
CHROMATOGRAPHY

Diethylaminoethyl cellulose columns were prepared according to the method of Boman (1957). The development was carried out using one step elution.

FIGURE 1. Spectra of phenyl phosphate and hydrolysate.

3 μmole of phenylphosphate, 21 units of enzyme, 250 μmole acetate buffer pH 5.5

.-.-.-	phenyl phosphate	10^{-3} M
.....	phenol	10^{-3} M
-x-x-x-	hydrolysate	10^{-3} M


1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Δ O.D. at 270 mμ
210
230
250
270
290
310
mμ

CHAPTER IV

EXPERIMENTAL RESULTS

PURIFICATION

Norland potatoes were peeled and homogenized with one volume of 0.02 M acetate buffer, pH 5.2 containing 0.01 M KCN in a Waring blendor for 3 minutes at 5°C. All operations were performed at this temperature. The homogenate was squeezed through four layers of cheese cloth to remove the cell debris and centrifuged at 3,500 x *g* for 5 minutes. The precipitate containing starch and cell debris was discarded. The pH of the supernatant solution was adjusted to 5.0 and the precipitate centrifuged off. The supernatant solution was brought to 0.45 saturation with the gradual addition of solid $(NH_4)_2SO_4$ and allowed to stand for two hours with slow stirring. The precipitate was removed by centrifugation and discarded. The supernatant was brought to 0.65 saturation with more solid $(NH_4)_2SO_4$ and allowed to stand overnight. The precipitate was taken up in 0.02 M acetate buffer, pH 5.2, and dialysed overnight against forty volumes of the same buffer. The contents of the dialysis bag were recentrifuged to remove any insoluble proteins present. The supernatant was deep brown in colour due to the presence of polyphenol oxidases. A part of the enzyme was treated

with Darko G-60 (activated charcoal; 25 mg per ml enzyme) to remove the excess of pigment developed. This step in itself lead to a considerable degree of purification (Table V).

FRACTIONATION ON DEAE CELLULOSE COLUMN

Preparation of the column: 5 g of Diethylaminoethyl cellulose was mixed with 200 to 300 ml of the buffer, pH 5.2, and stirred for fifteen minutes. The mixture was centrifuged and the supernatant liquid discarded. The cellulose was again stirred with the buffer for 15 minutes and centrifuged. This step was repeated at least six times and the cellulose left overnight in approximately 400 ml of the buffer. A column (2.5 x 18 cm) was filled with the buffer and a pad of glass wool was inserted into it. The slurry of cellulose-buffer was slowly poured from the top as the buffer runs out below. As soon as all the ion exchanger had been transferred, a flask containing the starting buffer was connected at the column, which was packed under gentle pressure.

Pressure unit

The pressure on the column was maintained by compressing the air on top of the reservoir containing the developing buffer (Figure 2). A Mariotte bottle "A" containing about 12 litres of water was set up about two feet high from the table

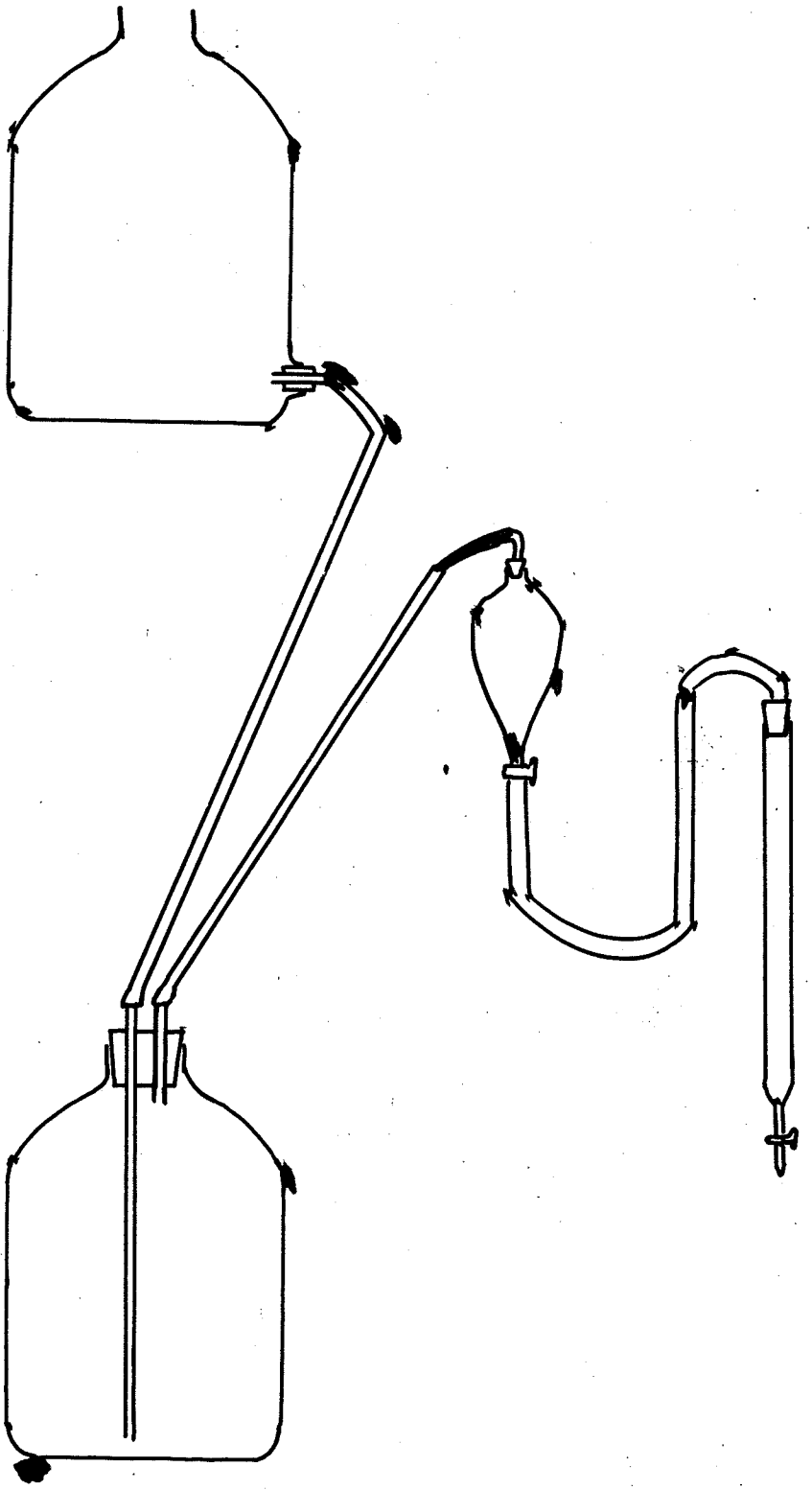
TABLE V. Purification of Enzyme.

Fraction	Total Volume	Total Protein	Total Action	Specific Activity	% Recovery
Crude Juice	1000 ml	10,500	15,750	1.5	100
Ammonium Sulphate	200	1,000	7,500	7.5	47.6
G-60 Treated	50	45	1,057	23.5	56.3
DEAE Cellulose	2	1.56	156	100	41.6*

* Peak I alone was measured.

FIGURE 2. Pressure Unit.

Legend required here.



top and connected to bottle "B" on the table. Bottle "B" has an inlet from "A" and an outlet leading to the reservoir. As water flows from "A" to "B" under the force of gravity the air in "B" is compressed which transfers the pressure through the buffer in the reservoir, to the column.

STEPWISE DEVELOPMENT

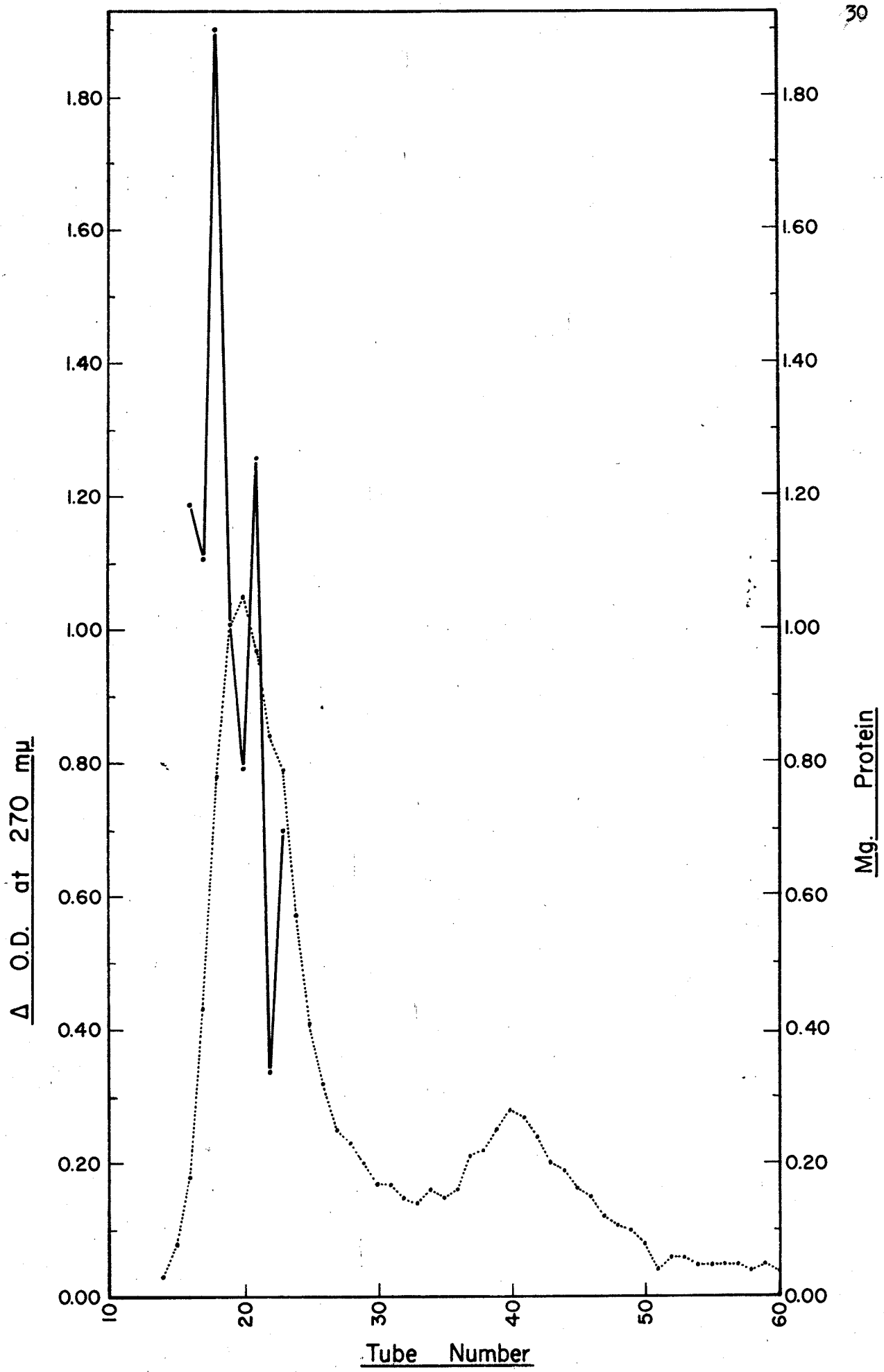
Enzyme solution from the previous step containing 50 mg protein was adsorbed on the column. The column was washed with the starting buffer using one dead volume in order to ensure a proper adsorption of the protein. Development was started with 0.1 M acetate buffer (pH 5.2) under pressure. Fractions were collected at intervals of five and ten minutes. The volume of each fraction was 2 to 3 ml or 4 to 5 ml. In the preliminary runs about a hundred fractions were collected, and their protein content determined. No measurable protein was present after the 60th fraction. In the subsequent experiments therefore only the first sixty fractions were collected.

Two methods were employed for the collection of fractions. The first one was based on drop counting and the other on time interval. In the later experiments, however, fractions were collected on a time basis (Figure 3).

FIGURE 3. Chromotography on DEAE cellulose.

Stepwise development. Buffer acetate 0.1 M pH 5.5.

.-.-.-.- phosphatase activity
..... protein content.


Δ O.D. at 270 mμ
Mg. Protein
Tube Number
0.00
0.20
0.40
0.60
0.80
1.00
1.20
1.40
1.60
1.80
10
20
30
40
50
60

REGENERATION OF THE COLUMN

After each run the ion exchanger was washed with a strong eluant to get rid of the firmly adsorbed solutes. After a thorough washing with one or several eluants the ion exchanger was again equilibrated with a desired buffer. The washing was done *in situ* or the column was dismantled and the cellulose regenerated. The ion exchanger was regenerated by passing 0.1 M NaOH (about twice the dead volume) through the column. With elution the cellulose turns a clear white colour. Pigments (if any) present in the adsorbed solute can be seen to move in a zone along with the starting buffer (acetate 0.02 M, pH 5.2) by continuous flow of the buffer through the column until the outflow attained the same pH as the inflow. In practice this procedure was found to be time consuming (especially when the columns packed under pressure) and in the later experiments the column was dismantled, regenerated and the slurry was then poured into a column. If a column was not required immediately, the ion exchanger was dried and stored in a sufficient quantity of the buffer. Before use, the columns were checked for fungal contaminants.

SPECIFICITY

The acid phosphatase from potato is nonspecific (Table VI). All the substrates, as shown in this table were hydrolysed at

TABLE VI. Substrate Specificity of Acid Phosphatase

Substrate	μmole	Pi liberated per cent
Glucose-1-phosphate	10	5.8
Glucose-6-phosphate	10	20.3
Fructose-6-phosphate	10	17.6
Fructose-1-6-diphosphate	10	31.2
Ribose-5-phosphate	10	30.5
Galactose-6-phosphate	10	3.0
Adenosine-5-diphosphate	10	30.0
Adenosine-5-monophosphate	10	18.1
Adenosine-5-triphosphate	10	44.9
2,3-propanediol phosphate	10	40.8
3 D(-) phosphoglycerate	10	58.3

The system for the assay consisted of 200 μmoles of sodium acetate buffer pH 5.5; substrate 10 μmoles, G-60 treated enzyme 10 units in a total volume of 2.0 ml. Incubation period 10 minutes. Protein was denatured with 1.0 ml, 20% cold trichloracetic acid and inorganic phosphate determined on aliquots.

varying rates under the experimental conditions (10 minutes incubation, 30°C, pH 5.5). The maximal rate of hydrolysis of phenyl phosphate occured at a substrate concentration of 3.0 µmoles with 21 units of the enzyme (Figure 4). At higher concentrations, the substrate was inhibitory to the enzyme. The Michaelis-Menten constant (K_m) calculated from a Lineweaver-Burk plot (Figure 5) of the data is 11.1×10^{-4} M. The rate of hydrolysis was directly proportional to the enzyme concentration (Figure 6). The hydrolysis of phenyl phosphate, followed spectrophotometrically, was verified with the method of determination of inorganic phosphate (Waygood 1950). Under the conditions 100% hydrolysis occured in 25 minutes (Figure 7).

TEMPERATURE EFFECT

The potato phosphatase is similar to other phosphatases in thermostability. A dialysed ammonium sulphate fraction of the phosphatase was heated in a waterbath at 50°C for one hour. Aliquots of 2.0 ml were withdrawn at 10 minute intervals. The denatured protein was centrifuged off. No loss of activity was found. On the contrary the activity of the enzyme was enhanced by approximately 120 percent on heating for twenty minutes. This activation decreased on heating further (from 20 to 60 minutes), although it showed a higher activity than the control (Figure 8).

FIGURE 4. Substrate Concentration.

20 units enzyme, 250 μmoles of acetate buffer pH 5.5. Increasing concentrations of substrate in 3.0 ml

A = 0.5 μmoles	B = 1.0 μmoles	C = 1.5 μmoles	D = 2.0 μmoles
E = 2.5 μmoles	F = 3.0 μmoles	G = 3.5 μ moles	H = 4.0 μmoles

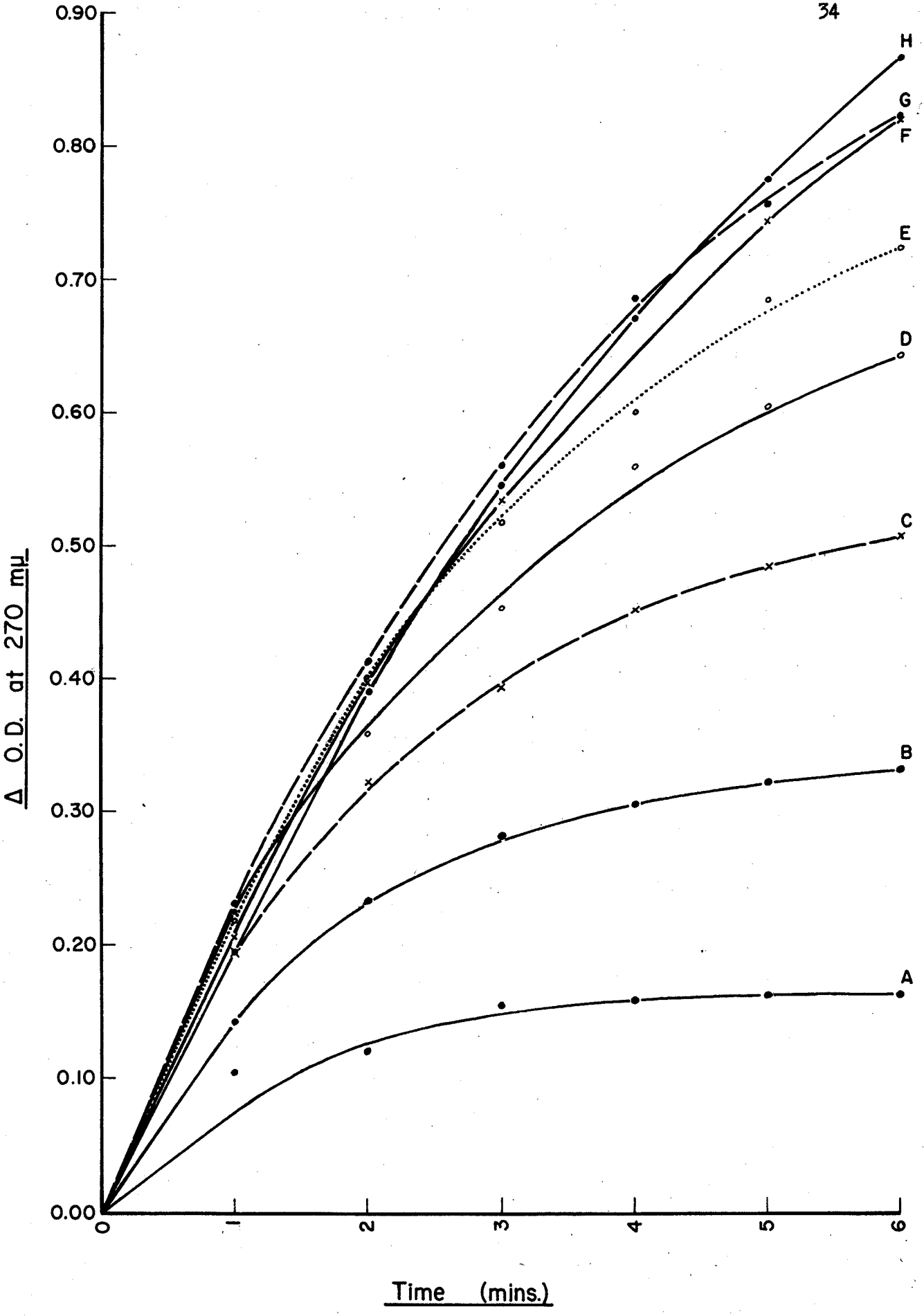

0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Δ O.D. at 270 mμ
0
1
2
3
4
5
6
Time (mins.)
H
G
F
E
D
C
B
A

FIGURE 5. Michaelis-Menten Constant.

$K_{m(1)} = 11.1 \times 10^{-4}$ M.

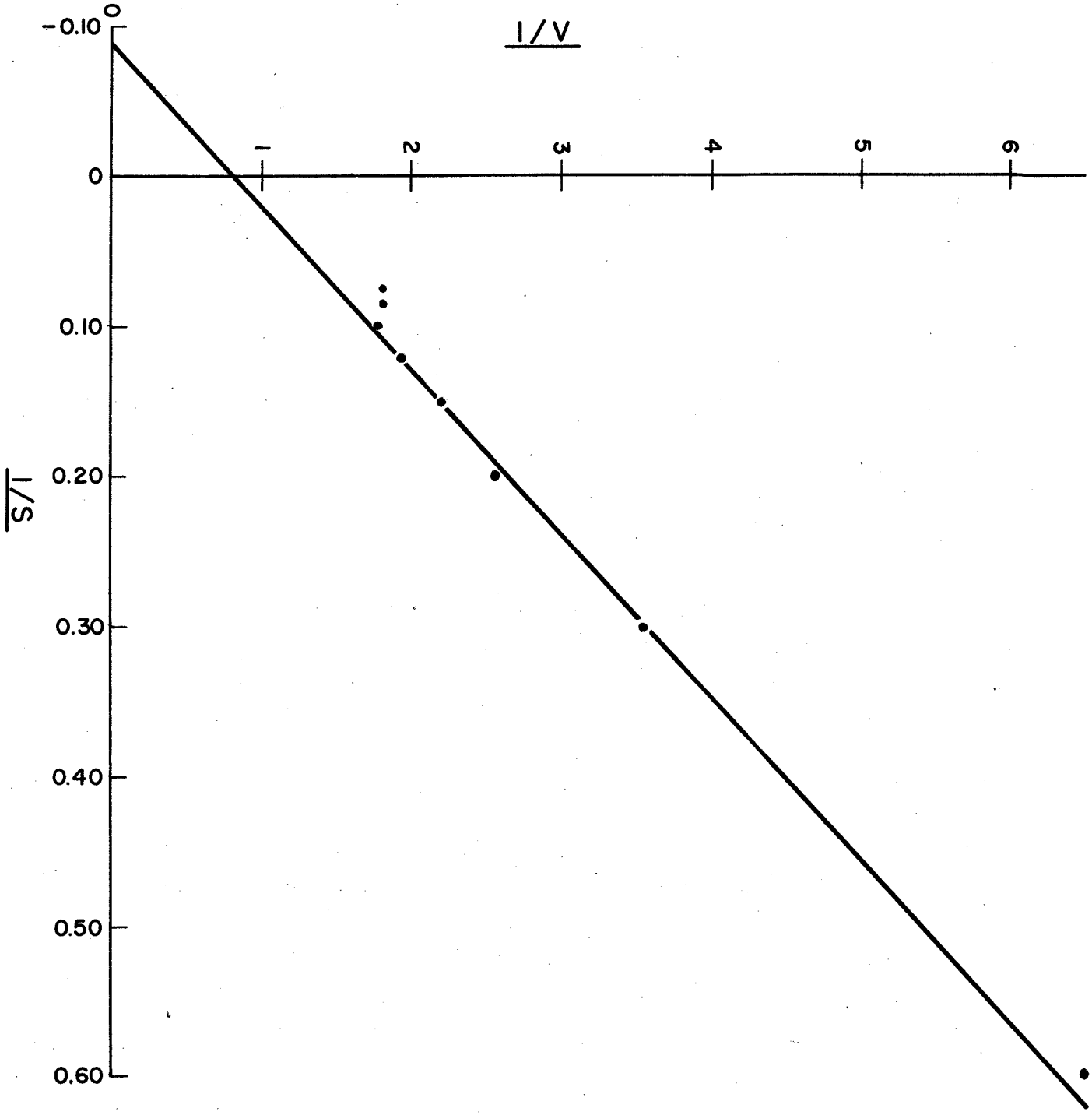


FIGURE 6. Enzyme Concentration.

3.0 μmoles phenyl phosphate, 250 μmoles acetate buffer pH 5.5, and enzyme
A = .05 ml (5 units). B = 0.1 ml C = 0.2 ml
D = 0.3 ml E = 0.4 ml, in a total volume of 3.0 ml.

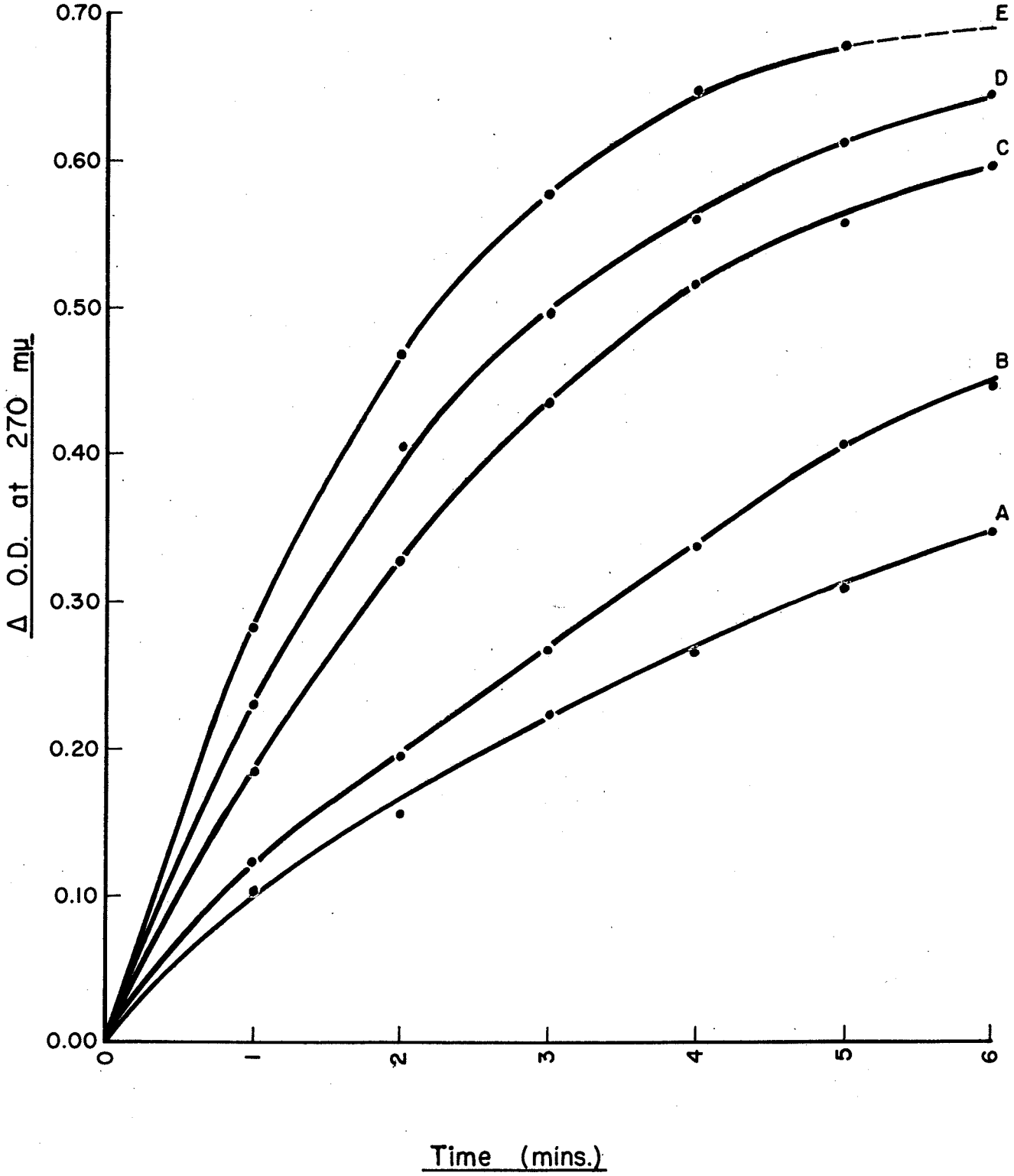

0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Δ O.D. at 270 mμ
0
1
2
3
4
5
6
Time (mins.)
E
D
C
B
A

FIGURE 7. Time x Hydrolysis

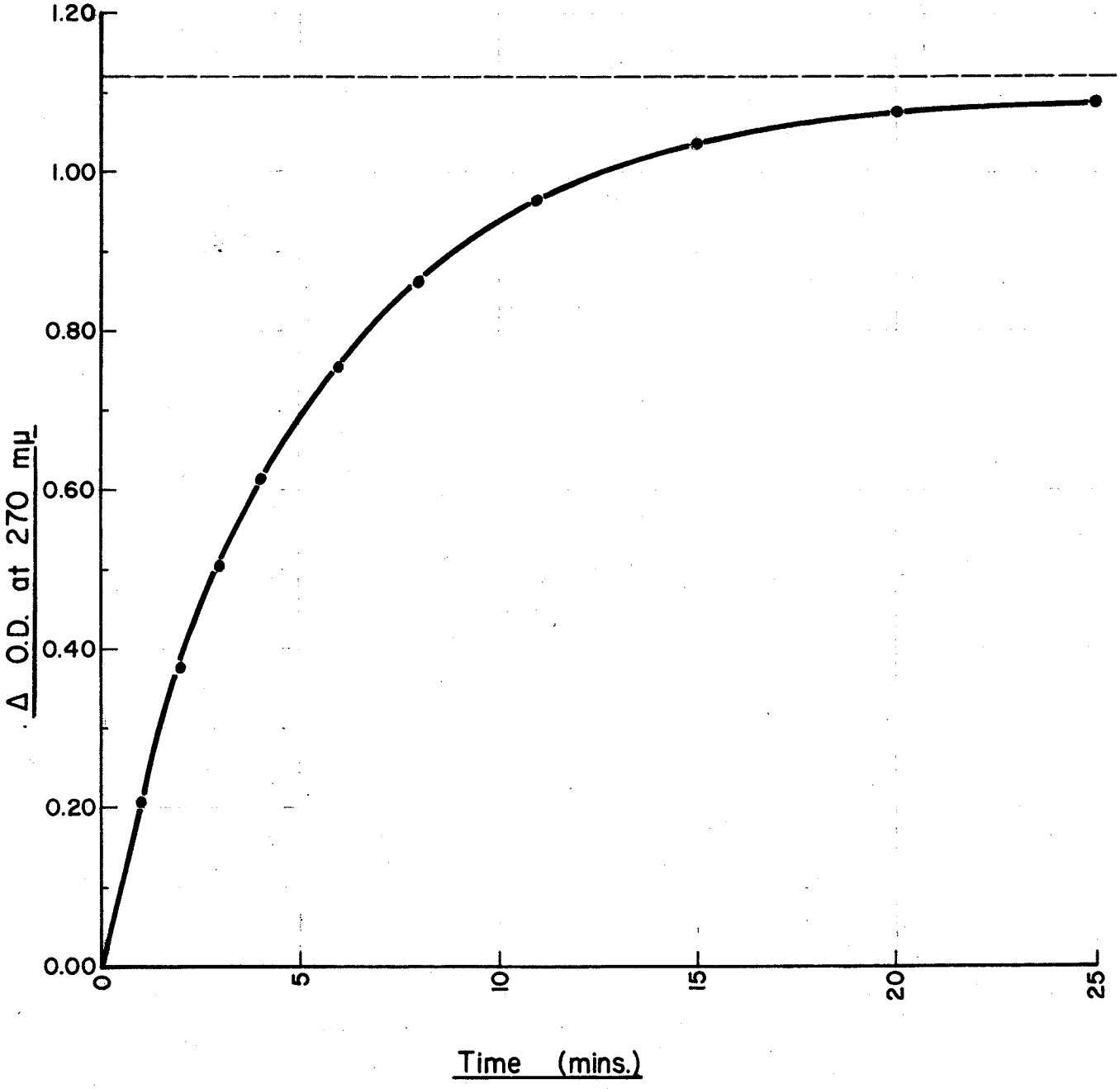
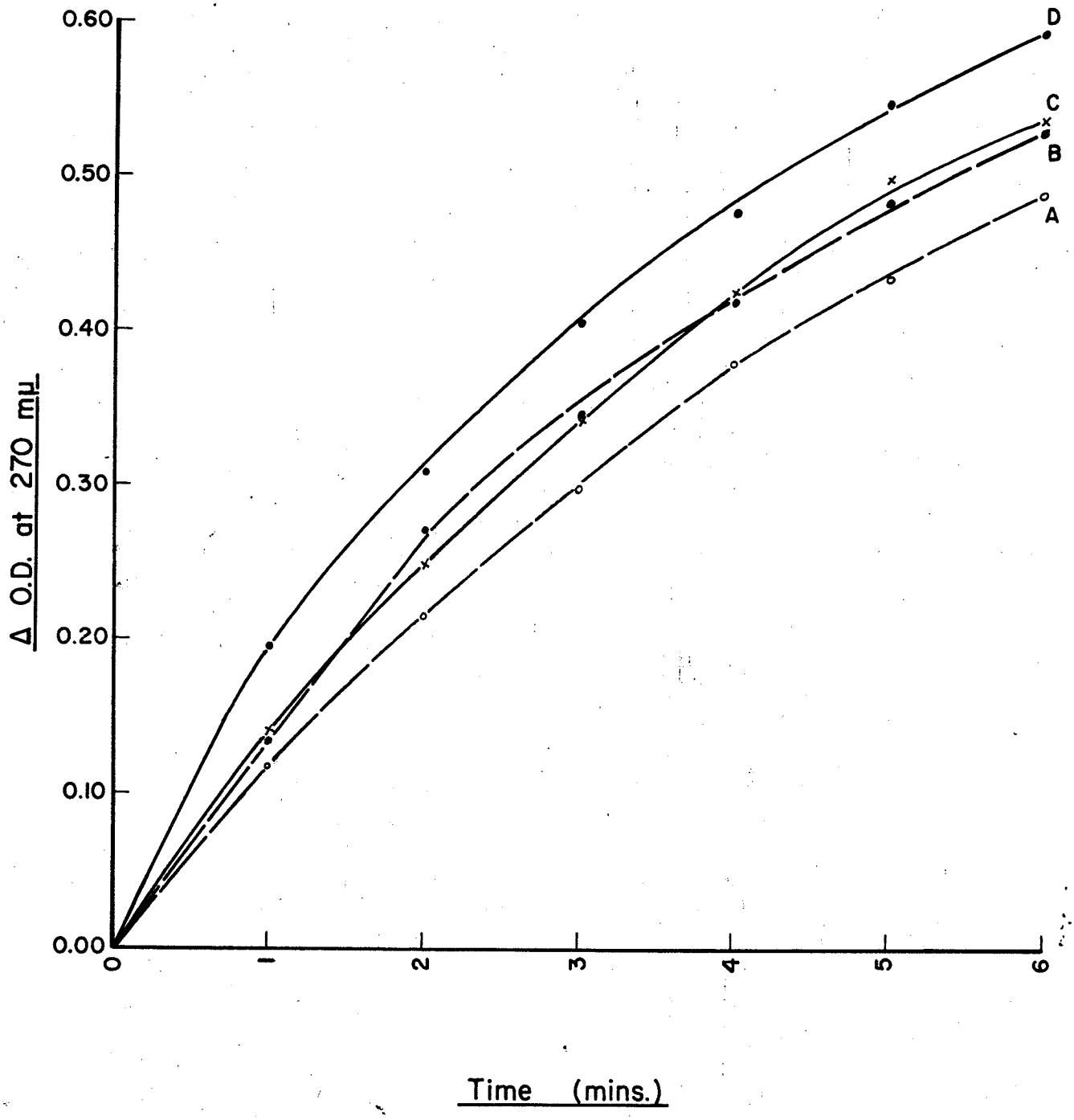

1.20
1.00
0.80
0.60
0.40
0.20
0.00
Δ O.D. at 270 mμ
0
5
10
15
20
25
Time (mins.)

FIGURE 8. Effect of Temperature.

A = Control
B = Heated for 60 minutes.
C = Heated for 40 minutes
D = Heated for 20 minutes.


0.60
0.50
0.40
0.30
0.20
0.10
0.00
Δ O.D. at 270 mμ
0
1
2
3
4
5
6
Time (mins.)
D
C
B
A

pH OPTIMUM

The activity of the acid phosphatase was determined using 0.1 M sodium acetate buffer over a pH range 4.0 to 7.0. The rate of hydrolysis was followed spectrophotometrically. The pH optimum of the acid phosphatase activity is 5.5. There is a sharp increase in the activity on the acid side, decreasing more slowly between 6.0 to 7.0 (Figure 9).

EFFECT OF IONS

The effects of various ions were studied to verify if the acid phosphatase from potato would behave in conformity with other phosphatases reported in the literature. The ions were added to the system and the rate of reaction followed without any preincubation. The final concentration of the ion was 10^{-4} M in 3.0 ml total volume.

Ferric ion (as a chloride), activated the enzyme 18.6% over the control. Manganous ion had a similar effect (+14.5%) whereas Zinc and Magnesium were inhibitory (86.2% and 84.1% respectively). Similarly Copper and Mercury (both added as chlorides) were inhibitory, and decreased the activity to 74.6% and 18.1% respectively, of the control (Table VII).

As shown in Table VII, oxalate (Sodium > Ammonium) activated the enzyme 20 - 50% over the control. Sodium azide increased the activity by 30%. Tartarate and Formate similarly increased

the activity 13 - 14%. Whereas EDTA inhibited the enzyme up to 14%.The phosphatase was inhibited (34%) by Arsenate.

Fluoride (as Potassium salt) activated the initial activity slightly over the control, yet after two minutes the rate of reaction decreased (6% inhibition). The Phosphatase action was completely inhibited by Molybdate.

FIGURE 9. Effect of pH.

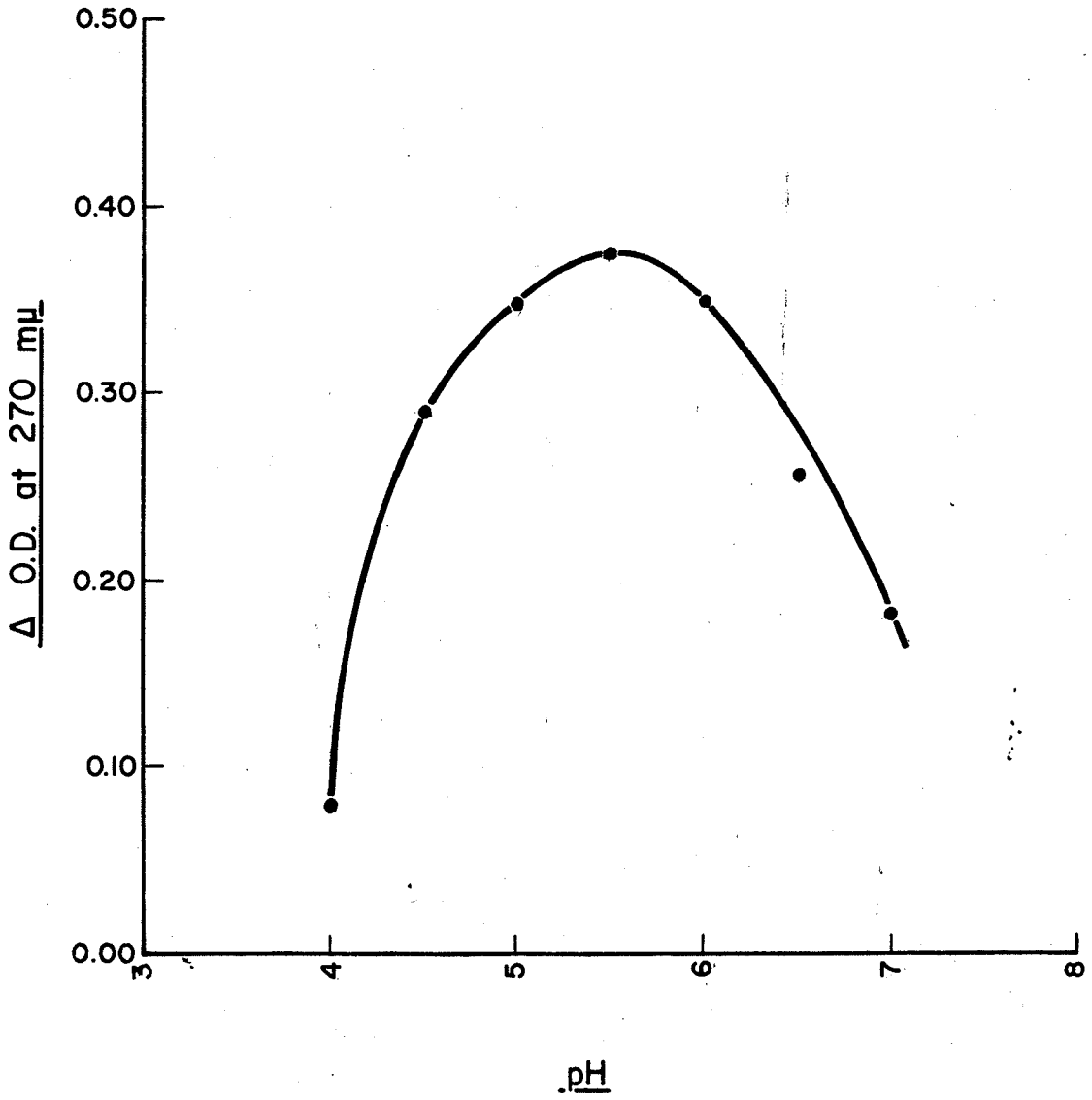


TABLE VII. Effect of ions on the acid phosphatase.

Ion	Concentration	% Activity
Oxalate (Na)	10^{-4} M	149.2
Azide	10^{-4} M	130.6
Oxalate (Amm.)	10^{-4} M	121.8
Ferric chloride	10^{-4} M	118.6
Manganous chloride	10^{-4} M	114.5
Tartarate (Sod.)	10^{-4} M	114.0
Formate (Sod.)	10^{-4} M	113.2
Fluoride	10^{-4} M	94.9
EDTA	10^{-4} M	86.2
Zinc chloride	10^{-4} M	84.1
Magnesium chloride	10^{-4} M	77.6
Cupric chloride	10^{-4} M	74.6
Arsenate (Sod.)	10^{-4} M	64.5
Mercuric chloride	10^{-4} M	18.1
Molybdate (Sod.)	10^{-4} M	0

Rate of reaction was followed without preincubation. Reaction mixture contained: 250 µmoles of Sodium acetate buffer, 3 µmole Phenyl phosphate and 21 units enzyme, 0.3 µmole of the ion in a 3.0 ml volume.

CHAPTER V

DISCUSSION

Several acid phosphatases have been isolated from the higher plants. While the phosphatases are present generally in all tissues, rapidly growing tissues form a rich source. All organs of the higher plants have been source material for the preparation of phosphatases. Roche (1950) suggested seeds and tubers as preferred sources of phosphomonoesterases, type II. Axelrod (1947) extracted a phosphatase from citrus fruits. Spinach leaves were the source material for a phosphatase (Borough 1954; Chakravorty et al 1959). Shuster and Kaplan (1953) extracted β-nucleotidase from germinating barley and rye grass leaves. Wheat leaves were used by Roberts (1956, 1957). Krishnan (1949 a,b.) prepared an apyrase from potato tubers. Naganna et al (1955) extracted phosphatases from potato tubers. The experimental results reported herein are based on an acid phosphatase extracted from potato tubers which will be referred to as the potato phosphatase. Sweet potato tubers were the source material for a phosphatase (Ito et al 1955). Newmark and Wenger (1960) extracted a non specific phosphatase from white lupine seedlings. Pea seeds were used by Turner and Turner (1960). Joyce and Grisolia

(1960) purified a non specific phosphatase from wheat germ.

The potato phosphatase closely resembles the other phosphomonoesterases, in its properties. It has a pH optimum at 5.5. Phosphomonoesterases 11 have an optimum in the range 5.0 to 6.0. Turner and Turner (1960) reported a glucose monophosphatase from pea seeds with pH optimum between 5.4 and 5.7. The optimum pH for a nonspecific acid phosphatase from wheat germ (Joyce and Grisolia 1960) was found to be pH 5.8. The lupine seedling phosphatase (Newmark and Wenger 1960) has an optimum range between 5.2 and 5.5. Similar optima have been reported for phosphatases from spinach leaf (Borough 1954) and citrus juice (Axelrod 1947). Roberts (1956a) suggested that the wheat leaf phosphatase extracted by him with an optimum pH 5.7 was a mixture of several phosphatases. Chakravorty et al (1959) described an acidic C-1 Diphosphatase active between pH 5.0 and 6.0. A 3'-nucleotidase from rye grass (Shuster and Kaplan 1955) has a broad range from pH 6.0 to 9.0. Toward more acidic side (pH 4.5) it shows 100% activity with KCN and Glutathione both of which normally inhibit the enzyme.

The potato phosphatase hydrolysed at different rates all the substrates tested (Table VI). This nonspecificity is general among the phosphomonoesterases. The lupine seedling phosphatase (Newmark and Wenger 1960) hydrolysed hexose phosphates, nucleotides and polynucleotides.

Purified preparation of phosphatase from sweet potato, according to Ito et al (1955) hydrolysed not only phosphomonoesterases but also pyrophosphates. Newmark and Wenger (1960) and Axelrod (1947) also reported pyrophosphatase activity with their phosphomonoesterases. Roche (1950) suggested that phosphomonoesterases and pyrophosphatases of similar pH optima and other physical characteristics are found together in tissues and that often pyrophosphatases are natural contaminents of phosphatase preparations. He suggested further that adsorption and thermal inactivation may be to separate the two activities.

The potato phosphatase is similar to other phosphomonoesterases in its capacity to withstand heating at moderate temperatures. Heated at 50°C for one hour the enzyme did not lose any activity. Newmark and Wenger (1960) reported that their lupine phosphatase retained 90% of the original activity when it was heated at 37°C for two hours or for one hour at 50°C while rapidly being inactivated at 60°C. Similar results were reported by Roberts (1957) (Table III). Thermal inactivation of extraneous proteins was one of the steps of purification used in the preparation of acid phosphatases (Newmark and Wenger 1960).

Phosphomonoesterases 11 are not activated by Mg^{++} ions (Roche 1950). This holds true in the case of the potato phosphatase. Mg^{++} was ineffective on the rate of hydrolysis of glucose

monophosphates by a phosphatase from pea seeds (Turner and Turner 1960). Mg^{++} has generally either no effect or is only slightly stimulatory (Roberts 1956; Joyce and Grisolia 1960; Newmark and Wenger 1960). However these experiments show in the case of the potato phosphatase, there appears to be a slight inhibition by magnesium.

Manganese was slightly stimulatory in its effect on the potato phosphatase. Joyce and Grisolia (1960) also reported such an effect of Mn^{++} on their wheat germ phosphatase.

In the present investigation 26% inhibition of the potato phosphatase was noted in the presence of Zn^{++} (1×10^{-4} M. Table VII). For the lupine seedling phosphatase the inhibition values reported by Newmark and Wenger (1960) are 83% and 45% at levels of 1×10^{-3} M and 1×10^{-4} M respectively.

The potato phosphatase was inhibited up to 36% by copper (1×10^{-4} M. Table VII). Joyce and Grisolia (1960) recorded 75% inhibition by Cu^{++} (1.2×10^{-2} M). of the activity of a nonspecific phosphatase from wheat germ. Newmark and Wenger (1960) observed approximately 98% and 70% inhibition of lupine seedling phosphatase in the presence of Cu^{++} at concentrations of $1x\ 10^{-3}$ M and 1×10^{-4} M respectively.

Mercury caused an inhibition of approximately 82% in the present work with the potato phosphatase (Table VII). Turner and Turner (1960) observed an inhibition of about 70% of the pea seed

phosphatase activity by Hg^{++} with both glucose-1-phosphate and glucose-6-phosphate as substrates. The hydrolysis of 3-phosphoglycerate, 2-phosphoglycerate and 2,3-diphosphoglycerate by a nonspecific phosphatase, obtained from wheat germ, was totally inhibited by Hg^{++} (Joyce and Grisolia 1960).

In the present work with the potato phosphatase EDTA has shown a slight inhibition (14% Table VII), in contradiction to the reported protective action on the lupine seedling phosphatase (Newmark and Wenger 1960).

Oxalate activated the potato phosphatase (Table VII). In this it differs from Roche's claim that oxalate inhibits the phosphomonoesterases 11.

Similarly azide,formate and tartarate stimulated the potato phosphatase activity (30%, 13% and 14% respectively). Tartarate was suggested to be phosphatase inhibitory (Delory and Hetherington 1961).

Fluoride was inhibitory to the potato phosphatase to about 7% (Table VII), at a concentration of 1×10^{-4} M. This conforms with Roche(1950).

The potato phosphatase was completely inhibited by molybdate (1×10^{-4} M, Table VII). Turner and Turner (1960) reported inhibition of their pea seed phosphatase by molybdate. Roberts (1956) demonstrated molybdate inhibition (20 - 70% of activity) of wheat leaf phosphatase.

Thus in general behaviour towards the inhibitors of phosphomonoesterase 11, the potato phosphatase reacts as a typical acid phosphatase.

One of the interesting features that came to light when this phosphatase was subjected to heating at 50°C was that this enzyme is heat activated. When held at 50°C for 20 minutes this enzyme has shown an increase of 20% activity over the control. On further heating while there was a decrease in activity the treated enzyme was slightly more active than the control even after one hour. No explanation or similar results were reported in the literature about the phosphomonoesterase.

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