ACYLPHOSPHATASE

STUDIES ON ACYLPHOSPHATASE OF *Vigna Catjang*

A Thesis
submitted to the
UNIVERSITY OF POONA
for the degree of
DOCTOR OF PHILOSOPHY

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October 1 9 7 3

ACKNOWLEDGMENT

I take this opportunity to express my deep sense of gratitude to Dr. V. Jagannathan, National Chemical Laboratory, Poona for his assistance and guidance throughout the course of this investigation.

I wish to record my grateful thanks to Mr H. G. Vartak, Dr. S. V. Paranjpe and Mr A. M. Bodhe for help in preparing large batches of enzyme and Mr Mazhar Husain in estimation of sulfhydryl groups.

I gratefully acknowledge the help given by Dr. C.SivaRaman in carrying out the ultracentrifugal studies.

My thanks are also due to the Council of Scientific and Industrial Research for the award of a fellowship and the Director, National Chemical Laboratory, Poona for permission to submit this work in the form of a thesis for the degree of Doctor of Philosophy.

V. V. Deshpande

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CHAPTER I

INTRODUCTION

INTRODUCTION

SECTION I

Acylphosphatase phosphohydrolase (E.C.3.6.1.7) (acylphosphatase) catalyzes the hydrolysis of acyl phosphates. This enzyme was discovered by Lipmann during the course of his studies on acetyl phosphate. The enzyme is specific for acylphosphates and does not act on other phosphate anhydrides or esters. Several workers have purified the enzyme from animal tissues. It has been obtained in highly purified form from horse muscle, bovine brain, pork heart and human erythrocytes and the enzyme from rabbit muscle has been crystallized. Its occurrence and role in plants have, however, not been studied. The present work deals with the isolation in pure form of a specific acylphosphatase from the seeds of *Vigna catjang* and a study of its properties, kinetics and biological role.

SECTION II

HISTORICAL

Acylphosphatase was discovered by Lipmann in 1945. He found that acetyl phosphate was rapidly destroyed by the action of an unknown enzyme from animal tissues. Later the enzyme was identified as a specific acylphosphatase, which could be readily obtained in soluble form from animal tissues. Muscle was shown to have high acylphosphatase activity, whereas the common phosphatases are absent or present only in small amounts in this tissue. The enzyme was found to have no action on phosphate esters. Its enzymic nature was established by the fact that it was nondialyzable and was completely destroyed by the action of pepsin. The enzyme was found to be heat stable, especially in acid solution. It was also not denatured by trichloroacetic acid. These properties of the enzyme were used for its partial purification and by heating, trichloroacetic acid precipitation and acetone fractionation a preparation was obtained which showed the properties of a basic protein. This enzyme was inhibited by several polyvalent anions. Inorganic phosphate was a potent inhibitor and less strong inhibition was observed with sulphate, citrate and oxalate. Fluoride had very little effect on its activity.

Shapiro and Wertheimer (1945) also demonstrated rapid enzymic hydrolysis of acetyl phosphate by extracts and homogenates of liver,

brain, muscle and kidney. All these tissues had approximately the same activity. There was no enzymic activity in serum. The hydrolysis of acetyl phosphate was shown to be inhibited by phosphate but not by fluoride or cyanide. The activity was totally destroyed by heating to 100° for 5 min.

The enzyme from horse muscle was purified by Koshland (1955), Harary (1963) and Ramponi, Guerritore, Treves, Nassi and Baccari (1969). Extensive purification of the enzyme from bovine brain was reported by Raijman, Grisolia and Edelhoch (1960) and Diederich and Grisolia (1969). The latter have also obtained the pork heart enzyme in highly purified form (Diederich and Grisolia, 1971). Other animal tissue enzymes which have been purified are those from chicken muscle (Pechere, 1957) and human erythrocytes (Rakitzis and Mills, 1969). Shiokawa and Noda (1970) obtained the rabbit muscle enzyme in crystalline form.

The metabolic function of this enzyme is not well understood. Bacteria, which synthesize and metabolize acetyl phosphate generally contain very little or no acylphosphatase. Animal tissues, on the other hand, do not form acetyl phosphate. (Acetyl phosphate and formyl phosphate can be formed by the action of carbamyl phosphate synthetase but they are not important metabolic intermediates in animal tissues). Possible substrates for this enzyme are the glycolytic intermediate, 1,3 diphosphoglyceric acid and carbamyl phosphate. The observation that acylphosphatase is inhibited by a very low concentration of thyroxine is of considerable interest.

The isolation of the enzyme in highly purified form and its very low molecular weight have stimulated considerable interest in acylphosphatase. In recent years studies on ultracentrifugation and electrophoresis, amino acid composition, molecular weight and other properties and kinetics of the enzyme have been described.

SECTION III

OCCURRENCE

Acylphosphatase is widely distributed in animal tissues. It has been reported (Harary, 1963) to be present in guinea pig, rabbit and bovine retina, brain, kidney, spleen, bone marrow and testes.The occurrence of this enzyme in plants has not hitherto been reported.

Animal tissues:

1) Muscle. The demonstration of the enzyme in horse muscle (Lipmann, 1945) and its purification (Koshland, 1955; Harary, 1963; Ramponi, Guerritore, Treves, Nassi and Baccari, 1969) have been described earlier. Reference has also been made to the isolation of crystalline acylphosphatase from rabbit muscle (Shiokawa and Noda, 1970). Pechere (1957) purified the enzyme from chicken muscle and obtained three different fractions with enzyme activity.

Heart. Diederich and Grisolia (1971) purified the enzyme from pork heart.

Brain. The enzyme from this tissue has also been obtained in highly purified form (Diederich and Grisolia, 1969).

Erythrocytes. The enzyme was shown to be present in human erythrocytes and was purified by Rakitzis and Mills (1969).

Placenta. The acylphosphatase of human placenta was studied by Guerritore and Dellepiane (1955). The acetylphosphatase content of placenta was less than that of other organs.

Tumours. Dinescu and Kâtulescu (1964) observed that there was a higher acetylphosphatase activity in Jensen sarcoma, Guerin carcinoma, Walker carcinoma 256 and Yoshida sarcoma than in normal rat kidney, muscle, spleen and liver and negligible activity in ascitic Ehrlich carcinoma. There was no difference in the levels of the enzyme in whole blood of healthy and cancer hearing individuals.

Acylphosphatase of house-fly

An extract of acetone powder of house-flies hydrolyzed acetylphosphate and was shown to be a mixture of acetylphosphatase and phosphotransacetylase (Sucker, 1965).

Acylphosphatase and Na^+-K^+ ATPase.

A K^+ stimulated acetylphosphatase of brain microsomes was described (Sachs, Rose and Hirschowitz, 1967). Microsomal Na^+-K^+ ATPase was compared with K^+ acetylphosphatase (Yedy Israel and Elwood Titus, 1967). On the basis of differences in the inhibitory effects of ouabain, oligomycin and N-methylmaleimide it has been suggested that the K^+-acetyl phosphatase activity of beef brain may represent an entity different from Na^+-K^+ ATPase.

This survey will not deal with the extensive literature on Na^+-K^+ ATPase which lies outside the scope of the present work.

2) Localization of acylphosphatase

Very little work has been done on the distribution of the enzyme between nuclei, mitochondria and other cell components.

Zanobini, Ramponi and Guerritore (1962) observed that when mitochondria are kept for 15 min in 0.3 M sucrose or in hypotonic solution there is a parallelism between the release of acetylphosphatase activity and the swelling effect.

The distribution of carbamyl phosphatase activity in cells differs with the tissue studied (Melani, Ramponi and Guerritore, 1961). Of the total activity of homogenates of skeletal muscle and brain of rats 85% was in the soluble fraction, whereas from the homogenates of liver and kidney about 45% was in the soluble fraction. The percentage of soluble enzyme in liver and kidney appears to be less than that in muscle and brain. But no conclusive evidence has been presented for the occurrence of particulate acylphosphatase.

SECTION IV

Types of acylphosphatase

(1) Acid-stable and acid-unstable types

The acylphosphatase activities obtained from liver mitochondria and muscle differ from each other in their behaviour to heat at low pH and in their reactions to anions (Zanobini, Ramponi and Guerritore, 1961). Apparently acylphosphatase occurs in both heat-stable and heat-labile forms. The heat-labile form predominates in liver while the heat-stable form is preponderant in muscle. The heat-labile form is not inhibited by phosphate in contrast to the inhibition of the heat-stable form by phosphate as well as by a number of polyvalent anions. Further work is needed to establish that both forms are true acylphosphatases.

(2) Carbamyl phosphatase and acylphosphatase

A phosphatase which hydrolyzes carbamyl phosphate has been demonstrated in a number of tissues. It was noted by Grisolia (1960) that a purified preparation from bovine brain acted both upon acetylphosphate and carbamyl phosphate. Thus carbamylphosphatase was shown to be the same enzyme as acetylphosphatase previously described by Lipmann (1945). The enzyme was found to be widespread in animal tissues. Until recently very little was known about the physiological function of acylphosphatase. The fact that carbamyl phosphate and 1,3-diphosphoglyceric acid could be the naturally occurring substrates

for this enzyme suggests a possible role for acylphosphatase.

Purified preparations of acylphosphatase from muscle, brain and heart act upon carbamyl phosphate. The ratio of the activity with acetyl phosphate to that with carbamyl phosphate for purified preparations of acylphosphatase from horse muscle, beef brain, pig heart and human erythrocytes were 11, 10, 11 and 0 respectively. The ratio is the same for the first three enzymes whereas acylphosphatase from human erythrocytes does not act upon carbamyl phosphate.

An extract of liver mitochondria has been reported to have acetylphosphatase activity which is twice that of carbamylphosphatase activity (Melani et al. 1961).

(3) Isoenzymes

During the purification of acylphosphatase from rabbit muscle (Shiokawa and Noda, 1970) the enzymic activity was separated into three fractions A, B and C by chromatography on carboxymethyl cellulose at pH 4.8. The fractions B and C were obtained by eluting the enzyme at 0.16 M and 0.25 M NaCl respectively. The specific acgivities of these two fractions were nearly the same. Fraction A was obtained by eluting the enzyme at 0.1 M NaCl and its specific activity was about half that of fractions B and C. The crystalline enzyme was obtained from fraction B which contained most of the activity. The activities represented by peaks A and C were postulated to be isoenzymes of the crystalline enzyme.

The existence of a second acetylphosphatase was also reported in human erythrocytes by Rakitzis and Mills (1969). The final specific activity of acylphosphatase purified from human erythrocytes ranged from 700 to 6,800 (umoles of acetylphosphate hydrolysed per mg protein per hour at pH 5.4). Preparations of low specific activity usually had traces of hemoglobin. The enzyme obtained after the CM-cellulose step could be adsorbed on DEAE-cellulose at pH 9.4 but at least one fourth of the original activity of the crude hemolysate was retained on a DEAE-cellulose column at pH 6.9. These observations suggested the presence of an enzyme which is different from the more basic purified acylphosphatase. The former enzyme was not further investigated.

Pechere (1957) obtained three different fractions with enzyme activity from chicken muscle but whether they are isoenzymes or not is not known.

These reports suggest the existence of isoenzymes of acylphosphatase in different tissues but none of them is well characterized.

SECTION V

METHODS OF ESTIMATION

(1) Differential precipitation method

Lipmann and Tuttle in 1944 introduced a method based on the differential precipitation of calcium acetylphosphate and inorganic phosphate. With the molybdate reagent of Fiske and Subbarow complete decomposition of acetylphosphate occurs at room temperature in less than 10 min. Hence direct colorimetry yields an apparent phosphate value which represents the sum of acetylphosphate and inorganic phosphate. True inorganic phosphate is estimated after precipitation with alcoholic calcium chloride solution which leaves the soluble calcium acetylphosphate in the supernatant liquid. The difference between apparent and true inorganic phosphate is regarded as acetylphosphate. This method has not been widely used.

(2) Lipmann and Tuttle's hydroxylamine method (1945)

This method is based on the measurement of residual acetylphosphate. On treatment with hydroxylamine acylphosphates are converted into hydroxamic acids as follows:

$$RCOOPO_3^{=} + NH_2OH \longrightarrow R.CONHOH + HPO_4^{=}$$

acylphosphate — hydroxylamine — hydroxamate

The hydroxamic acid forms a colored complex with trivalent iron.

The color varies from orange-brown to purplish brown depending on the concentration of acylphosphate. The main absorption of the purple iron complex is between 480 to 540 mm in which region ferric chloride does not absorb. A number of anions such as fluoride, phosphate, oxalate, sulphate and citrate depress the intensity of the color by forming iron complexes, but they do not interfere with the reaction between acylphosphate and hydroxylamine.

This method is widely used because it is rapid and convenient and applicable over a wide range of conditions such as pH, enzyme concentration and different buffer systems.

(3) Spectrophotometric method

Ramponi et al. (1966) introduced this method for the rapid estimation of acylphosphatase and the study of its properties. It makes use of aromatic substrates such as benzoyl phosphate or p-nitrobenzoyl phosphate, since the phosphorylation of these compounds is accompanied by marked change in absorption in the ultraviolet. This method is rapid and sensitive and, since the progress of the reaction can be followed without stopping it as in the hydroxamate method, it is particularly suitable for kinetic studies.

(4) Micromethod for acetylphosphate determination

Recently Satchell and White (1970) have developed a new method based partly upon Lipmann's method (1945) and partly upon that introduced by Mission (1908) for the determination of inorganic phosphate. The basis of the new micromethod is to convert acylphosphate to hydro-

procedure and then to estimate colorimetrically not the hydroxamic acid but the inorganic phosphate. This was accomplished by forming the phosphomolybdovanadate complex, extracting it with butanol and measuring the colour of the butanol layers at 310 nm against a reagent blank (Parvin and Smith, 1969).

SECTION VI

PURIFICATION

Animal tissues

Horse muscle

Lipmann (1945) partially purified the enzyme from horse muscle by heating, trichloroacetic acid precipitation and acetone fractionation and Koshland (1955) obtained a 37-fold preparation by a slightly modified procedure from the same source. Harary (1963) reported a 635-fold purification of acylphosphatase from horse muscle. His purification procedure consists of heating the enzyme in acid, trichloroacetic acid precipitation and ammonium sulphate fractionation. The final specific activity of the enzyme was 3,810 units* per milligram (at 37° and pH 5.4). Ramponi et al. (1969) have described a procedure for the purification of acylphosphatase from horse muscle, which consists of extraction with acid and two successive chromatographic separations on CM-Sephadex C-25. They used benzoyl phosphate as substrate. The final specific activity of this highly purified preparation from horse muscle was 72,000 units/mg. The homogeneity of the final product was established by starch gel electrophoresis, acrylamide gel electrophoresis, gel filtration on Sephadex G-75 and ultracentrifugation.

(*Enzyme activities have been calculated in terms of/umoles of acetylphosphate hydrolyzed per hour).

Rabbit muscle

A crystalline acylphosphatase was obtained from rabbit muscle by Shiokawa and Neda (1970). The crystalline enzyme had a specific activity of 51,600 units per mg (at 25° and pH 5.4). This purification was achieved by acid extraction, chromatography on CM-cellulose and ammonium sulphate fractionation. The enzyme was homogeneous by ultracentrifugation and disc electrophoresis on acrylamide gel.

Brain

Bovine brain. Raijman, Grisolia and Edelhoch (1960) purified the enzyme from bovine brain. Their purification procedure involved acidification to pH 4, precipitation with sodium picrate, precipitation with acetone, removal of impurities with sodium sulfosalicylate, acetone precipitation, chromatography on DEAE-cellulose and adsorption on bentonite. The final specific activity of this preparation was 7,800 units per mg. Diederich and Grisolia (1969) obtained a highly purified preparation of the enzyme from the same source. Their purification procedure involved the following steps: extraction of the tissue at pH 4, acetone precipitation, removal of proteins by sulfosalicylate and two subsequent fractionations on columns of Bio-Rex-70 resin. The specific activity of the purified preparation was 75,000 units per mg. Acrylamide gel electrophoresis of the purified enzyme indicated a single band at pH 8.3 and pH 4.5.

Erythrocytes

A specific acylphosphatase was purified by Rakitzis and

Mills (1969) from human erythrocytes. Blood was centrifuged and the plasma and buffy coat were removed. The packed cells were lysed and the hemolysate was applied to a CM-cellulose column in 0.005 M phosphate buffer at pH 7.5 and eluted with 0.5 M NaCl. It was then transferred to a DEAE-cellulose column at pH 9.4 in 0.01 M Na_2HPO_4 solution and eluted with 0.01 M phosphate buffer at pH 6.0. The specific activity of the final preparation ranged from 700 to 6,800 units per mg. Preparations of low specific activity usually had traces of hemoglobin as the major protein contaminant. The variation in activity was attributed to contamination with hemoglobin, but no data on inhibition of the enzyme by hemoglobin were presented.

Heart

Pork heart. Acylphosphatase from pork heart was purified by Diederich and Grisolia (1971). The purification procedure involved heating the acid extract of the enzyme at 70°, acetone fractionation and chromatography on two successive columns of Bio-Rex-70 resin. The final specific activity of the purest fraction was 36,000 units per mg and it appeared as a single homogeneous band when subjected to acrylamide disc electrophoresis at pH 8.3 and pH 4.5.

The results on the purification of acylphosphatase from animal tissues are summarized in Table 1. It will be seen that the maximum specific activity of the enzyme varies with the tissue. It is highest for the enzyme from brain and the lowest for the enzyme from erythrocytes and vanes from 700 to 75,000. The reason for this variation is not known, especially in the case of the erythrocyte

enzyme the activity of which differs by one to two orders of magnitude from that of the other enzymes. But it should be noted that the erythrocyte enzyme also differs in its specificity and, unlike the other enzymes, has no action on carbamyl phosphate.

TABLE 1

SUMMARY OF PURIFICATION OF ACYLPHOSPHATASE FROM ANIMAL TISSUES

Nature of the tissue	pH	Temp.	Maximum specific activity
			units/mg protein
Horse muscle	5.3	25°	30,000
Rabbit muscle	5.4	25°	51,600
Bovine brain	-	27°	75,000
Human erythrocytes	5.4	37°	700 - 7,000
Pork heart	-	27°	36,000

Activities are expressed as μmoles of acetylphosphate hydrolyzed per hour.

SECTION VII

PROPERTIES

(1) Effect of temperature

Acylphosphatase from muscle is heat-stable. At acid pHs the enzyme withstands heating to 60° for 20 min without loss in activity. Heating under the same conditions at pH 8.6 in tris buffer leads to a loss of approximately 33 to 50 per cent of the activity.

Partially purified preparations of acylphosphatase from brain are also very heat-stable. The partially purified brain enzyme could be heated to 80° for 15 min between pH 1 to 7 with only little (10 to 20%) loss in activity. The thermal stability of purer fractions is less than that of the crude fraction. Heating of the dialyzed purified preparation at 37° for 20 min results in 27 per cent loss in activity.

In contrast to the muscle and brain acylphosphatases, the enzyme from erythrocytes is thermolabile. The stability of erythrocyte acylphosphatase was tested with the crude hemolysate as well as with the purified enzyme. Heating for 10 min at 60° over a pH range of 5-9 completely destroyed the activity of the crude hemolysate. Heating of the purified preparation at 70° for 5 min destroyed 40% of its activity.

Thus stability decreases for the muscle and brain enzymes on purification but increases for the erythrocyte enzyme. The enzyme is more stable to acid than to alkaline conditions.

(2) Substrate specificity

Acylphosphatase of animal tissues does not catalyze the hydrolysis of pyrophosphate, ATP, ADP, AMP, acetyl adenylate, acetylcholine, acetyl CoA or glycerol phosphate. It catalyzes the hydrolysis of propionyl, butyryl, succinyl phosphate as well as acetylphosphate.

Muscle

Horse muscle (Ramponi et al. 1969)

Pure acylphosphatase from horse muscle catalyzes the hydrolysis of compounds, which are anhydrides of carboxyl and phosphate groups. However, compounds of the type acyl-AMP (such as amino acid-AMP anhydrides formed by amino acid activation during protein synthesis) are not hydrolyzed. The phosphate group should presumably be unsubstituted. The enzyme showed activity with p-nitrobenzoyl phosphate, benzoyl phosphate, 3-phosphoglyceryl phosphate, acetylphosphate and carbamyl phosphate. There is obviously less specificity regarding the nature of the group attached to the carboxyl group. The enzyme possesses very little activity towards phosphocreatine, adenosine-5'-triphosphate, pyrophosphate, and p-nitrophenylphosphate at pH 5.3. It does not split p-nitrophenylphosphate at pH 10.4, phosphoenolpyruvate, acetyl AMP or phosvitine. The enzyme is, therefore, specific for compounds of the type $R.CO.O.PO_3H_2$. Further work is, however, needed to clarify the effect of chain length and substituents in "R". The relative rates of hydrolysis of p-nitrophenyl phosphate, benzoyl phosphate, 3-phosphoglyceryl phosphate, acetylphosphate and carbamyl phosphate were 21, 6, 1.2, 1 and 0.09 respectively. Relative activities varying from

5.8×10^{-3} - 7.6×10^{-5} (relative to 1 for acetylphosphate) were reported for phosphocreatine, ATP, pyrophosphate and p-nitrophenyl phosphate, but these activities are very low and may be due to impurities.

Bovine brain (Diederich and Grisolia, 1969)

Acylphosphatase from bovine brain hydrolyzes acetylphosphate, carbamyl phosphate and 1:3 diphosphoglycerate. It was noted that the ratios of activity with acetylphosphate and carbamyl phosphate were essentially the same during 100-fold purification. The same enzyme, therefore, catalyzes the hydrolysis of all the three compounds. The acetylphosphate/carbamyl phosphate activity ratio for the purified enzyme was 10 and the activity ratio of acetylphosphate/diphosphoglycerate was found to be 9. The enzyme has no effect on ATP, AMP and phosphoenol pyruvate.

Human erythrocytes (Rakitzis and Mills, 1969)

Acylphosphatase purified from human erythrocytes hydrolyzes acetylphosphate and 1,3 diphosphoglycerate but not carbamyl phosphate. Moreover no enzyme activity could be detected with 3-phosphoglycerate, phosphoenol pyruvate, fructose-6-phosphate, ATP, ADP, 3',5' cyclic AMP, 6-phosphogluconate, phosphoserine, inorganic pyrophosphate and p-nitrophenyl phosphate.

Pork heart (Diederich and Grisolia, 1971)

Purified pork heart acylphosphatase acts on acetylphosphate, carbamyl phosphate and 1,3 diphosphoglycerate. The ratio of activity

with acetylphosphate to that with carbamyl phosphate or diphosphoglycerate was 11.

It will be seen that all animal tissue acylphosphatases act both on acetylphosphate and 1,3 diphosphoglyceric acid and that with the exception of the erythrocyte enzyme they also act on carbamyl phosphate.

(3) Activators and inhibitors

(i) Horse muscle (Ramponi et al. 1969)

Acylphosphatase from muscle does not seem to require a metal for its activity. This was shown by the lack of effect of metal ions and metal-binding inhibitors such as EDTA or α ,α -dipyridyl. Iodobenzoate, iodoacetate and pCMB do not inhibit the enzyme indicating that -SH groups are not necessary for activity. The enzyme is competitively inhibited by ortho- and pyrophosphate and irreversibly by preincubation with 10^{-5}M thyroxine.

(ii) Bovine Brain (Diederich and Grisolia, 1969)

Caffeine. Up to 2.5×10^{-2}M caffeine had no effect on enzyme activity.

Adrenaline. Adrenaline at 1.25×10^{-2}M inhibited the enzyme 65%.

Inosinic acid. Inosinic acid had no effect upto 2.3×10^{-3}M; 33% activation was noted at 4.6×10^{-3}M but further increase in concentration (upto 7×10^{-3}M) did not produce further activation.

2,4-Dinitrophenol. DNP at 2.5 x 10^{-4}M when preincubated with the enzyme inhibited 50% of the activity, but it was approximately only half as effective when added directly to the incnbation mixture.

Phosphate. With 2 x 10^{-2}M phosphate 50 to 60% inhibition was observed. 4 x 10^{-2}M phosphate inhibited 70 to 80% of the activity.

Urea and guanidine. When acylphosphatase was dissolved in 5 M urea or 5 M guanidine and assayed, there was complete inhibition of enzyme activity. This type of inhibition was, however, reversible since 95% of the original activity was recovered upon lowering the concentration of the denaturant by dilution. Any unfolding of the peptide chain caused by urea or guanidine is, therefore, completely reversible.

Proteolytic enzymes

Preincubation of the enzyme with 2% pepsin at pH 2 and at 37° for 10 min inactivated the enzyme entirely. Trypsin and papain at 0.5 and 1% concentration respectively, when preincubated with the enzyme at pH 7 and 37° for 10 min, had no effect on enzyme activity. A slight activation of about 15% was sometimes observed after papain digestion. However, when the papain digested acylphosphatase was dialyzed for 30 min at 3-4° against very small volumes of deionized water, approximately 10% of the activity was found in the diffusate whereas none appeared in the controls. No further work has been reported on this interesting observation.

Mercuric chloride and iodoacetate

Mercuric chloride at 4 x 10^{-5}M and iodoacetate at 4 x 10^{-3}M in

the assay system showed no inhibition.

KCl and $MgCl_2$

15 mM KCl and 5 mM $MgCl_2$ were found to enhance enzyme activity when tris-acetyl phosphate was used as a substrate. This is the only report of metal activation of this enzyme.

(iii) Human erythrocytes (Rakitzis and Mills, 1969)

The effect of several metabolic intermediates on the erythrocyte enzyme was determined using acetyl phosphate and 1,3 diphosphoglyceric acid as substrates.

At 5 mM inhibition by phosphoenol pyruvate, 6-phosphogluconate, fructose-6-phosphate, fructose 1:6 diphosphate and glucose-6-phosphate was 12, 23, 32, 44 and 50 per cent respectively. Inhibition in the presence of either acetyl phosphate or 1,3-diphosphoglyceric acid was nearly the same with ATP and phosphoenolpyruvate, 3-Phosphoglyceric acid showed 20 per cent inhibition with 1,3 diphosphoglycerate and only 5 per cent with acetylphosphate. NADH showed negligible effect on activity. Further work is needed to assess the significance of these inhibitions at physiologically occurring concentrations of these compounds.

pCMB and iodoacetate do not inhibit the enzyme appreciably. Thyroxine and triiodothyronine are both inhibitory (74, 54 and 57 per cent inhibition by 0.1 mM D, L-thyroxine and triiodothyronine). Sodium fluoride and EDTA inhibit significantly. Mg^{++} at 10 mm concentration did not have any effect on erythrocyte acylphosphatase at pH 7.5. Further work is needed on the reason for EDTA inhibition. The erythrocyte enzyme differs from other animal tissue enzymes not only in

its lack of action on carbamyl phosphate and low specificity but also in its inhibition by NaF and EDTA.

(iv) Pork heart (Diederich and Grisolia, 1971)

The effect of phosphate on pork heart acylphosphatase was studied using acetyl phosphate as substrate. Pi, ATP and 3-phosphoglycerate showed 37%, 11% and 8% inhibition respectively when 1,3-diphosphoglyceric acid was the substrate. $HgCl_2$ (2 mM) pCMB (1 mM) and iodoacetate (10 mM) showed negligible effect on acylphosphatase activity.

(4) Optimum pH

The optimum pH for the brain enzyme was 7.4 - 7.6 as against 5.3 for the horse muscle enzyme. Rakitzis and Mills (1969) found the optimum pH of the human erythrocyte enzyme to be 5. The optimum pH range for the hydrolysis of acetyl phosphate by heart acylphosphatase as determined by Diederich and Grisolia was also 5.4 to 5.6.

(5) Isoelectric pH

Harary (1963) found an isoelectric point of pH 8.6 for horse muscle acylphosphatase by paper electrophoresis. However, Ramponi et al. (1967) by the method of thin layer gel filtration on Sephadex G-75 obtained a value of 11.4 for the isoelectric pH of muscle acylphosphatase. The isoelectric pH for the erythrocyte enzyme as determined by Rakitzis and Mills (1969) was 8.9 and that for the heart enzyme was 7.25 to 7.3 as determined by electrofusing.

(6) Ultracentrifugation studies (Table 2)

Horse muscle (Ramponi et al. 1969)

The sedimentation constants at 5.4 mg/ml and 8.8 mg/ml were $S_{20,w}$ = 1.23 x 10^{-13} and 1.32 x 10^{-13} and the diffusion constants were 10.71 x 10^{-7} and 12.38 x 10^{-7} respectively. The molecular weights as determined by the Archibald method were 8,450 and 10,300 respectively at the above two concentrations.

Rabbit muscle

The acylphosphatase from rabbit muscle crystallized by Shiokawa and Noda (1970) sedimented as a single peak in the analytical ultracentrifuge. The sedimentation coefficient $S_{20,w}$ was calculated to be 2.1 and the molecular weight as determined by the sedimentation equilibrium method was 23,500.

Bovine brain (Diederich and Grisolia, 1969)

The bovine brain enzyme sedimented as a single peak in the ultracentrifuge with $S_{20,w}$ of 1.25 at a concentration of 0.1 and 0.5% protein in 0.1 M acetate at pH 4.7. The molecular weight was calculated to be 13,000.

(7) Amino acid composition

Amino acid analyses have been reported for the purified enzymes from three sources (Table 3).

Horse muscle (Rampeni et al. 1969).

From the specific volumes of constituent amino acid residues the partial specific volume $\bar{V}$ was calculated to be 0.725 ml/g. The ratio of polar to apolar amino acid residues was found to be 2.05, which places the enzyme in the group of proteins with a large proportion of polar residues.

TABLE 2

SEDIMENTATION CONSTANTS AND MOLECULAR WEIGHT OF ACYLPHOSPHATASE FROM DIFFERENT SOURCES

Tissue	Sedimentation constant	Molecular weight
	$S_{20,w} \times 10^{13}$	Daltons
Horse muscle	1.32	10,300
Rabbit muscle	2.1	23,500
Bovine brain	1.25	13,000
Pork heart	-	11,095

TABLE 3

AMINO ACID ANALYSIS: DATA FOR ANIMAL TISSUE ACYLPHOSPHATASES

Amino acid	Residues per mole of enzyme		
	Rabbit muscle	Horse muscle	Bovine brain
Cysteic acid	-	2	-
Aspartic acid	17	6	7
Threonine	15	5	5
Serine	24	10	4
Glutamic acid	17	9	11
Proline	7	3	3
Glycine	20	7	7
Alanine	6	3	4
Valine	22	8	7
Ieoleucine	8	2	3
Leucine	7	3	5
Tyrosine	8	3	2
Phenylalanine	8	3	4
Lysine	-	8	7
Histidine	1	1	2
Arginine	10	5	3
Tryptophan	3	-	2
Methionine	4	2	1
	Ammonia	7	

Calculation on

Rabbit muscle

The amino acid composition of crystalline acylphosphatase from rabbit muscle was determined by Shiokawa and Noda. It was noted that only one histidine residue is present in a molecule of the enzyme (Shiokawa and Noda, 1970).

Bovine brain (Diederich and Grisolia, 1969)

The molecular weight of bovine brain acylphosphatase determined on the basis of amino acid composition was found to be 8,732. The tryptophan content was calculated to be 2.1 moles/mole of the enzyme. The sulfhydryl content was 1×10^{-6} mole of -SH groups per 8×10^{-5} mole of enzyme which is equivalent to only about 0.01 mole of -SH groups per mole of enzyme. It was not possible to detect an NH_2-terminal amino acid derivative by either the fluorodinitrobenzene or the fluoresceinisothiocyanate method indicating that this group is substituted.

(8) Kinetics

Horse muscle

Harary (1953) found that the Michaelis-Menten constant for acetylphosphatase from muscle was 8×10^{-3} M for acetylphosphate and about 10^{-5} M for 1,3-diphosphoglyceric acid.

Human erythrocytes

A rather broad range of K_m values from 7.4 to 12.7 was noted with acetyl phosphate as substrate (Rakitzis and Mills, 1969). This variation in K_m values may be attributed to the presence of different proportions of isoenzymes in different enzyme preparations or to the presence of small and variable amounts of inorganic phosphate as

impurity in different samples of acetylphosphate. Determination of K_m with a crude hemolysate using acetylphosphate as substrate gave a value of 8.6 mM and the maximal velocity was 300 µmoles/ml/h at pH 7.5. A K_m of 117 µM was obtained for acylphosphatase from erythrocytes using 1:3-DPGA and the maximal velocity with 1:3-DPGA was 47.5 µmoles/ml/h.

The inhibition of acylphosphatase by ATP at pH 7.5 was studied using acetylphosphate as substrate. ATP at the level of 10 mM did not affect the maximum velocity, but the K_m was increased in the presence of ATP. A purely competitive type of inhibition was observed with a Ki of 4.4 mM. Inorganic phosphate was also a competitive inhibitor of acylphosphatase with a Ki of 3 mM with acetylphosphate as substrate. Carbamyl phosphate was also found to inhibit competitively with a Ki of 6.9 mM with acetylphosphate as substrate. The K_m and Ki for erythrocyte acylphosphatase are summarized in Table 4.

TABLE 4

KINETIC PROPERTIES OF ERYTHROCYTE ACYLPHOSPHATASE

Substrate	V_{max}	K_m	Ki (ATP)	Ki(P_i)	Ki(carbamyl phosphate)
Acetylphosphate	300	10.3 mM	4.4 mM	3 mM	6.9 mM
1:3 Diphospho-glycerate	45	0.12 mM			

From the Table 4 it will be seen that the K_m is much lower (by 2 orders of magnitude) for 1,3-diphosphoglycerate than for acetylphosphate, though V_{max} is greater with the latter substrate. The physiological concentration of 1,3-diphosphoglycerate is very low and the low K_m with this substrate is hence of significance for the enzyme to be effective in regulating glycolysis.

SECTION VIII

EFFECT OF THYROXINE

The tissue levels of acylphosphatase are increased by injection of thyroxine although the enzyme itself is inhibited by thyroxine *in vitro*. There was no inhibition by 1×10^{-5}M thyroxine at 35° or by 1×10^{-6}M at 16° but nearly 50 per cent inhibition was observed at 5×10^{-6}M thyroxine at 16° and 5×10^{-5}M at 35°.

Harary (1957) studied the inhibition of horse muscle acylphosphatase by L-thyroxine. The enzyme used for this purpose was a partially purified preparation from horse skeletal muscle. The effect of 5×10^{-5}M thyroxine on enzyme activity was only slight if both the substrate and L-thyroxine were added at the same time. However, upon preincubation of the enzyme with 5×10^{-5}M thyroxine complete inhibition of the hydrolysis of acetylphosphate was observed. Further experiments with varying concentrations of enzyme and L-thyroxine indicated an apparent stoicheiometric inhibition of the enzyme. Both DL-3-3'-5 triiodothyronine and DL-3-5-di-iodo-thyronine at the same concentration inhibited the enzyme but were 60% as effective as thyroxine. No inhibition was observed with 5×10^{-5}M sodium iodide. Benzoic acid, DNP, L-thyroxine and DL-phenylalanine were not inhibitory at 5×10^{-5}M, but at 6×10^{-4}M an inhibition resulted with these compounds which was 80% of that observed with 5×10^{-5}M thyroxine. Except in the case of benzoic acid this inhibition did not depend upon preincubation with the enzyme.

Attempts were made to investigate the mechanism of this inhibition (Harary, 1957). It is known that thyroxine has metal complexing properties. It was suggested that the inhibition of the enzyme may be due to the binding of thyroxine with the metal rather than due to the inactivation of enzyme by thyroxine. However, all attempts to show a metal requirement for this enzyme were unsuccessful. EDTA, NaF, NaCN, α,α-dipyridyl, Na-diethyldithiocarbamate or o-phenanthroline did not inhibit the enzyme. Dialysis of acetylphosphatase against distilled and demineralized water resulted in a loss of 20% of the activity and this loss in activity could not be restored by $MgCl_2$, $CaCl_2$, $MnCl_2$, $Cu(NO_3)_2$, $CoCl_2$, $FeCl_3$, $Zn(acetate)_2$, $FeSO_4$ or by the addition of concentrated dialysate. Thus binding of thyroxine with an essential metal is unlikely to be the cause of thyroxine inhibition.

Addition of zinc acetate (10^{-3}M) to the preincubation mixture at zero time prevented inhibition by thyroxine, but addition after the preincubation period did not reverse the already established inhibition. It is possible that zinc protects the enzyme by binding with thyroxine and prevents it from binding with enzyme.

Additions of upto 40 µmoles of acetylphosphate after preincubation of thyroxine and enzyme without substrate had no effect on reversing the already established inhibition. However, addition of 5 µmoles of acetylphosphate in the preincubation mixture was effective in preventing 50% of the inhibition. The substrate therefore protects the enzyme, perhaps by combining with the susceptible site.

Grisolia (1960) studied the effect of preincubation of thyroxine with brain acylphosphatase at two temperatures. He found that thyroxine was more inhibitory when preincubation was carried out at 16° than at 25°.

It is not clear whether in all the experiments cited above, thyroxine caused inhibition or inactivation. As has been noted earlier, erythrocyte acylphosphatase is inhibited 50 per cent or more by D and L-thyroxine and triiodothyronine. Very little is known about the physiological role of the inhibition *in vitro*. Further elucidation of the mechanism of the inhibition may throw some light on the possible role of acylphosphatase in regulating metabolic processes.

SECTION IX

PHYSIOLOGICAL ROLE OF ACYLPHOSPHATASE

It is not known whether phosphatases have a role in the cell other than the hydrolysis of phosphate compounds. It has been postulated that these enzymes may in some cases function also as transferases. But isotope and chemical studies indicated that acylphosphatase does not catalyze the transfer of acetate from acetylphosphate to a group of acetyl acceptors or the transfer of phosphate to glucose or creatine. Thus acylphosphatase does not possess acetyl transferase or phosphotransferase activity and it appears to act in the cell only as a hydrolase with specificity for acylphosphates.

Since there is no evidence for the occurrence of acetylphosphate in mammalian tissues, the role of acylphosphatase remains in doubt. There are a few theories postulated to explain the role of acylphosphatase.

(1) The hydrolysis of 1:3 diphosphoglyceric acid by acylphosphatase

Harary (1957) showed that acylphosphatase catalyzes the hydrolysis of 1:3-diphosphoglyceric acid. 1:3-DPGA can be generated by the phosphorylation of 3-phosphoglyceric acid by ATP catalyzed by phosphoglyceric acid kinase. 1:3-DPGA in turn is hydrolyzed by acylphosphatase to yield phosphoglyceric acid and inorganic phosphate.

(i) ATP + 3 phosphoglyceric acid $\underset{\leftarrow}{\overset{\text{PGA kinase}}{\rightarrow}}$ ADP + 1:3-DPGA

(ii) 1:3-DPGA $\xrightarrow{\text{AcPase}}$ 3 phosphoglyceric acid + Pi

The sum of equations (i) and (ii) gives

(iii) ATP $\xrightarrow[\text{AcPase, PGA}]{\text{PGA kinase}}$ ADP + Pi

Equation (iii) would mean a PGA dependent hydrolysis of ATP catalyzed by PGA kinase and acylphosphatase.

At low levels of tissue inorganic phosphate the rate of glycolysis may be limited by the inorganic phosphate content. At this stage acylphosphatase may enhance the rate of hydrolysing 1:3-diphosphoglyceric acid and thereby uncoupling glycolysis from phosphorylation.

(ii) Acylphosphatase in Na^+-K^+ transport

Data from several laboratories suggest that Na^+-K^+ ATPase which has acetylphosphatase is involved in the active transport of sodium and potassium. It is possible that acylphosphatase in erythrocytes etc. may have a role in transport of ions across cell membranes. There is, however, no evidence to support this suggestion. The difference in substrate specificity of the erythrocyte enzyme from that of enzyme from other animal tissues suggest the possibility that their roles may be different.

(iii) Acylphosphatase as a chemotropic effector

A very interesting and novel suggestion has been made by Grisolia (1968) regarding the physiological role of acylphosphatase. It was shown that a non-enzymic carbamylation of glutamic dehydrogenase occurs when carbamyl phosphate is incubated with the enzyme, thereby inactivating the enzyme. This may have an important bearing on regulation of enzymic activity making proteins more susceptible to degradation by cathepsins or lysozomal enzymes. The function of acylphosphatase may be to regulate the concentration of the acyl-phosphates (carbamyl phosphate, formyl phosphate and acetylphosphate,

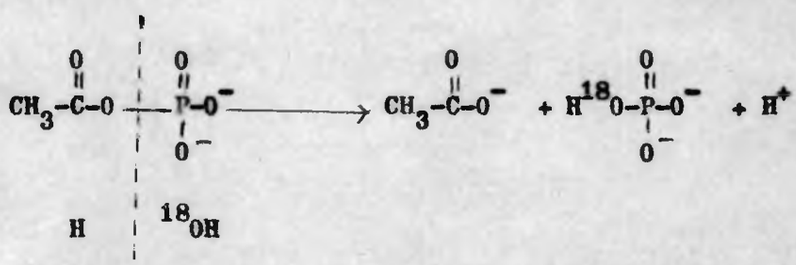
which are all known to be produced by carbamyl phosphate synthetase and 1:3-DPGA) to protect unduly high levels of these compounds being formed, which would have a harmful effect. Prevention or regulation of non-enzymic acylation of enzymes (and possibly other proteins) would be a function of acylphosphatase which is termed a chemotropic effector.

Acylphosphatase of erythrocytes acts on 1:3-DPGA but not on carbamyl phosphate, since the latter is not formed or is present only in negligible amounts in erythrocytes. It would be interesting to determine whether avian erythrocytes, which are nucleated, contain enzymes which hydrolyze both 1:3-DPGA and carbamyl phosphate.

SECTION X

MECHANISM OF ACTION

Acylphosphatase catalyzes the hydrolysis of a variety of acylphosphates such as acetylphosphate, 1:3-diphosphoglyceric acid and carbamyl phosphate. Whether the enzyme cleavage takes place between carbon and oxygen or between phosphorus and oxygen was studied by Bentley (1949) with water enriched with the ^{18}O isotope. Since an analytical method for the estimation of ^{18}O in phosphate was not readily available only the acetate portion of the molecule was studied. At the end of the reaction the products were analyzed. Only a very small amount of ^{18}O was found in the isolated acetic acid indicating that the enzymic hydrolysis proceeds with the splitting of the phosphorus-oxygen bond as shown in the following scheme.



Koshland (1952) studied the nonenzymic hydrolysis of acetylphosphate catalyzed by acid and base, metal ions and pyridine. In these experiments it was observed that at the extremes of pH the dominant pathway was a nucleophilic attack by water on the carbonyl carbon, whereas at neutrality the major reaction was an attack on the phosphorus atom. The enzymic reaction resembles this latter spontaneous process. Koshland suggested that the accelerated rate of the enzymic reaction

is due to polarization of the electrons in the substrate molecule at the active site of the enzyme.

Recently Satchell and White (1972) have studied the chemical mechanism of the reaction in detail and have supported the earlier studies of Bentley and Koshland. They observed that the enzymic catalysis differs significantly from the nonenzymic hydrolysis of acylphosphates. The substrate is held primarily by the phosphate group. During the surface reaction the phosphorus atom is uniquely located by bonds to three of the phosphate oxygen atoms and suffers a slow nucleophilic attack by an adjacent water molecule. The mechanism explains the observed unsuitability of species $ROPO_3^{-2}$, $RCO.OPO_3H$ and $RCO.OPO_3R'$ as substrates for muscle acylphosphatase.

SECTION XI

PRESENT WORK

The work reported in this thesis deals with the isolation in pure form of acylphosphatase from the seeds of Vigna catjang and the study of its properties and kinetics.

The enzyme was separated from non-specific phosphatases and purified by fractionation with ammonium sulphate and chromatography on DEAE-cellulose, CM-cellulose and Sephadex-G-100. The purified enzyme was homogeneous by ultracentrifugation and gel electrophoresis. The maximum specific activity obtained by this procedure was 300 to 1200 umoles of acetylphosphate hydrolyzed per mg protein per hour at 30° at pH 5.7.

The study of the properties and kinetics of the enzyme include the effects of pH, temperature, substrate concentration and inhibitors, ultracentrifugation, acrylamide gel electrophoresis and amino acid composition.

Chapter II of the thesis deals with the materials and experimental methods used in these studies.

Chapter III deals with the isolation of acylphosphatase, its separation from other phosphatases and its purification.

Chapter IV describes properties and kinetics of purified acylphosphatase.

Chapter V deals with the discussion of the results of these studies.

Chapter VI contains a summary of the results and conclusions of this work.

A bibliography is presented at the end.

CHAPTER II

MATERIALS AND METHODS

CHAPTER II

MATERIALS AND METHODS

Materials:

Seeds: *Vigna catjang* seeds were purchased from the local market and stored at 0° till use.

Chemicals: All common chemicals as well as Tris were of analytical grade. The following chemicals were obtained from Sigma Chemical Company, U.S.A. : ATP, diisopropylfluorophosphate, dithiothreitol, oxidized glutathione, reduced glutathione, 5-5' dithio-bis-2-nitro-benzoic acid, p-hydroxymercuribenzoate. Sodium borohydride and cysteine hydrochloride were obtained from Fluka (AG).

Acetylphosphate was either obtained from Sigma Chemical Company or from Biochemicals Unit, Delhi or prepared in this laboratory (Stadtman, 1957(a)). Carbamyl phosphate was obtained from Sigma Chemical Company and recrystallized before use (Metzenberg, Marshall and Cohen, 1960).

Amberlite IRC-50 (XE-64) (mesh 200 to 400) was obtained from Rohm and Haas. DEAE-cellulose (100 to 200 mesh 0.5 meq per g), CM-cellulose (0.7 meq per g) and cellulose phosphate were obtained either from Bio-Rad Laboratories or from Sigma Chemical Company. Calcium phosphate gel was prepared according to the procedure of Swingle and Tiselius (1951). Celluloses were washed according to the method described by Peterson and Sober (1956). CM-Sephadex, Sephadex G-75 and Sephadex-G-100 were obtained from Pharmacia Fine

Chemicals, Sweden. Sephadex was suspended in water, kept on a boiling water bath for 5 hours, cooled and deaerated before use. All the chromatographic columns were prepared with flow of liquid under gravity without application of external pressure. Stepwise change in molarity of the buffer was used for the elution of the enzyme from the column.

The chemicals used for acrylamide gel electrophoresis, acrylamide, N-N'-methylene-bis-acrylamide and tetramethyl-methyl-ethylene-diamine and Amido Black 10B were obtained from Eastman Kodak Company, U.S.A.

Methods:

Definition of unit of activity and specific activity

The unit of acylphosphatase activity is defined as the amount of enzyme that hydrolyzes one μmole of acetylphosphate per hour at 30° at pH 5.7. The specific activity of the enzyme is defined as the activity per mg of protein.

Estimation of acylphosphatase activity

Hestrin's (1949) colorimetric method was followed for assaying acylphosphatase. The details of the method are as follows.

The assay system consisted of 100 μmoles of K-acetate buffer pH 5.7, acetylphosphate (9 μmoles) and enzyme in a final volume of 1.5 ml. The final pH of the reaction mixture was 5.7 and the temperature was 30°. The reaction was started by adding enzyme and the reaction mixture was incubated for 30 min. The amount of acetylphosphate hydrolyzed was not greater than 25 to 30% of the initial quantity. The reaction was stopped by adding 3 ml of alkaline hydroxylamine (prepared by mixing equal volumes of 3.5 M NaOH and 2 M hydroxylamine hydrochloride). After 5 min this was followed by the addition of 1.5 ml of hydrochloric acid (2 volumes of concentrated HCl mixed with 3 volumes of water) and 1.5 ml of a 10 per cent solution (w/v) of ferric chloride in 0.1 N HCl. The colour was read at 540 nm. An optical density change of 0.120 for a 1 cm light path was taken as equivalent to one μmole of acetylphosphate hydrolyzed. A blank with substrate alone without enzyme was always run. Blanks with enzyme

were negligible except with crude extracts. While determining the effects of some compounds on enzyme activity controls were run to ensure that the compound did not interfere with the color given by acetylphosphate. In the case of reaction mixtures containing more than 100 µg of protein, the samples were filtered before taking the readings.

In some of the experiments acylphosphatase activity was determined by Lipmann's method (1945). There was no difference in the activities obtained by the two methods. Hestrin's method was used for most of this work.

Estimation of ATPase, glucose-6-phosphatase and β-glycerophosphatase activity and units of activity

Fiske and Subba Row's (1925) colorimetric method was followed for assaying ATPase, G-6-Pase and β-glycerophosphatase activities. The reaction mixture consisted of 10 µmoles of ATP, G-6-P or DL-β-glycerophosphate, 100 µmoles of K-acetate buffer pH 5.7 and enzyme in a final volume of 1.5 ml. The reaction was stopped by adding 1.5 ml of 10% trichloracetic acid and the amount of inorganic phosphorus formed was estimated. Blanks without enzyme and without substrate were also run. The unit of ATPase and glucose-6-phosphatase was defined as the amount of enzyme that liberates 1 µmole of inorganic phosphorus from the corresponding substrate per hour at pH 5.7 at 30°.

Estimation of protein

Protein was usually determined by the following method based on a modification of that of Warburg and Christian (1941). A correction for nucleic acid and other ultraviolet absorbing impurities

is made by the following equation (Jagannathan et al. 1956). It was assumed that a 0.1 per cent solution of protein has an optical density of 1 at 280 mm for a light path of 1 cm.

$$\frac{4}{7} \times 2.3 \times (\text{O.D. 280 mm} - \text{O.D. 340 mm}) - (\text{O.D. 260 mm} - \text{O.D. 340 mm})$$

$$= \text{mg protein per ml}$$

If necessary the enzyme solutions were diluted with 0.01 M K-acetate buffer, pH 5.7 and the optical densities at 260, 280 and 340 mm were determined. A buffer of the same composition was used as the blank.

Protein was also determined by the method of Lowry et al (1951) using bovine serum albumin as the standard. It was found that protein values as determind by Lowry's (1951) method were twice those obtained by the spectrophotometric method (with purified enzyme preparations). For the accurate estimation of protein in the final purified preparation only Lowry's method was used. The spectrophotometric method was routinely used during purification of the enzyme.

Centrifugations were carried out at 0° in an international centrifuge (Model PR-1 and PR-2), Sorvall (Model SS-1), Spinco (Model L) or Sharples supercentrifuge (AS 12 clarifier). Chromatographic fractions were collected on a Technicon automatic fraction collector.

All glassware was routinely washed with sodium carbonate and then with nitric acid, rinsed successively with tap water, distilled water and glass distilled water and dried. No grease or silicone was used for ground glass joints and stopcocks in chromatographic columns.

Glass-distilled water was used for the preparation of all solutions.

Spectrophotometric determinations were carried out either with a Model DU Beckman spectrophotometer or a Unicam spectrophotometer SP 500 model with cuvettes with a light path of 1 cm.

pH estimations were carried out with the glass electrode. The pH of concentrated salt solutions was determined after diluting the solution four times with water.

Ammonium sulphate precipitations

Ammonium sulphate saturations were carried out at 0° and were calculated according to Jagannathan et al. (1956). The following equations were used for calculating ammonium sulphate saturations.

For solid ammonium sulphate -

$$X = \frac{50\ (S_2 - S_1)}{1 - 0.28 \times S_2}$$

For saturated ammonium sulphate solution

$$Y = \frac{100\ (S_2 - S_1)}{1 - S_2}$$

where X is the g of solid ammonium sulphate to be added for every 100 ml of enzyme solution and Y is the ml of saturated ammonium sulphate to be added for 100 ml of solution. S_1 in the initial saturation and S_2 is the required saturation of ammonium sulphate at 0°.

Solid ammonium sulphate was added slowly over a period of 30 min with gentle stirring. Formation of froth was avoided. The precipitates were dissolved in a known volume of buffer and the final volume was then noted. The increase in volume was assumed to

be due to ammonium sulphate at the saturation at which the precipitate was obtained and a correction was made for the ammonium sulphate concentration of the enzyme solution.

Ultracentrifugation

The molecular weight of the enzyme was determined by the Archibald (1947) method. Runs were carried out on a Spinco Model 'E' analytical instrument equipped with a phase plate and a rotor temperature indicator and control system capable of maintaining a constant temperature during the run. The phase plate was used at an angle of 80°. The speeds of centrifugation for linear extrapolation of the gradient curve were calculated according to La Bar (1966). A synthetic boundary cell was used and readings at the meniscus were taken. All solutions were routinely spun at 13,000 x g for 30 min before analysis by ultracentrifugation.

Photographic plates were measured on a Hilger L-50 two-way measuring micrometer with a sensitivity of 1 u. Photographic plates were read at 0.1 mm intervals for molecular weight determinations and areas determined by trapezoidal analysis. The partial specific volume of the enzyme was assumed to be 0.725.

Polyacrylamide gel electrophoresis

Acrylamide gels were prepared by following the method of Reisfeld *et al.* (1962) with a slight modification. 15% acrylamide gels were used. The pH of the gel and the running buffer was 4.5. 0.35 M K-acetate buffer, pH 4.5 was used. The current applied was 4 milliamps per tube and the period of run was 6 h. Protein was

stained by using 0.5% Amido Black 10 B in 7% acetic acid for 30 min and it was destained by keeping overnight with 3% acetic acid.

The phosphatase bands were located by staining the gel with Lowry-Lopez (1946) reagents. After the run the gel was incubated for 30 min with 6 ml of a reaction mixture containing 50 µmoles of acetylphosphate and 400 µmoles of acetate buffer, pH 5.7. It was then removed and placed in a reaction mixture containing 6 ml of 0.1 M acetate buffer pH 4.5, 1 ml of 1% ascorbic acid and 1 ml of 1% ammonium molybdate and kept for 30 min. Only the phosphatases which hydrolyze acetylphosphate gave bands by this method.

By staining the gel by the above two methods it was possible to distinguish the enzyme from other protein impurities.

Determination of free -SH groups

DTNB titrations for the determination of free -SH groups were carried out according to the procedure of Ellman (1958). The protein was treated with an excess of reagent and the net absorption at 412 nm was employed to calculate the sulfhydryl content. A molar extinction coefficient of 1.36×10^4 $cm^2 \times mole^{-1}$ was assumed for the free thiol ion of the reagent at pH 8.0. A reagent blank was used to correct for the absorption of the reagent. The accuracy of the method was checked with glutathione.

Determination of total sulfhydryl and disulfide groups

Total -SH groups and -S-S- linkages in acylphosphatase were determined by two different procedures.

1) Reduction of -S-S- linkages using $NaBH_4$ in 8 M urea followed by DTNB titrations after removal of excess of $NaBH_4$.

2) Oxidation of -SH and -S-S- groups to cysteic acid by the standard procedure of performic acid oxidation followed by acid hydrolysis and cysteic acid estimation using an amino acid analyzer.

1) Reduction of enzyme by $NaBH_4$ in urea

The method of Cavallini, Graziani and Dupre (1966) was used. The enzyme was reduced with borohydride in the presence of urea, EDTA, and phosphate buffer, pH 7.5 at 38°. After incubation for 45 min excess borohydride was destroyed by the addition of potassium dihydrogen phosphate containing acid. The color was then developed with DTNB in an atmosphere of nitrogen and read at 412 nm. The number of sulfhydryl groups (N) was calculated using the following formula.

$$N = \frac{Mw \times A \times V}{12{,}000 \times m}$$

where Mw = molecular weight of the protein

A = absorbancy

V = volume of the final solution

m = weight in mg of the protein sample analyzed

The accuracy of the assay was checked with bovine serum albumin as standard.

Amino acid analysis

Amino acid analysis of performic acid oxidized sample of acylphosphatase was carried out according to the method described by Hirs (1967).

Estimation of tyrosine and tryptophan

Tyrosine and tryptophan in the enzyme were determined by (1) Goodwin and Morton's (1946) method and (2) Bencze and Schmid's method (1957). The values of tryptophan and tyrosine obtained by

the two methods were in good agreement.

Goodwin and Morton's method (1946)

Proteins show selective absorption in the ultraviolet region and the position of the absorption maximum varies with pH. The majority of the constituent amino acids do not show any absorption in the region 250-320 mm and it is known that phenylalanine, tyrosine and tryptophan are responsible for the observed ultraviolet absorption of protein solutions. In 0.1 N NaOH the absorption by tyrosine and by tryptophan is much stronger and that by phenylalanine is negligible. Under these conditions the protein solutions may be treated as two-component system for spectrophotometric analysis. The intensity of absorption at the point where the curves for tyrosine and tryptophan intersect is a direct measure of the total molar solute concentration and will be the same however the proportions are varied. At other wave lengths the intensity of absorption will vary with the relative proportions of the components. Using 0.1 N NaOH as solvent the two absorption curves intersect at 294.4 mm (ϵ =2375) and 257.15 mm (ϵ = 2748). By determining the absorption of the protein in 0.1 N NaOH at the above two wavelengths and at one other wavelength (e.g. at 280 mm) it is possible to determine the relative proportions of tyrosine and tryptophan in the protein.

Thus, if x = total mol / l in solution

y = g mol / l of tyrosine

x-y = g mol / l of tryptophan

at any wavelength other than the point of intersection

let ϵ tyrosine be A and ϵ tryptophan be B and the observed intensity of absorption be E

then,

$$E = yA + (x-y)B$$

$$\text{or } y = \frac{E - xB}{A - B}$$

$$x = \frac{\text{E value at an intersection}}{\epsilon \text{ tyr. at an intersection}}$$

(2) Bencze and Schmid's method (graphical method)

This method is based upon measuring the absorbance of the protein in 0.1 N NaOH in the range between 278 and 294 nm at 2 nm intervals. The readings are plotted against the wavelength and a line is drawn tangentially to the two characteristic peaks. From the slope of the tangent, the maximum absorption between 270 and 290 nm and the molecular weight of the protein the tyrosine and tryptophan content can be determined.

CHAPTER III

EXPERIMENTAL AND RESULTS

SECTION I

Acylphosphatase of plant tissue cultures

Preliminary work on acylphosphatase was carried out with tissue cultures of plants. Acylphosphatase was found to be present both in normal and tumor tissue cultures of Parthenocissus. In addition ATPase and glucose-6-phosphatase were also present in the tissue and by extraction with 0.2 N HCl-0.2 N KCl, followed by ammonium sulphate fractionation an acylphosphatase preparation was obtained free from the other phosphatases. These results, which will not be described in detail, showed the presence of a true acylphosphatase in plant tissues free from phosphatase activity with other substrates.

Acylphosphatase of seeds

Since plant tissue cultures take a very long time to grow in vitro and are difficult to obtain in large quantities, extraction of the enzyme from a plant source, such as seeds, which is available throughout the year, was tried.

In the case of Vigna catjang (black eyed peas), maize, and sorghum 1 g of handgroundseeds was extracted with a mixture of 5 ml of 0.01 M K-acetate buffer pH 5.7, 1 ml of 0.1 M HCl and 75 mg of solid KCl. The extract was allowed to stand for 30 min and neutralized to pH 5.7 by 0.1 M $KHCO_3$. The supernatant liquid obtained after centrifugation was assayed for acylphosphatase activity. In the case of mung beans instead of 0.1 M HCl, 0.2 M HCl was used for extraction. Acylphosphatase was present in all the different seeds which were tested

(Table 5), but the activity varied considerably. The highest activity was found in seeds of Vigna catjang.

TABLE 5

ACYLPHOSPHATASE ACTIVITY OF SEEDS

Seed	Activity
	(u/g dry seed)
Cholai (Vigna catjang)	243
Soya beans (Glycine soja or Glycine max)	90
Maize (Zea mays)	80
Sorghum (Sorghum vulgare)	18
Mung beans (Phaseolus mungo or Phaseolus radiatus var. mungo)	36

The results are not corrected for other non-specific phosphatases which may act on acetylphosphate.

Acylphosphatase of cholai seeds

(The local name cholai is used for Vigna catjang)

Since cholai was the richest source of acylphosphatase, cholai seeds were chosen for further work on the enzyme.

An extract of cholai seeds was prepared as before and the acylphosphatase activity as well as the liberation of inorganic phosphate from ATP, glucose-6-phosphate and β-glycerophosphate were determined.

TABLE 6

CHOLAI SEED PHOSPHATASES

	Activity (u/g seed)
Acylphosphatase	284
ATPase	108
Glucose-6-phosphatase	14
β-Glycerophosphatase	16

The extract contains high ATPase in addition to acylphosphatase as well as relatively low activities with glucose-6-phosphate and β-glycerophosphate. The relative amounts of acylphosphatase and ATPase varied with different lots of seeds and different methods of extraction.

SECTION II

SEPARATION OF ACYLPHOSPHATASE FROM OTHER PHOSPHATASES

The following experiments were carried out to establish whether the acylphosphatase was specific or whether it was a nonspecific phosphatase.

Heating

Acylphosphatase was found to be fairly stable towards heat and acid. Heating of the acid extract of the seeds at 50° for 5 min results in marked destruction of ATPase, whereas only 40 to 50% of acylphosphatase is lost (Table 7).

TABLE 7

EFFECT OF HEATING ON AcPase AND ATPase

	AcPase	ATPase
	u/g	u/g
Before heating	243	392
After heating at 50° for 5 min	117	31

Effect of fluoride

The effect of fluoride on the different phosphatases was tested. The phosphatase activities with different substrates with and without fluoride are shown in Table 8.

TABLE 8

EFFECT OF FLUORIDE ON CHOLAI SEED PHOSPHATASES

	AcPase units/g	ATPase units/g	G-6-Pase units/g
Without fluoride	56	36	9
With 10 mM fluoride	38	3	3
Inhibition (%)	33	92	66

It was found that 10 mM fluoride inhibited acylphosphatase activity only 30% whereas G-6-Pase was more strongly inhibited and ATPase almost completely inhibited. These results also suggested that acylphosphatase is different from the other phosphatases.

Acid extraction

The amounts of different phosphatases extracted when the concentration of acid used for extracting the seeds was varied were examined. In three different experiments 10 g of seeds were extracted with 50 ml of 0.01 M K-acetate buffer, pH 5.7 containing 0.75 g KCl and 10 ml of 0.2 M, 0.4 M or 0.6 M HCl. The extracts were centrifuged for 30 min at 3,000 x g. The supernatant liquids were neutralized to pH 5.7 by the addition of 2M $KHCO_3$. The precipitates were centrifuged off and the clear supernatant liquids were assayed for different phosphatase activities. Table (9).

TABLE 9

EFFECT OF ACID ON CHOLAI SEED PHOSPHATASES

Concentration of acid for extraction	AcPase	ATPase	G-6-Pase
	u/g	u/g	u/g
0.2 M HCl	192	144	42
0.4 M HCl	96	32	8
0.6 M HCl	72	16	2

It will be seen that with increasing amounts of acid the amounts of ATPase and G-6-Pase extracted are more markedly reduced than in the case of acylphosphatase. With 0.6 M HCI, very little G-6-Pase was present in the extract. The differences in the activities of the enzymes in the extracts were not due to differences in salt content after neutralization since the relative activities with the different substrates were unchanged even after precipitation of the enzymes with ammonium sulphate followed by dialysis.

The above results also indicate that a specific acyl-phosphatase is present in these seeds. Extraction with 0.6 M HCl-KCl was used in all further experiments. The amounts of ATPase and G-6-Pase were, however, variable with different batches and in some cases much higher amounts of ATPase relative to AcPase were present in the extract.

SECTION III

PURIFICATION

Preliminary experiments on the extraction of the enzyme indicated that acylphosphatase was extracted best with a solution consisting of five ml of 0.01 M K-acetate buffer pH 5.7- 0.2 M KCl and one ml of 0.6 M KCl per g of seed. Under these conditions the extraction of nonspecific phosphatases was markedly reduced.

Extraction

600 g of seeds were washed repeatedly first with tap water and then with distilled water and soaked in glass-distilled water at room temperature for 30 min. Washing of the seeds was necessary for the removal of preservatives which were sometimes added to the seeds. All subsequent operations were at 0° unless otherwise stated. Soaked seeds (equivalent to 150 g of dry seeds) were blendorized at a time with 300 ml of 0.01 M K-acetate buffer, pH 5.7, for 3 min. 1,8000ml of 0.01 M K-acetate buffer, pH 5.7, 600 ml of 0.6 M HCl and 4.5 g of KCl were then added to the homogenate. The mixture was stirred for 30 min and squeezed through muslin cloth. The filtrate was centrifuged for 30 min at 3,000 x g. The supernatant liquid was brought to a pH of 5.7 by the addition of 2 M $KHCO_3$. The precipitate formed was collected by centrifugation for 30 min at 3,000 x g and discarded and the clear supernatant liquid was used for purification.

SECTION IV

AMMONIUM SULPHATE FRACTIONATION

Preliminary experiments showed that when the enzyme extract prepared as described in the previous section was fractionated with ammonium sulphate and the fractions obtained at 0–0.5, 0.5–0.7 and 0.7–0.9 saturation, tested for activity, most of the activity was present in the 0.5–0.7 fraction. Other experiments showed that the precipitate obtained between 0.4 and 0.7 saturation contained almost all the acylphosphatase activity. Based on these results the following procedure was used for ammonium sulphate fractionation.

0 to 0.4 saturation. The crude enzyme was fractionated between 0 to 0.4 saturation. To every 100 ml of enzyme solution 22.5 g of powdered ammonium sulphate were added with stirring and after 30 min at 0°, the solution was centrifuged at 3,000 x g for 30 min. The sediment was discarded.

0.4 to 0.7 saturation. To every 100 ml of the supernatant liquid 19 g of ammonium sulphate were added to increase the saturation to 0.7. The precipitate was collected by centrifugation at 13,000 x g for 30 min.

Washing with 0.7 saturated ammonium sulphate. The precipitate obtained by 0.4 to 0.7 saturation was washed with 0.7 saturated ammonium sulphate solution to remove the adhering mother liquor.

Fractionation at pH 8.5 (0.4 - 0.9 saturation). Ammonium sulphate precipitation at an alkaline pH was carried out, since the enzyme precipitated at pH 5.7 was unstable on storage in some experiments.

This fraction also showed lack of linearity of activity with enzyme concentration. Precipitation at alkaline pH increased the purity only 2 to 3-fold but it gave an enzyme preparation, which could be stored for several weeks without loss of activity. The activity of the preparation was also proportional to enzyme concentration due possibly to the removal of inhibitory impurities.

The precipitate obtained after washing with 0.7 saturated ammonium sulphate was suspended in 0.1 M Tris-HCl buffer, pH 8.5, which was 0.4 saturated with respect to ammonium sulphate. It was centrifuged at 13,000 x g and the sediment was discarded. To every 100 ml of the above supernatant liquid 33 g of ammonium sulphate were added in order to increase the saturation from 0.4 to 0.9. The precipitate obtained by centrifugation at 13,000 x g for 30 min was dissolved in 0.01 M K-acetate buffer pH 5.7 and dialyzed against two or three changes of 100 volumes of the same buffer.

The results of a typical ammonium sulphate fractionation are presented in Table 10.

It is evident from the Table that about 7-fold purification is achieved in this fractionation. The final specific activity with different batches ranged between 30 to 50.

TABLE 10

AMMONIUM SULPHATE FRACTIONATION OF ACYLPHOSPHATASE

Fraction	Volume	Activity	Total activity	Protein	Total protein	Specific activity
	ml	units/ml	units	mg/ml	mg	units/mg
Extract	1,700	11	18,700	1.6	2,720	7
Ammonium sulphate saturation						
0 to 0.4 supernatant	1,925	7.5	13,700	1.4	2,695	5
0.4 to 0.7 ppt	65	200	13,000	10.0	650	20
0.4 to 0.9 ppt at pH 8.5	19	800	12,800	17.4	330	47

SECTION V

Miscellaneous adsorbents

IRC-50-XE-64

The enzyme obtained after ammonium sulphate precipitation was found to be adsorbed completely by the cation exchange resin IRC-50-XE-64 in 0.01 M K-acetate buffer, pH 5.7, 20 ml of the enzyme containing 10,000 units were treated with 10 g of resin in 0.01 M K-acetate buffer, pH 5.7. The mixture was stirred for 30 min and centrifuged. The residue was washed successively with 20 ml of 0.03 M K-acetate buffer, pH 5.7, 200 ml of 0.08 M K-acetate buffer, 15 ml of 0.15 M K-acetate buffer, 20 ml of 0.2 M K-acetate buffer and 40 ml of 0.3 M K-acetate buffer, pH 5.7. It was then eluted with 40 ml of 0.5 M K-acetate buffer, pH 5.7 in two lots. The enzyme was present in the 0.5 M eluates. Table 11 presents the summary of results on adsorption and elution of the enzyme from IRC-50-XE-64.

It will be seen from the Table 11 that enzyme of very high purity was obtained merely by batchwise chromatography of the enzyme on IRC. These results were obtained in three successive experiments, but it was not possible to reproduce them subsequently. Several experiments were tried to ascertain the reason for this lack of reproducibility. These include washing of IRC at different pHs and with phosphate buffer, washing the seeds to remove any preservatives added to the seeds, passing the enzyme through Sephadex-G-25 before IRC-treatment etc. The results of all these experiments were negative and it was not possible to reproduce the earlier results. The reasons

TABLE 11

ADSORPTION AND ELUTION OF ACYLPHOSPHATASE ON IRC

Fraction	Volume	Activity	Total activity	Protein	Total protein	Specific activity
	ml	units/ml	units	mg/ml	mg	units/mg
Before adsorption	20	500	10,000	76	1,520	7
0.5 M eluate	40	260	10,400	0.2	8	1,300

for this lack of reproducibility is not known. It may be due to the change in the strain of seeds used for these experiments. This method was not further used.

Cellulose phosphate

It was found that 1 mg (dry weight) of cellulose phosphate was required to adsorb 5 units of crude enzyme in 0.01 M K-acetate buffer, pH 5.7 and at pH 4.8. The enzyme could be eluted with 0.1 M K-acetate buffer, pH 5.7 or with 0.1 M K-acetate buffer, pH 4.8. But there was no significant increase in specific activity. This adsorbent was not used for further work.

Calcium phosphate gel

The partially purified enzyme (i.e., the enzyme obtained after CM-cellulose chromatography) was used for these experiments. 50% adsorption was observed when 4 mg of the adsorbent was used per unit of enzyme in 0.01 M K-acetate buffer, pH 5.7 containing 0.01 M NaCl. Elution with 0.1 M K-acetate buffer, pH 5.7 did not result in any increase in specific activity.

SE-Cellulose

50% adsorption of the partially purified enzyme (the enzyme obtained after CM-cellulose chromatography) was observed on SE-cellulose in two to three batchwise experiments in 0.01 M K-acetate buffer, pH 5.7 and about 4-fold increase in the specific activity of the unadsorbed enzyme was observed. But it was not possible to reproduce these results.

Alumina - C_γ

Adsorption of partially purified enzyme on alumina-C_γ was tried using two different amounts of adsorbent (1 mg of alumina per 40 units and 1 mg of alumina per unit of enzyme) in 0.01 M tris, pH 7.5. Only 50% adsorption was observed in both the cases. There was no increase in the specific activity of the unadsorbed enzyme. The enzyme could not be eluted with 0.2 M tris, pH 7.5. This method was not used for further purification.

CM-Sephadex

Preliminary experiments with this adsorbent (1 mg per 10 units, 5 units or 2 units) showed complete adsorption of the partially purified enzyme in all the three cases. But no enzyme could be eluted with 0.2 M K-acetate buffer, pH 5.7 and only 30% of the enzyme could be eluted with 0.5 M K-acetate buffer, pH 5.7. In view of the low recovery of enzyme and absence of any significant purification column chromatography was not tried with CM-Sephadex.

Sephadex G-75

Chromatography of enzyme obtained after CM-cellulose chromatography on columns of Sephadex G-75 in 0.05 M K-acetate buffer, pH 5.7 and in 0.05 M K-acetate buffer, pH 5.7, containing ammonium sulphate (7 g/100 ml = 0.1 saturation) did not result in any increase in the specific activity of the enzyme in any of the fractions.

SECTION VI

CHROMATOGRAPHY ON CARBOXYMETHYL CELLULOSE

The enzyme obtained after ammonium sulphate fractionation was used for studies with CM-cellulose. Preliminary batchwise experiments indicated that at pH 5.7, 10 units of the enzyme were adsorbed completely by 1 mg of CM-cellulose in 0.01 M K-acetate buffer at pH 5.7. The activity could be eluted by 0.3 M K-acetate buffer, pH 5.7. At pH 7.5, 5 units of enzyme were adsorbed completely by 1 mg of CM-cellulose and the enzyme could be eluted with 0.2 M Tris buffer pH 7.5. About two to three-fold purification was achieved in both the cases. Since at pH 7.5, the amount of impurities that were left in the unadsorbed fraction was more than at pH 5.7, chromatography at pH 7.5 was preferred. ATPase and G-6-Pase were completely removed in the unadsorbed fraction. Separation of acylphosphatase from other non-specific phosphatases as well as significant purification was obtained by chromatography of the enzyme on CM-cellulose.

The adsorbent was washed according to the method of Petersen and Sober (1962) and finally washed with 0.001 M EDTA, pH 7.5. A column (3.8 x 10 cm) was prepared and washed with 0.01 M Tris buffer, pH 7.5, 1 mg of adsorbent being used for 1 unit of enzyme. 19 ml of the enzyme (corresponding to 5,700 units) obtained after ammonium sulphate fractionation and dialysis were loaded on the column. The column was washed with about 400 ml of 0.01 M Tris buffer, pH 7.5 and with 250 ml of 0.1 M Tris buffer, pH 7.5. The column was then

eluted with about 300 ml of 0.2 M Tris buffer, pH 7.5. 20 ml fractions were collected and the activity and protein in each fraction were determined. The results are presented in Table 12 and the activity and protein patterns are shown in Fig. 1.

Fractions 6 to 10 were pooled and precipitated with ammonium sulphate at 0.9 saturation by adding 60 g of ammonium sulphate for 100 ml of liquid. The activity and protein of the dialyzed enzyme were determined. About 25 to 50 per cent of the initial activity was recovered with about 35-fold increase in specific activity. Since other non-specific enzymes were removed here, the actual recovery of true acylphosphatase is higher.

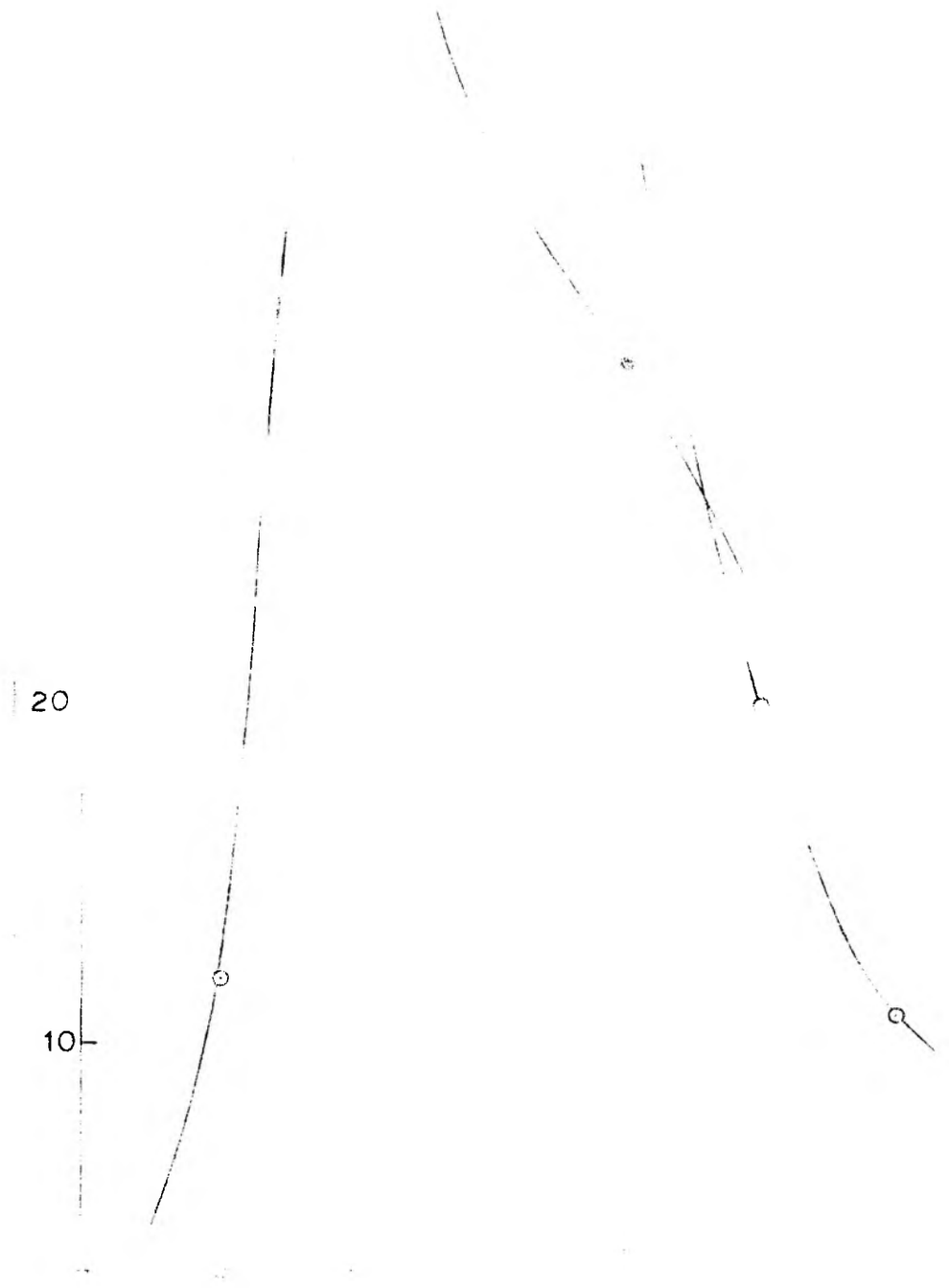
While precipitating the enzyme from dilute solutions very high losses in recovery were observed. About 30 to 40 per cent of the activity was lost in this step. This was avoided by concentrating the enzyme two to four times by lyophilization before precipitation at 0.9 saturation with ammonium sulphate. 80 to 90 per cent of the activity was recovered by this procedure.

During this chromatography, ATPase and other nonspecific phosphatases remain in the unadsorbed supernatant fraction and 0.1 M buffer washings. 0.2 M buffer eluates are completely free from ATPase and G-6-Pase. Only true acylphosphatase is present after CM-cellulase chromatography (Table 13).

TABLE 12

COLUMN CHROMATOGRAPHY OF ACYLPHOSPHATASE ON CM-CELLULOSE

Fraction	Volume	Activity	Total activity	Protein	Total protein	Specific activity
	ml	units/ml	units	mg/ml	mg	units/mg
Before loading	19	300	5,700	26	494	12
0.2 M eluted fractions						
1 to 4	80					
5	20	12	240	0.14	2.8	86
6	20	48	960	0.20	4.0	240
7	20	50	1,000	0.17	3.4	294
8	20	38	760	0.11	2.2	345
9	20	20	460	0.06	1.3	350
10	20	11	220	0.05	1.0	220
11	20	8	160	0.05	1.0	160
12	20	5.2	104	0.04	0.8	125
13	20	1.2	24	0.04	0.8	30
Before precipitation (6 to 10)	100		3,500			
Fractions 6 to 10 precipitated and dialyzed	4.5	560	2,500	1.3	5.85	430



ELUTION PATTERN [illegible] PHOSPH

TABLE 13

SEPARATION OF ACYLPHOSPHATASE FROM OTHER PHOSPHATASES BY CHROMATOGRAPHY ON CM-CELLULOSE COLUMN AT pH 7.5

Fraction	AcPase	ATPase	G-6-Pase
	units	units	units
Before CM-cellulose chromatography	9,600	5,500	1,000
0.01 M washings	1,300	1,100	
0.1 M washings	3,850	3,100	
0.2 M eluates	2,300	0	0

SECTION VII

SEPHADEX-G-100

The enzyme obtained after CM-cellulose chromatography showed three bands (and in some cases a few other faint bands) on disc electrophoresis when the gels were stained with Amido Schwarz. However, when the gels were stained for acylphosphatase (by first incnbating the gel with acetyl-phosphate and then treating with a mixture of 1% ascorbate and 1% ammonium molybdate at pH 4.5, Chapter II) only one phosphatase bands was observed. This indicated that the impurities present in the enzyme were protein impurities other than phosphatases. From the position of the bands on the gel it could be seen that the impurities were higher molecular weight impurities. Due to this difference in molecular weights of the three components, it appeared to be worthwhile to attempt the separation of the enzyme from the impurities by chromatography on Sephadex G-100. 0.05 M K-acetate buffer at pH 4.5 was used because at this pH the protein showed good separation on gel electrophoresis. The stability of the enzyme at this pH was determined separately. The enzyme was found to lose no activity in 24 hr at 0° at pH 4.5.

15 g of Sephadex-G-100 were allowed to swell in a sufficient volume of 0.05 M K-acetate buffer, pH 4.5 at room temperature for about three to four days and a column (1.8 x 100 cm) was prepared. The column was equili-brated with the same buffer at 0°.

3 ml (2,250 units and 9 mg) of enzyme (in 0.05 M K-acetate buffer, pH 4.5) obtained after CM-cellulose chromatography were loaded on the column. 0.05 M K-acetate buffer at pH 4.5 was passed through the column. 1.5 ml fractions were collected and the protein in all the fractions was

determined. The fractions having protein were assayed for enzyme activity. The protein was present in fractions 24 to 40. The activity was also present in fractions 24 to 40. The purity of the active fractions was also determined by gel electrophoresis. Fractions 28 to 40 showed a single band. They were pooled (18 ml) and precipitated with ammonium sulphate at 0.9 saturation. The precipitate was dissolved in 3 ml of 0.01 M K-acetate buffer, pH 5.7 and dialyzed against two changes of 500 ml of the same buffer. The volume after dialysis was 4 ml. 55% of the initial activity was recovered in this chromatography. The results are shown in Table 14 and Fig. 2.

From the Table 14 it will be seen that there is relatively little increase in purity in this fractionation, but the enzyme obtained after CM-cellulose chromatography showed several bands on acrylamide gel electrophoresis, whereas after sephadex chromatography only one enzyme band was obtained.

TABLE 14

FRACTIONATION OF ACYLPHOSPHATASE ON SEPHADEX-G-100

Fraction No.	Volume	Activity	Total activity	Protein	Total protein	Specific activity	No. of bands on gel electrophoresis
	ml	units/ml	units	mg/ml	mg	units/mg	
Before Sephadex chromatography	3	750	2,250	3	9	250	Three
Fractions 0+1 to 23	135						
24	1.5	26	39	0.090	0.135	290	Two
26	1.5	44	66	0.150	0.225	290	Two
28	1.5	60	90	0.190	0.285	320	One
30	1.5	84	126	0.250	0.375	336	One
32	1.5	90	135	0.330	0.495	272	One
34	1.5	92	138	0.365	0.548	250	One
36	1.5	92	138	0.355	0.533	250	One
38	1.5	80	120	0.285	0.428	276	One
40	1.5	44	66	0.210	0.315	210	One
Fractions 28 to 40 pooled, precipitated and dialyzed	4	300	1,200	1.0	4.0	300	One

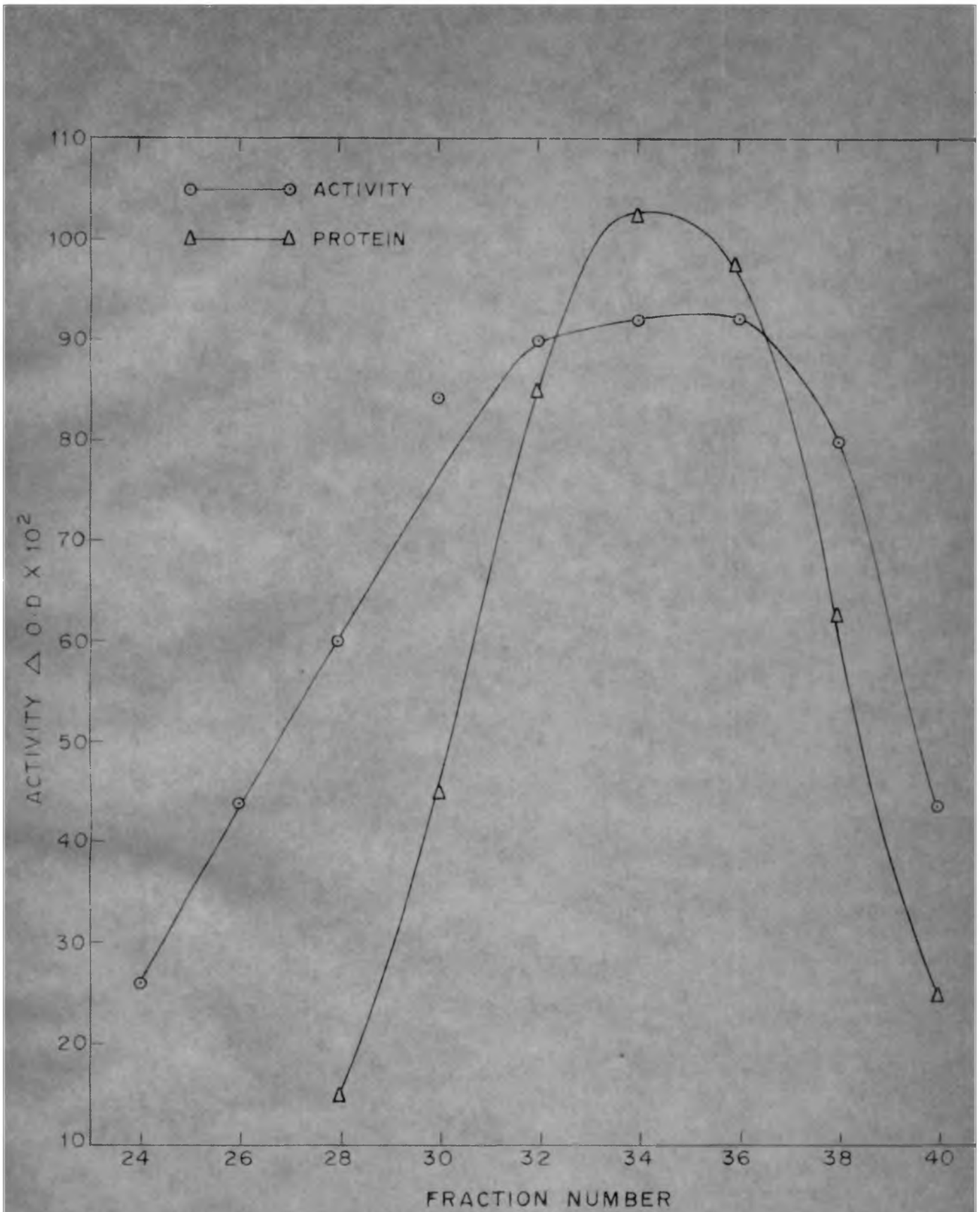


FIG. 2 CHROMATOGRAPHY OF ACYLPHOSPHATASE ON SEPHADEX-G-100
(1.8 x 100 cm)

SECTION VIII

MODIFIED PURIFICATION PROCEDURE

The extraction procedure described in the beginning of thie chapter was suitable for the extraction of enzyme on a small scale. However, for more rapid work and to get larger amounts of enzyme it was essential to modify the earlier procedure. The following changes were made in the new purification procedure.

1) Seed extraction

Only 150 g of seeds could be blendorized at a time in the Waring blendor. For processing big batches of 3 kg of seeds blendorizing was too time-consuming. The blendor was, therefore, replaced by a motorized meat mincer. The soaked seeds were passed through a meat mincer and the minced material was suspended in buffer. The mincing operation required 30 min whereas blendorizing would have taken a much longer period. Moreover, the enzyme is unstable in strongly acid solution if the temperature is not low and it was difficult to carry out blendorizing without undue increase in temperature.

2) Ammonium sulphate fractionation

In the previous procedure the extract was first precipitated at 0.4 ammonium sulphate saturation. The solution was centrifuged and the precipitate was discarded. The ammonium sulphate saturation of the supernatant liquid was then raised to 0.7. This involved two centrifugations of a large volume of liquid. It was easier directly to precipitate the enzyme at 0.7 saturation and then to refractionate

it at 0.3-0.9 saturation in a smaller volume rather than fractionate a large volume between 0.4-0.7 saturation.

In the modified purification procedure the fractionation at pH 8.5 was deleted and chromatography on DEAE-cellulose was introduced.

Chromatography on DEAE-cellulose

In some of the experiments, even after ammonium sulphate fractionation, the ATPase content of the extract was high. If a direct chromatography on CM-cellulose was performed with such enzyme preparations, the specific activity of the enzyme obtained after CM-cellulose chromatography was rather low (120-150). Further purification of this enzyme was difficult. To overcome this difficulty, negative adsorption of the enzyme on DEAE-cellulose was introduced before chromatography on CM-cellulose.

In a preliminary batchwise experiment it was found that when 20 mg of DEAE-cellulose were used for 1 mg of protein, acylphosphatase remained unadsorbed while almost all the ATPase was adsorbed on the cellulose. Column chromatography of the enzyme on DEAE-cellulose was then carried out.

30 g of DEAE-cellulose were washed and equilibrated with 0.01 M potassium phosphate buffer, pH 7.5. A column (6 x 10 cm) was prepared and washed with 0.01 M phosphate buffer, pH 7.5. 30 ml of the ammonium sulphate precipitated and dialyzed enzyme corresponding to 21,000 units and 1.5 g of protein were loaded on the column. The column was washed with 600 ml of 0.01 M K-phosphate buffer, pH 7.5. 50 ml fractions

were collected and the activity and protein of each fraction were determined. (Phosphate is inhibitory to acylphosphatase activity and dialysis is required to obtain true acylphosphatase activity; but for routine work dialysis of each fraction was not carried out.) The results are presented in Table 15 and Fig. 3. The results are not corrected for inhibition by phosphate. Most of the ATPase is removed in this step. Fractions 4 to 8 were pooled and precipitated with ammonium sulphate at 0.9 saturation. The precipitate was dissolved and dialyzed against 0.01 M K-acetate buffer, pH 5.7. About 50% of the initial activity was recovered and about two to three-fold increase in the specific activity was observed. The specific activity of the enzyme obtained after fractionation on DEAE-cellulose ranged from 40 to 70 units per mg protein.

For large-scale preparation of the enzyme three such columns were run simultaneously.

On the basis of the experiments described in previous sections the purification procedure was modified and is described in the next Section.

TABLE 15

FRACTIONATION OF ACYLPHOSPHATASE ON DEAE-CELLULOSE COLUMN (6 x 10 cm) IN 0,01 M PHOSPHATE BUFFER, pH 7,5.

Fraction No.	Volume	Activity	Total activity	Protein	Total protein	Specific activity
	ml	units/ml	units	mg/ml	mg	units/mg
Before DEAE cellulose chromatography	30	700	21,000	50	1,500	14
Fractions (undialyzed)						
1 to 3	150					
4	50	10	500	1.6	80	6
5	65	60	3,900	1.7	110	35
6	65	45	2,925	1.2	78	38
7	50	13	650	1.0	50	13
8	50	13	650	1.0	50	13
9	50	-	-	0.6		

SECTION IX

FINAL PURIFICATION PROCEDURE

Step I. Extraction

Three kg batches were usually processed. The seeds were washed with glass-distilled water and soaked in water at room temperature for 20 min. All further operations were carried out at 0°. The seeds were passed through a meat mincer and the minced material was suspended in the extraction medium consisting of 15,000 ml of 0.01 M K-acetate buffer, pH 5.7, 225 g of KCl, and 3,000 ml of 0.6 M HCl. The mixture was allowed to stand for 30 min with occasional stirring and then squeezed through muslin. The liquid was neutralized to pH 5.7 by the addition of 2 M $KHCO_3$. It was passed through a Sharples centrifuge and the precipitate was discarded (Fraction I). All oerations upto neutralization of the extract to pH 5.7 should be carried out as rapidly as possible.

Step II. Ammonium sulphate fractionation

0 to 0.7 saturation. The supernatant liquid from Step I (14,200 ml) was precipitated with ammonium sulphate by adding 6177 g (43.5 g for every 100 ml solution). It was passed through a Sharples centrifuge. The precipitate was collected and the supernatant liquid was discarded.

Washing with 0.7 saturated ammonium sulphate. The above precipitate was suspended in 0.7 saturated ammonium sulphate. It was thoroughly mixed to break up lumps. (All operations upto this stage should be carried out on the same day). The next day the

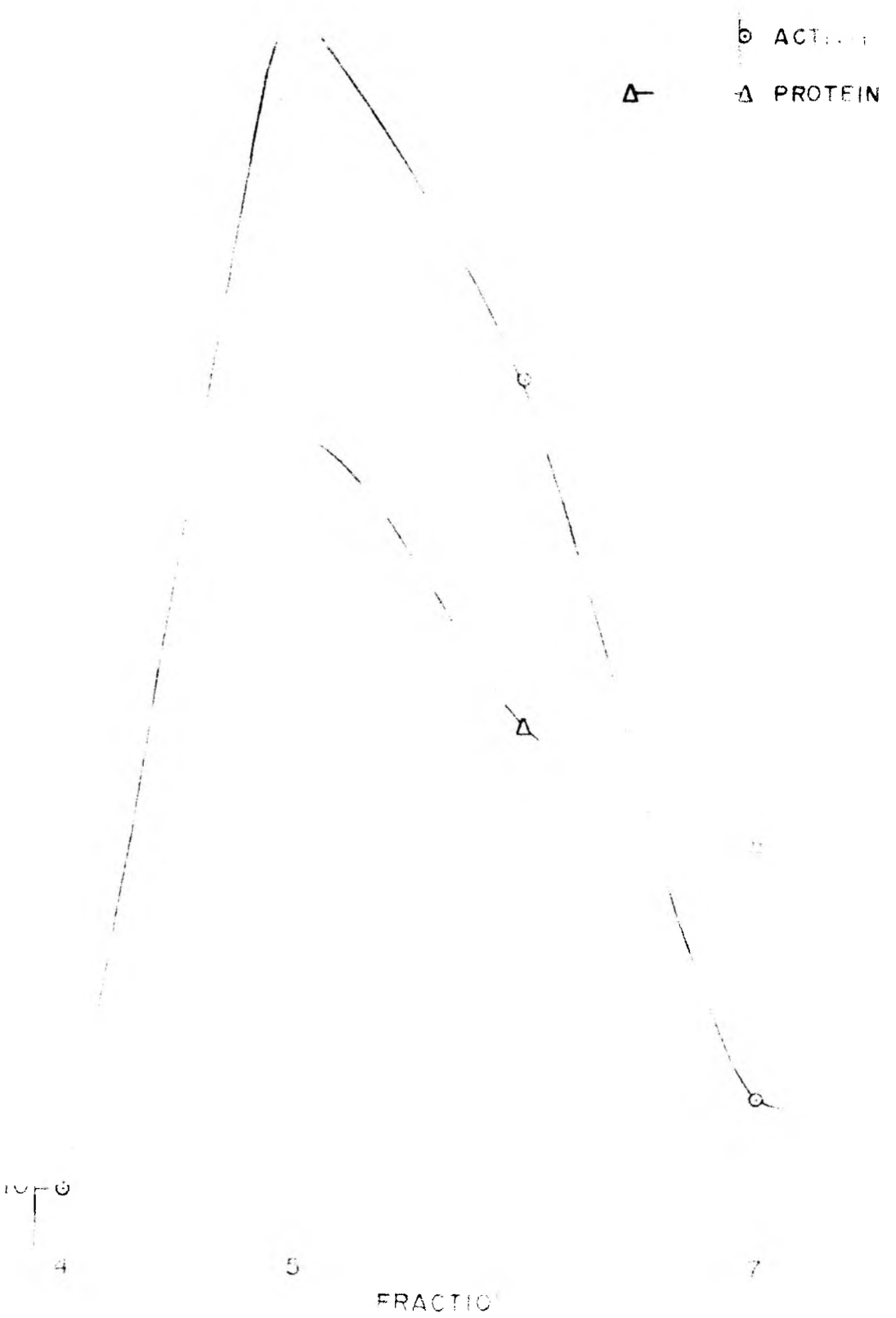


FIG. 3 ACTIVITY AND PROTEIN PATTERN OF ACYLPHOSPHATASE ON DEAE-CELLULOSE CHROMATOGRAPHY

suspension was centrifuged for 30 min at 13,000 x g. The precipitate was dissolved in 500 ml of 0.01 M K-acetate buffer, pH 5.7. The final volume of the solution was noted (540 ml). (Fraction II). The final ammonium sulphate saturation was calculated to be about 0.05.

0.05 to 0.3 saturation. The ammonium sulphate saturation of the above solution was raised from 0.05 to 0.30 by adding 13.6 g of ammonium sulphate to every 100 ml of the solution. It was allowed to stand for 30 min and centrifuged for 30 min at 13,000 x g. The precipitate was discarded and the supernatant liquid (volume 525 ml - Fraction III) was collected.

0.3 to 0.9 saturation. Fraction III was precipitated with ammonium sulphate by increasing the saturation from 0.3 to 0.9 by adding 40 g of ammonium sulphate for every 100 ml of solution. The precipitate was dissolved in 120 ml of 0.01 M K-acetate buffer, pH 5.7 and dialyzed against three changes of 2 l each of the same buffer. The volume after dialysis and centrifugation was 165 ml (Fraction IV).

Step III. Chromatography on DEAE-cellulose.

30 g of DEAE-cellulose were washed and equilibrated with 0.01 M K-phosphate buffer, pH 7.5. A (6 x 10 cm) column was prepared and washed with 0.01 M phosphate buffer, pH 7.5. 30 ml of ammonium sulphate precipitated and dialyzed enzyme (Fraction IV) were loaded on the column. The column was washed with 600 ml of 0.01 M phosphate buffer, pH 7.5. 50 ml fractions were collected and the activity and protein of the fractions were determined. The active fractions were pooled and precipitated at 0.9 saturation. The precipitate was dissolved and dialyzed against 0.01 M K-acetate buffer, pH 5.7 (Fraction V). Three such columns were usually run simultaneously.

About 50% of the initial activity was recovered and about 2-3 fold increase in the specific activity was obtained. The specific activity of the enzyme obtained after fractionation on DEAE-cellulose ranged from 30 to 70 units per mg.

Step IV. Chromatography on CM-cellulose.

14 g of CM-cellulose were washed and equilibrated with 0.01 M Tris buffer, pH 7.5. A (15 x 4.4 cm) column was prepared and washed with the same buffer. Two such columns were usually run simultaneously. 35 ml of the enzyme from Step III (Fraction V) were loaded on the column. The column was washed with 400 ml of 0.1 M Tris buffer, pH 7.5 and eluted with 400 ml of 0.2 M Tris buffer, pH 7.5. 50 ml fractions were collected and estimated for activity and protein. The active fractions with specific activity higher than 200 units/mg were pooled (total volume 275 ml) and concentrated by lyophilisation to less than one-fourth of the initial volume (60 ml). The enzyme was then precipitated with ammonium sulphate at 0.9 saturation by adding 60 g of ammonium sulphate for every 100 ml solution. The precipitate was dissolved in 3 ml and dialyzed overnight against 1 litre of 0.01 M K-acetate buffer, pH 5.7 (Fraction VI). About 10-fold purification was achieved by this fractionation and 40% of the activity was recovered. The specific activity of the enzyme after fractionation ranged from 300 to 1,500 units per mg. This step was carried out with 10 batches and the final enzyme was free from other phosphatases.

Step V. Chromatography on Sephadex-G-100.

Acrylamide gel electrophoresis of the enzyme obtained after CM-cellulose chromatography showed that it needed further purification. As stated previously staining the gels with Amido Schwarz and for acylphosphatase showed that the enzyme from Step IV (Fraction VI) contained at least two protein impurities other than acylphosphatase. Chromatography on Sephadex-G-100 was carried out for further purification. It should be noted that the main criterion for purity of the eluate fractions was not merely specific activity but the absence of any other protein impurities on acrylamide gel electrophoresis.

A (100 x 1.8 cm) column was prepared with 15 g of Sephadex-G-100 and equilibrated with 0.05 M K-acetate buffer, pH 4.5. 4.5 ml of Fraction VI containing 2,700 units and 10 mg protein were loaded on the column. The column was washed with 0.05 M K-acetate buffer, pH 4.5 and 1.5 ml fractions were collected. The fractions containing protein were assayed for acylphosphatase activity and the fractions having acylphosphatase activity were tested for their purity by acrylamide gel electrophoresis. The fractions having acylphosphatase activity and showing a single band on gel by both the staining techniques were pooled and precipitated at 0.9 ammonium sulphate saturation. The precipitate was dissolved in 3 to 4 ml of 0.01 M K-acetate buffer, pH 5.7 and dialyzed overnight against two changes of 1 litre each of 0.01 M K-acetate buffer, pH 5.7 (Fraction VII).

Four such (3 kg) batches were processed upto the last step of purification. The final specific activity of the enzyme ranged from

300 to 1200 units per mg and the final yield of the enzyme was 4%. The yield is higher if correction is made for other phosphatases present in the initial extract. The purified enzyme (Fraction VII) showed a single band on gel electrophoresis and was homogeneous in the ultracentrifuge.

The results of a typical fractionation procedure are given in Table 16.

TABLE 16

PURIFICATION OF ACYLPHOSPHATASE

Fraction	Volume	Activity	Total activity	Protein	Total protein	Specific activity
	ml	units/ml	units	mg/ml	mg	units/mg
Neutralized extract	15,000	16	240,000	8	120,000	2
0 to 0.7 saturation	540	340	184,000	36	19,440	9
0.3 to 0.9 saturation	165	680	112,000	50	8,250	14
DEAE-cellulose chromatography	112	400	45,000	13	1,456	31
CM-cellulose chromatography	30	600	18,000	2.2	66	273
Sephadex-G-100 chromatography	30	300	9,000	1	30	300

(3 kg seeds were used)

SECTION X

LOCALIZATION OF ENZYME

Acylphosphatase activity of the seed embryos was estimated in order to determine whether the activity was localized mainly in the embryos. The activity of the root nodules on the plant was also estimated in order to determine whether the bacteria are responsible for the high activity of enzyme in this legume.

Embryos

Seeds of V.catjang were washed with water to remove preservatives and soaked in glass-distilled water for 30 min at room temperature. The seedcoats were removed and the embryos were separated from the seeds. 1 g of embryos was obtained from 20 g of seeds. The embryos were ground and extracted with a mixture of 5 ml of 0.01 M K-acetate buffer, pH 5.7, 75 mg of KCl (solid) and 1 ml of 0.6 M HCl. The extract was allowed to stand for 30 min and centrifuged at 13,000 x g for 30 min. The supernatant liquid was neutralized to pH 5.7 with 2 M $KHCO_3$ and was tested for acylphosphatase activity. It was observed that the acylphosphatase activity of embryos was 43 units per g of embryos. 20 g of seeds which corresponded to 1 g of embryos had a total acylphosphatase activity of 1,000 units. This showed that the embryo has negligible acylphosphatase activity. These results are not corrected for other phosphatases, since true acylphosphatase activity is not less than 25% of the total activity whereas the activity of the embryos is only 4% of the total activity.

Nodules

300 mg of nodules from a one month old plant were washed thoroughly with glass-distilled water and were extracted with a mixture of

1.5 ml of 0.01 M K-acetate buffer pH 5.7, 0.3 ml of 0.6 M HCl and 22 mg of solid KCl. The mixture was thoroughly ground using a pestle and mortar till the plant cells appeared broken under the microscope (only the plant cells and not the Rhizobia were tested for the enzyme). The extract was allowed to stand for 30 min and centrifuged at 13,000 x g for 30 min. The supernatnat liquid was neutralized to pH 5.7 with 2 M $KHCO_3$ and was tested for acylphosphatase activity. It was found that nodules of V.catjang possess no acylphosphatase activity.

Localization of enzyme in the cell

50 g of seeds were washed and soaked in water for 30 min. The soaked seeds were extracted with 500 ml of 0.05 M Tris buffer, pH 7.5 (containing 0.061 M Mg^{++}, 0.001 M thioethanol and 0.25 M sucrose) by blendorizing for 3 min. The homogenate was allowed to stand for 30 min and centrifuged at 800 x g. The residue contained nuclei, cell debris and unbroken seeds. It was suspended in 125 ml of 0.01 M K-acetate buffer, pH 5.7 and tested for acylphosphatase activity. Only 20% of the total activity was found to be present in the "800 x g residue".

The supernatant liquid was further centrifuged at 20,000 x g. The mitochondria sedimented in the residue. The residue was suspended in 40 ml of 0.05 M Tris buffer, pH 7.5 and tested for acylphosphatase activity. Mitochondria had negligible acylphosphatase activity. The residue was sonicated to see whether they release enzyme upon breaking. There was no difference in activity before and after sonication.

The supernatant liquid obtained after centrifugation at 20,000 x g contained microsomes and the soluble fraction of the seeds. It was assayed for acylphosphatase activity and it was found to have 80% of the

total activity. Acylphosphatase and ATPase activities of the different fractions are summarized in Table 17.

In a separate experiment the extract of the seeds was centrifuged at 100,000 x g. All the activity was found to be present in the supernatant liquid. The microsomes are, therefore, inactive and the acylphosphatase of V.catjang is a soluble enzyme.

TABLE 17

CHOLAI SEED FRACTIONATION

	Residue 800 x g			Residue 20,000 x g						Supernatant 20,000 x g		
				Before sonication			After sonication					
	Vol. ml	u/ml	Total units	Vol. ml	u/ml	Total units	Vol. ml	u/ml	Total units	Vol. ml	u/ml	Total units
AcPase	225	19	4,275	42	15	630	42	15	620	360	44	15,840
ATPase	225			42	19	798	42	21	882	360	48	17,280

SECTION XI

ATPase FRACTION WITH ACYLPHOSPHATASE ACTIVITY

The crude extract of V.catjang contained besides acylphosphatase other non-specific phosphatases such as ATPase and G-6-Pase which also had acylphosphatase activity. During the purification of acylphosphatase on CM-cellulose column (in 0.01 M Tris-HCl buffer, pH 7.5) the unadsorbed fraction contained ATPase activity (which also had phosphatase activity) while "true" acylphosphatase could be adsorbed completely and was eluted with 0.2 M Tris-HCl buffer, pH 7.5. The latter was free from ATPase.

The effect of some compounds on ATPase and "acylphosphatase" activity of the ATPase fraction was determined. The ATPase fraction obtained during CM-cellulose fractionation was used for these experiments. It is not known whether it contained small amounts of true acylphosphatase. The ATPase fraction was tested for its activity with ATP and acetylphosphate in the presence of different inhibitors.

Effect of p-CMB and iodoacetate

6.6×10^{-4} M pCMB inhibited true acylphosphatase activity by 66% and 6.6×10^{-3} M iodoacetate inhibited it by 40%. pCMB and iodoacetate at the same concentrations had no effect on ATPase activity of the ATPase fraction whereas the "acylphosphatase" activity of this fraction was inhibited 25% by both the compounds.

DFP

Diisopropylfluorophosphate at 6.6×10^{-4} M had no effect on true AcPase or on the "AcPase" activity of the ATPase fraction whereas ATPase activity was inhibited 25%.

$HgCl_2$ and $Na_2S_2O_3$

$HgCl_2$ at 6.6×10^{-4} inhibited true acylphosphatase 80% and "acylphosphatase" activity of the ATPase fraction 100%. There was only 16% inhibition of the ATPase activity. Similarly sodium metabisulphite inhibited true AcPase and "AcPase" of the ATPase fraction 66% and 75% respectively. ATPase was inhibited 40%. The inhibition of true acylphosphatase by Hg^{++} and by sodium metabisulphite was irreversible, whereas the inhibition of "acylphosphatase" activity of the ATPase fraction could be reversed by dialysis or by treatment with cysteine or thioethanol (5 moles of reducing agent per mole of Hg).

Effect of heat and $HgCl_2$ on the ATPase fraction

The following experiment was carried out in order to determine whether the two activities of ATPase (with ATP and acetylphosphate) could be separated by selective destruction of one. The enzyme was treated with 0.002 M $HgCl_2$ at pH 5.7 for 15 min. After the treatment the pH was brought down to 3.5 and the enzyme was heated at 50° for 5 min at this pH. The heated enzyme was neutralized to pH 5.7, centrifuged and the supernatant liquid was dialyzed against one litre of 0.01 M K-acetate buffer, pH 5.7 containing 0.01 M EDTA. In the control experiment the enzyme was given exactly the same treatment but without treatment with mercuric chloride. The results are presented in Table 18.

TABLE 18

HEATING OF $HgCl_2$ TREATED AcPase/ATPase FRACTION

	$HgCl_2$ treated		Untreated	
	Before heating u/ml	After heating u/ml	Before heating u/ml	After heating upml
AcPase	2.6	2.6	5.4	4.2
ATPase	8.8	6.8	18.4	16.0

From Table 18 it will be seen that acylphosphatase is not destroyed completely by mercuric chloride treatment and by heating it at 50° for 5 min at pH 3.5. It was not possible to separate ATPase from AcPase by this method.

Effect of fluoride, phosphate and EDTA

True acylphosphatase activity is unaffected by fluoride (6.6×10^{-3} and 1.32×10^{-2} M) but there is 80% inhibition in the "acylphosphatase" activity of the ATPase fraction. 1.3×10^{-3} M phosphate inhibited "acylphosphatase" activity of the ATPase fraction by 30% whereas true acylphosphatase was unaffected at this phosphate concentration. 6.6×10^{-3} M EDTA inhibited "AcPase" activity of the ATPase fraction by 35%. EDTA had no effect on true acylphosphatase activity.

These experiments show that the seeds of Vigna catjang contain an enzyme which hydrolyzes both ATP and acetylphosphate. The two activities could not be separated and possibly reside in the same enzyme though more work is needed to establish this. The "acylphosphatase" activity of this fraction is different from true acylphosphatase.

No studies were carried out on the glucose-6-phosphatase which also had a fluoride sensitive acylphosphatase activity. The wide variation in the ratios of G-6-Pase and ATPase activities of different fractions showed that they are different enzymes.

CHAPTER V

DISCUSSION

DISCUSSION

Purification

Acylphosphatase from *V.catjang* was purified about 200-fold. In the crude extract, acylphosphatase activity formed only a part of the total activity which was also due to ATPase etc. Hence the actual purification is closer to 800 to 900 fold. The purified preparation was homogeneous by ultracentrifugation and gave a single band on gel electrophoresis. The final specific activity of 300 to 1200 units per mg obtained by this procedure is the lowest when compared to the specific activities obtained for the enzyme from animal sources. The preparations from animal sources have specific activities of 30,000 (horse muscle) to 75,000 units/mg (bovine brain) and only the human erythrocyte enzyme has a specific activity of 700-7000 units/mg.

The separation of acylphosphatase from other nonspecific phosphatases offered difficulty during purification. Heating of the crude extract was to a certain extent useful in destroying phosphatases other than acylphosphatase, but the results were not reproducible. Extraction of seeds with strong acid was more useful in keeping the amounts of nonspecific phosphatases to a minimum. Ammonium sulphate fractionation and chromatography on DEAE-cellulose removed the nonspecific phosphatases such as ATPase and G-6-Pase. The major purification was obtained by CM-cellulose chromatography. The enzyme obtained after CM-cellulose chromatography was completely free from ATPase and G-6-Pase, but contained two high molecular weight impurities as shown by acrylamide gel electrophoresis. These impurities were separated from the enzyme by chromatography on Sephadex-G-100. It is noteworthy that the enzyme

has no mobility at pH 5.7 or 8.5 in polyacrylamido gel at 7 per cent gel, whereas it was mobile in 15 per cent gel at pH 4.5. This appears to be characteristic of basic proteins of low molecular weight (Reisfield, 1962).

An unexplained finding during purification was the lack of reproducibility of the results during fractionation on IRC-50 XE 64 and SE-cellulose. In the earlier experiments on IRC, enzyme of high purity (1,000 units/mg) was obtained in one step. But later it was not possible to reproduce these results. Similarly with SE-cellulose in some experiments 50% of the enzyme was adsorbed on cellulose and the purity of the enzyme which remained unadsorbed was very high (1,000 units/mg). This result also could not be reproduced. The reason for these discrepancies is not known.

Another puzzling feature about the enzyme is the wide variation in the specific activity of the final purified enzyme. Though it was homogeneous in all cases by gel electrophoresis, different lots of seeds and even the same lot gave enzyme of maximum specific activity varying from 300 to 1,200 units/mg. The enzyme is quite stable and it is improbable that the variation in specific activity is due to the presence of denatured enzyme. In the case of the erythrocyte enzyme also the final specific activity varies from 700 to 7000 units/mg. The enzyme with low specific activity was found to contain traces of hemoglobin as an impurity. No such explanation is available for the variation in specific activity of acylphosphatase from V.catjang. It is possibly due to genetic inhomogneiety of the seeds used.

Stability

The enzyme (1 mg/ml) was remarkably stable at higher levels of purity when stored at -20°, pH 5.7. It was not very stable in the earlier stages of purification, probably because of the presence of proteolytic enzymes in the crude extract.

Molecular weight

The molecular weight of acylphosphatase as determined by ultracentrifugation is 6,000 daltons. It is lower than that reported for the enzyme from other sources: 10,000 (for horse muscle), 23,000 (rabbit muscle), 13,000 (bovine brain)and 11,000 (pork heart). The acylphosphatase of V.satiang appears to be one of the smallest enzymes described hitherto.

Substrate concentration

The V_m is above 4.5 mM and K_m at 30° at pH 5.7 is 0.85 mM. This value is lower than the values obtained for the enzyme from muscle (8 mM) and erythrocytes (7.4 - 12.7 mM). Inorganic phosphate inhibited acylphosphatase activity, the Ki of 2.2 mM being comparable with the Ki of 3 mM for the erythrocyte enzyme. The inhibition is of the competitive type in both the cases.

Sulphate was found to inhibit acylphosphatase activity non-competitively with a Ki of 1.7 mM.

Effect of temperature

There is no significant inactivation of the enzyme under the conditions of assay at temperatures ranging from 2-30°C. The increase

in activity for a 10° rise in temperature was only 30% in contrast to an average of 100% for several enzymes. The energy of activation was calculated to be about 5200 cal. The enzyme from V.catjang resembles the muscle and brain enzyme in being thermostable, whereas the erythrocyte enzyme is more thermolabile.

Effect of pH

A sharp pH optimum of 5.6 was observed for acylphosphatase of V.catjang. This is similar to the pH optimum reported for the muscle, erythrocyte and heart enzyme. The optimum pH of the enzyme from brain was, however, reported to be 7.5.

Amino acid analysis

Acylphosphatase from V.catjang contains 1 mole each of tyrosine and tryptophan per mole of enzyme whereas 2 moles of tryptophan and 3 moles of tyrosine per mole of enzyme have been reported for bovine brain and rabbit muscle enzymes respectively. Acylphosphatase of V.catjang was found to have one sulphide and one disulphide group per mole of enzyme. As the development of color with DTNB was very slow the sulfhydryl group appears to be buried inside the molecule and is relatively inaccessible. As stated earlier the sulfhydryl content of the enzyme from bovine brain (Diederich and Grisolia, 1969) was only 0.01 mole of -SH groups per mole of enzyme. With DTNB there was no difference in color of the blank and experimental sample after 15 and 30 min suggesting that there was no -SH group. However, in view of the results obtained for acylphosphatase from V.catjang, the above results with the brain enzyme will have to be rechecked and it would

be necessary to determine whether the enzyme from brain also possesses a buried sulfhydryl group.

The presence of a peptide of low molecular weight which is bound by a disulfide link to the enzyme was reported (Ramponi *et al.* 1971) for acylphosphatase from horse muscle. This peptide was later recognised to be glutathione. There is no such evidence of a mixed disulfide for acylphosphatase from *V. cattang*. If such a small peptide is attached to the enzyme by an -S-S- link and is required for activity, reduction with borohydride should have inactivated the enzyme, whereas enzyme activity is unaffected by borohydride. It is also noteworthy that in spite of the presence of an -S-S- link in the enzyme it is unaffected by dithiothreitol and borohydride. It was, however, not established whether reoxidation of -SH to form -S-S- takes place readily and is appreciable during the period of assay.

Inhibitors

pCMB and iodoacetate

The plant enzyme is different from the animal tissue enzymes in being inhibited by pCMB and iodoacetate. However attempts to show binding of pCMB to the enzyme by a spectrophotometric method (Boyer, 1954) were unsuccessful. It is doubtful whether pCMB binds with the sulfhydryl of the enzyme. As stated earlier, inhibition of the enzyme by pCMB may be similar to the inhibition by pCMB of pancreatic ribonuclease, which has no -SH group.

Cysteine, thioethanol and reduced glutathione

Cysteine, thioethanol and reduced glutathione had no effect on

enzyme activity. Incubation of the enzyme with cysteine or thioethanol or CoA together with H_2O_2 for 24 h at 0° had no effect on enzyme activity. H_2O_2 by itself has no effect on enzyme activity.

Oxidised glutathione

Addition of oxidized glutathione to the enzyme inhibited enzyme activity by 50%. 80% inhibition was obtained by incubating the enzyme with oxidized glutathione and pCMB, the inhibition being nearly additive.

Bisulphite

Incubation of the enzyme with bisulphite resulted in 50% loss in activity. Incubation of cysteine, thioethanol, CoA or sodium borohydride with bisulphite treated and dialyzed enzyme did not result in the reversal of the inhibition caused by bisulphite.

A possible explanation for these results is that G-S-S-G acts on the -SH group of the enzyme to form a derivative of the type G-S-S-Enz which has only half the activity of the enzyme. The lack of partial inactivation with cysteine and thioethanol may be due to the fact that the derivatives of the enzyme with these compounds have the same activity as the enzyme. Further work is needed, preferably with labeled compounds to determine whether the glutathione is linked to the enzyme. But this supposition does not explain why reduction with excess cysteine or borohydride followed by dialysis does not restore the activity of the GSSG treated enzyme.

The effect of bisulphite in inhibiting the enzyme and of Hg^{++} in inactivation of the enzyme may be related to breaking of the S-S bond

of the enzyme.

In this connection it is interesting to note recent work on the regulation of enzyme activity by linking the enzyme sulfhydryl group with another thio-derivative by a disulphide linkage. Fructose 1,6-diphosphatase which has been studied by Nakashima, Horecker, Traniello and Pontremoli (1970) is altered in activity and K_m value by incubation with CoA or acyl carrier protein under such conditions as to link these compounds by disulphide bonds to a sulfhydryl group in the enzyme. It was recently reported by Ramponi, Cappugi, Treves and Naesi (1971) that the horse muscle enzyme contains glutathione linked by a disulphide group to the enzyme. The alteration of activity and K_m by linkage of another sulfhydryl compound or protein by a disulphide link to the enzyme appears to be a new mechanism for the regulation of enzyme activity, which may be of physiological importance. Further work is needed to elucidate the nature of the compound linked to V.catjang acylphosphatase.

Metals and metal chelating agents

The enzyme does not appear to require a metal for its activity as determined by the lack of effect of added metal ions and metal binding agents such as KCN, - -dipyridyl and EDTA. Exhaustive dialysis of the enzyme against 0.01 M EDTA did not result in any loss in activity. Na^+, K^+, Mg^{++}, Ca^{++}, Mn^{++} and Co^{++} have no effect on enzyme activity. Acylphosphatase from brewer's yeast was reported to require Mg^{++} for its activity (Harary, 1957). No evidence could be obtained for a Mg^{++} requirement for acylphosphatase from V.catjang. However

the possibility of trace amounts of Mg^{++} or other metal being present in the substrate or buffer was not excluded. More rigorous purification of all reagents may be needed to establish unequivocally that this enzyme has no metal requirement. Whether different samples of acetylphosphate contain traces of Mg^{++} as impurity which activates the enzyme is not known. $MgCl_2$ (5 mM) was found to enhance the activity of the enzyme from bovine brain when tris-acetylphosphate served as a substrate (Diederich and Grisolia, 1969)

The erythrocyte enzyme is the only one which has been reported to be inhibited by EDTA. Hg^{++} strongly inhibited the plant enzyme and the inhibition was irreversible whereas none of the animal tissue enzymes has been reported to be inhibited by Hg^{++}.

Anions

Sulphate, oxalate and citrate inhibited the enzyme. However, chloride and tartrate had no effect on enzyme activity. Phosphate inhibits competitively and sulphate noncompetitively. Phosphate inhibition is characteristic of animal tissue acylphosphatases.

Miscellaneous compounds

A variety of other compounds such as nucleotides, coenzymes, amino acids and sugars had no effect on enzyme activity. Thyroxine (3.3×10^{-5} M) had no effect when it was added to the assay system but preincubation with enzyme showed 20% inhibition. The plant enzyme resembles the animal tissue enzymes in showing inhibition on incubation with thyroxine. DFP, KI, alcohol, abscisic acid, benzaldehyde, cadaverine and 2,4-dinitrophenyl hydrazine had no effect on enzyme activity.

The plant enzyme resembles the erythrocyte enzyme in being inhibited by carbamyl phosphate, but differs from it in showing no inhibition by fluoride.

Substrate specificity

Acylphosphatases from animal tissues, except the erythrocyte enzyme, act both upon acetylphosphate and carbamyl phosphate. The ratio of activity with acetylphosphate to that with carbamyl phosphate is the same (10:1) for the enzymes purified from bovine brain, pork heart, horse muscle and beef liver. Only the erythrocyte enzyme does not act on carbamyl phosphate. However, all enzymes from animal tissues tested so far were found to hydrolyze 1,3-diphosphoglycerate. The enzyme from V.catjang does not act either on carbamyl phosphate or on 1,3-diphosphoglycerate. It acts both on acetylphosphate and propionyl phosphate. Thus it appears to act on fatty acylphosphates unsubstituted in the acyl group.

The name acylphosphatase rather than acetylphosphatase has been used since it acts on both acetyl and propionyl phosphates. Other acylphosphates must be tested as substrates before a more specific name is given for the enzyme. In any case in view of the wide difference in specificity of known acylphosphatases, their nomenclature requires reconsideration. The acylphosphatases (excluding nonspecific phosphatases) act (a) on acetylphosphate, 1,3 DPGA and carbamyl phosphate, (b) acetylphosphate and 1,3 DPGA and (c) acetylphosphate only. The specificity of the Vigna catjang enzyme for acetylphosphate and propionyl phosphate and lack of activity either with ATP, G-6-P etc. or with

acylphosphates such as carbamyl phosphate and 1,3-diphosphoglycerate show that it is different from all other known phosphatases and acyl-phosphatases.

Role of acylphosphatase

The role of acylphosphatase is not known, but it has been postulated that the function of acylphosphatase may be to prevent or regulate the concentration of acylphosphates and thereby act as a chemotropic effector. It has also been postulated that it regulates the rate of glycolysis by hydrolysing 1,3 DPGA and thereby uncoupling glycolysis from phosphorylation.

The enzyme from plants acts neither on 1,3 diphosphoglycerate nor on carbamyl phosphate. Its physiological substrate and mode of action are, therefore, unknown and remain to be determined. The occurrence of acetylphosphate, acetokinase and phosphotransacetylase in plants requires to be reexamined. There is no evidence for the occurrence of acetyl phosphate except in microorganisms, but it is possible that acetylphosphate does occur in plants, possibly by the action of acetokinase (from acetate and ATP) or phosphotransacetylase (from acetyl-CoA and phosphate). The function of the enzyme may then be to regulate the concentration of acetylphosphate. But if acetyl-phosphate is not formed in plants, further work will be needed to determine which substrate is acted upon by acetylphosphatase of V.catjang.

Localisation

The acylphosphatase activity of root nodules of V.catjang was negligible. There was negligible activity in the seed embryos. The

major activity was found to be concentrated in the cotyledons. Fractionation of the seed extract by differential centrifugation showed that the nuclear, mitochondrial and microsomal fractions had very little activity, whereas the 100,000 x g supernatant liquid contained all the acylphosphatase activity. Thus acylphosphatase of V.catjang is a soluble enzyme located in the cytoplasm.

ATPase fraction with acylphosphatase activity

The crude extracts of seeds of V.catjang contained ATPase and G-6-Pase as well as acylphosphatase. Some of the properties of the ATPase were studied in detail. ATPase is not identical with acylphosphatase nor was it derived from acylphosphatase by acid treatment during extraction. The purified acylphosphatase has no ATPase activity. However, attempts to obtain ATPase without acylphosphatase activity were unsuccessful. The properties of true acylphosphatase and of ATPase were quite different. pCMB and iodoacetate inhibited acylphosphatase but not ATPase. DFP has no effect on the AcPase activity of either of the enzymes whereas ATPase was inhibited (25%). Hg^{++} was inhibitory to "acylphosphatase activity" of the ATPase fraction, but had little effect on its ATPase activity. The inhibition of true acylphosphatase by Hg^{++} and bisulphite was irreversible, whereas inhibition by these compounds of "acylphosphatase" of the ATPase fraction was reversible. EDTA and fluoride do not inhibit the specific acylphosphatase but inhibit the "acylphosphatase" activity of the ATPase fraction. The activities towards acetylphosphate and ATP of the ATPase probably exist in the same molecule but at different active centres.

In conclusion acylphosphatase of V.catjang which has been isolated in homogeneous form is a basic protein of unusually low molecular weight which is specific for acetylphosphate and propionyl phosphate and has no action on carbamyl phosphate or 1,3-diphosphoglycerate and the physiological substrate and role of which are unknown.

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