THE UNIVERSITY OF MANITOBA

PURIFICATION OF THE CONSTITUTIVE ALKALINE PHOSPHATASE From *Neurospora crassa*: Some Chemical and Physical Properties

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF MICROBIOLOGY

WINNIPEG, MANITOBA

October, 1973

"If instead of laughing at someone who has difficulty,
And instead of saying 'he will never make it',
You lend a helping hand,
Then you will have done a good,
Not only to him, but also to yourself,
And you will have made this world,
A happier place for someone to live in".

The Author.

ABSTRACT.

This work is a continuation of the work entitled " A Study of Some Alkaline Phosphatases of Neurospora crassa " by F.W.J.Davis (1970). In his work Davis purified the enzyme to about 50% and investigated its kinetics. The present work is an attempt to purify the enzyme to homo - geneity and to explore further some of its physical and chemical properties.

The enzyme was purified by Sephadex G - 200. On polyacrylamide gels three protein bands were seen, two of which were active with the substrate PNPP. The minor enzyme activity (Davis, 1970) was absent on gels. Homogeneity was tested by SDS - polyacrylamide gel electrophoresis, and by sedimentation analysis of both the native and SDS - treated enzyme on the Model E Analytical Ultracentrifuge. From these methods the enzyme was judged to be homogeneous.

The minimum molecular weight of the enzyme was found by 7.5% poly - acrylamide gel (SDS $\rightleftharpoons$ gel) to be 30,000 $\pm$ 2,000. The isoelectric point of the enzyme immediately before Sephadex purification was found to be at pH 4.15 with 0.10 N sodium acetate buffer. It was also found that the number of protein bands seen on gels was apparently affected by the use of different buffers used to elute the enzyme from the Sephadex column. It is suspected that the enzyme perhaps changes conformation as it passes down the Sephadex column and assumes different aggregation states at different pHs or with different buffers.

The amino acid composition of the enzyme was determined.

ACKNOWLEDGEMENTS

The author wishes to express his gratitude to Dr.F.W.J.Davis and Dr. Howard Lees for their guidance during the course of this investigation.

Thanks are also extended to Dr. P. Maeba and his group, Dr. D. Burton and his group and to Dr. H.B. LeJohn and his group for the use of their materials and instruments. Special thanks are extended to Henry Yu and Hern Shiung Wang for their help in numerous ways.

The author also wishes to thank Mr. Ray Batenchuk of the Department of Plant Science, University of Manitoba, for the amino acid analyses.

ABBREVIATIONS

Buffer A	0.02 M Tris, 0.004 M $MgCl_2$, 0.0001 M $ZnCl_2$.
BSA	Bovine Serum Albumin
EDTA	Ethylenediamine tetraacetic acid
Gl - 3 - P DeHase	Glyceraldehyde - 3 - phosphate dehydrogenase
OD	Optical Density
P_i	Inorganic phosphate
PNPP	p - nitrophenyl phosphate
SDS	Sodium Dodecyl Sulfate

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INTRODUCTION

Few non - specific phosphomonoesterases have been obtained in high purity with the exception of that from Escherichia coli which has been purified, crystallized, dissociated, reassociated, and many of its physical and chemical properties investigated.

Alkaline phosphatase is ubiquitous, being found in animals, bacteria and fungi. Moreover, its intracellular distribution varies widely making it difficult to assign any specific function to the enzyme if indeed it has any single specific function.

A number of papers (Davis and Lees, 1969 and 1972) from this laboratory have described some of the properties of an alkaline phosphatase from Neurospora crassa. Various purification techniques (Sephadex separation, calcium phosphate gel adsorption, DEAE column fractionation) have been used but at best these techniques yielded at least three bands of putative activity which suggested the possiblity that the enzyme might exist in three polymeric forms of a single monomeric unit. The present work represents an investigation of this possibility.

HISTORICAL REVIEW

The study of the non - specific phosphomonoesterases began a long time ago (Suzuki et al, 1906; McCollum and Hart, 1908). However, the interest in these non - specific phosphomonoesterases did not wane through the years even until today (Mushak et al, 1972; Miles et al, 1971; Ponomavera, 1971). Their presence has been noted in mammals, fungi and bacteria, and in the case of mammals in a multiplicity of places.

A. Classification of the phosphatases.

The simplest classification according to Roche (1950) is as follows:

Type I	optimum pH 8.6 - 9.4	Activation by magnesium ion, inhibition by - SH. More active on Beta than on Gamma glycero-phosphate. Optimal stability at pH 7.5 - 8.5.
Type II	Optimum pH 5.0 - 5.5	No activation by magnesium ion. Inhibition by fluoride ion. More active on Beta than on Gamma glycero-phosphate. Optimal stability at pH 5.0 - 6.0.
Type III	Optimum pH 3.4 - 4.2	Inhibition by magnesium ion. More active on Beta than on Gamma glycero-phosphate. Optimal stability at pH 6.5 - 7.5.
Type IV	Optimum pH 5.0 - 6.0	Activation by magnesium ion. More active on Gamma than on Beta glycero-phosphate. Optimal stability at pH 6.5 - 7.5

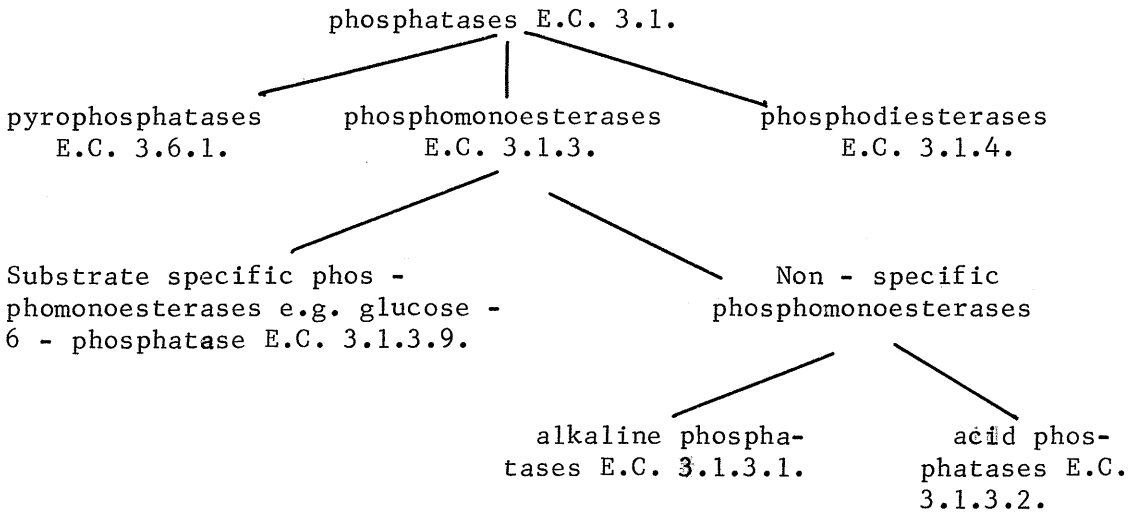
Type 1 enzymes are called by the trivial name alkaline phosphatases. The Commission on Enzymes of the International Union of Biochemistry (Florkin and Stotz, 1964) has given Type 1 the proper name of ortho - phosphoric monoester hydrolases and are numbered E.C. 3.1.3.1.

Types 11, 111, and 1V are called by the trivial name acid phos - phatases and by their proper name orthophosphoric monoester hydrolases E.C. 3.1.3.2.

Some attempt has also been made to classify these phosphomono - esterases according to substrate specificity (Schmidt and Laskowski, 1961).

The following "organizational chart" of the phosphatases illustrates the relationship between the various enzymes.

An "Organizational Chart" of the Phosphatases



This review will focus on the alkaline phosphatases and particularly on the alkaline phosphatase from E.coli since the great majority of work on alkaline phosphatase has been done on this enzyme.

B. Distribution of Alkaline Phosphatases in Nature

Alkaline phosphatase is found in bacteria and fungi, although it is often repressed by inorganic phosphate (Nyc et al, 1966). There is little information concerning its distribution in invertebrate tissues; it is present in the Indian leech (Fernley, 1971), in developing Drosophila (Schneiderman et al, 1966), and the surf clam (Strittmatter et al, 1965). It is relatively abundant in fishes (Bodansky et al, 1931) and mammals; it is absent from higher plants (Roche, 1950).

C. Possible Function of Alkaline Phosphatases

At the present time it is not possible to assign a precise function to any alkaline phosphatase. Undoubtedly bone phosphatase is concerned with ossification and two alternative roles have been proposed:

1. Precipitation of calcium phosphate is induced by the localized production of high concentrations of inorganic phosphate owing to phos - phatase activity (Fernley, 1971).

2. The enzyme permits crystal growth at nucleation sites in the matrix by ensuring the removal and continued absence of inorganic pyro - phosphate which is known to be a crystal "poison" (Fleisch et al, 1966). Other factors must be involved because tissues with high concentrations of alkaline phosphatase (e.g gut, kidney, and placenta) do not normally calcify, while tissues such as aorta, in which phosphatase is absent, can be made to calcify. Furthermore, rachitic cartilage has a high phosphatase activity yet it will not ossify.

In other tissues which are major sources of phosphate it is perhaps

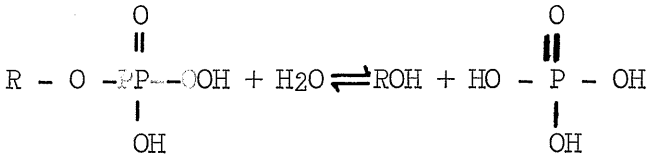
significant that the enzyme is localized at the absorptive surface suggesting a direct role in the transport of nutrients across the epithelial membrane. Bodansky found an increase in plasma phosphatase following ingestion of carbohydrate (Bodansky, 1934). Ingestion of fats by rats gave an increase synthesis of intestinal phosphatase which later appeared in the plasma and lymph ducts (Glickman et al, 1970). This may correlate with the histochemical finding that intestinal epithelial cells from rats on a high fat diet showed marked phosphatase activity in the Golgi region (Fernley, 1971). A careful investigation by Langman and collaborators (Langman et al, 1966) indicated that ingested fat (not carbohydrate) leads to the appearance of intestinal phosphatase in the plasma of certain groups of individuals.

Regulation of alkaline phosphate activity was discussed by Cox and Griffin (1967). Steroids such as hydrocortisone or prednisolone (1 ug/ ml of medium) can induce a 3 to 20 - fold rise in certain Hela cell cultures, while other cell lines are induced by 15 mM phenyl phosphate. Recently it has been shown that fibroblast cell cultures are also stimulated by prednisolone (Waters et al, 1969). According to one report inorganic phosphate may have a control function; here a decrease in the level of rat kidney inorganic phosphate produced by a low phosphate diet was accompanied by an increase in alkaline phosphatase. Nine other enzyme monitored were unaffected (Melani et al, 1967).

D. Catalytic Properties

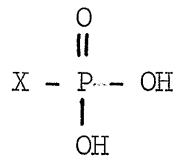
(a) Specificity

The equilibrium catalyzed by alkaline phosphatase is classically of the following general type:

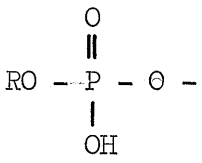


Since the equilibrium lies to the right it is customary to say that alkaline phosphatase hydrolyses phosphate esters, but some related compounds are also hydrolyzed.

The enzyme also catalyzes transphosphorylation reactions in which a different alcohol substitutes for H_2O as a phosphate acceptor. Compounds that are hydrolyzed have the general structure:



where X can be RO - , RS - , HS -



and F - , but P - N and P - C bonds are not cleaved. The enzyme has no diesterase activity.

(b) Inhibitors

The compounds that markedly inhibit the non - specific alkaline phosphatases can be classified under the following general headings;

(1) Metal chelating agents.

(2) Certain divalent cations.

(3) Phosphate and some related polyvalent anions.

The alcohol moieties of many phosphate ester substrates also are more or less potent competitive inhibitors of the hydrolytic reactions.

(c) pH - Activity Relationships

The number of reports concerning the effect of pH on activity of various alkaline phosphatases is legion (Motzok, 1959; Motzok et al, 1959). It has been shown that the optimum pH for a particular reaction is a function of the nature of the phosphate ester substrate, the concentration of the substrate, the type of enzyme preparation employed, the concentration of enzyme added, the nature and concentration of metal activator, and the type of buffer involved. From this it is apparent that any statements as to pH optima, Km values, or initial rates of hydrolysis by the non - specific alkaline phosphatases are almost meaningless unless conditions are explicitly defined. With impure enzyme preparations such values are applicable only to a given preparation.

E. Some Properties of Highly Purified Alkaline Phosphatases

(a) Subunit study of the E. coli enzyme

Most of the work on the subunit aspect of alkaline phosphatase was done on the E. coli enzyme.

Escherichia coli alkaline phosphatase can be reversibly denatured by thiol reduction in the presence of urea, a treatment which dissociates the dimer (Levinthal et al, 1962).

Proteins purified from alkaline phosphatase - negative mutants that

are antigenically related to alkaline phosphatase are readily and reversibly dissociated by acid (Schlesinger and Levinthal, 1963). Normal alkaline phosphatase is more stable; but at a lower pH, less than 3.0, it too forms monomers with release of zinc ions. However, chelating agents that remove zinc do not cause the dimer to dissociate. Also although the enzyme is metastable with respect to monomers at pH 4.4, there is no exchange of ^{65}Zn with the zinc bound to the enzyme. Other methods of forming monomers are treatment with 6 M guanidine-HCl and heating at 95°C for 15 minutes (Schlesinger and Barrett, 1965).

The properties of the subunit are as follows:

(1) They can be frozen and thawed several times at pH 2.0 but cannot be stored for long periods at -20°C.

(2) They are stable at 4°C and pH 2.0 for several days.

(3) They are unstable at concentrations less than 10 ug/ml at room temperature; however, BSA will stabilize dilute solutions.

(4) In contrast to dimer the subunit is denatured by periodate, Pauly reagent, and ionic detergents, and is readily digested by proteolytic enzymes.

(5) Subunits do not form a precipitate with antiphosphatase antibody; however, there appear to be some antigenic determinants common to both subunits and active enzyme since subunits interfere with the precipitation of alkaline phosphatase. The alkaline phosphatase-antibody complex has 70% of the original enzymic activity as a suspension in solution. Therefore the antibody does not bind to the active site of alkaline phosphatase, but it can still differentiate monomers and dimers.

Zinc increases the rate of dimerization (Schlesinger, 1965) but

it is not necessary for dimerization to occur. Starting at a high pH and slowly lowering the pH all the zinc is lost by the time the pH reaches 4.0, yet the molecule, though inactive, is still dimeric. However, upon in - creasing the pH of a solution of monomers, the dimer reforms by pH 5.0, yet the zinc does not bind completely until pH 6.0.

The subunits were found to be identical by the analysis of tryptic peptides. Since trypsin specifically cleaves at lysyl and arginyl residues, there will be as many peptides formed as there are arginine and lysine residues if the monomers are completely different. If the monomers are identical only half as many peptides will be formed. The latter was found to be true. Hence the subunits are said to be identical.

Recently, Reynolds and Schlesinger (1969) reported that between pH 7.0 and 8.0, and a zinc (11) concentration greater than 10^{-5} M, alkaline phosphatase forms an active tetramer. The tetramer binds 16 zinc ions and many phosphate ions.

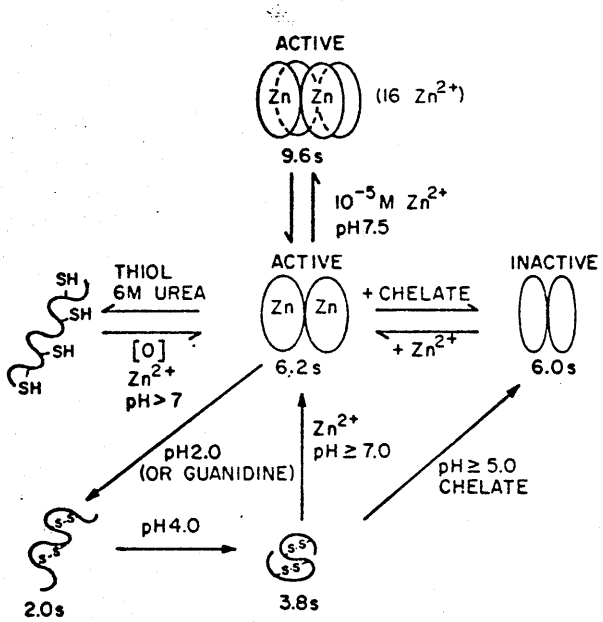
Diagram 1 depicts the molecular states of the E.coli alkaline phospha - tase under various conditions (please, see next page).

(b) Isoenzymes

Purified preparations of alkaline phosphatase from E.coli judged homogeneous when examined in the analytical ultracentrifuge, contain several isoenzymes, because several bands which contain enzymatic activity are obtained in starch - gel and disc - gel electrophoresis. Although most workers find three bands, four and five equally spaced bands have been found (Levinthal et al, 1962).

Lazdunski and Lazdunski (1967) separated three isoenzymes on DEAE -

Diagram 1 : This diagram depicts the molecular states of the E.coli alkaline phosphatase under various conditions.



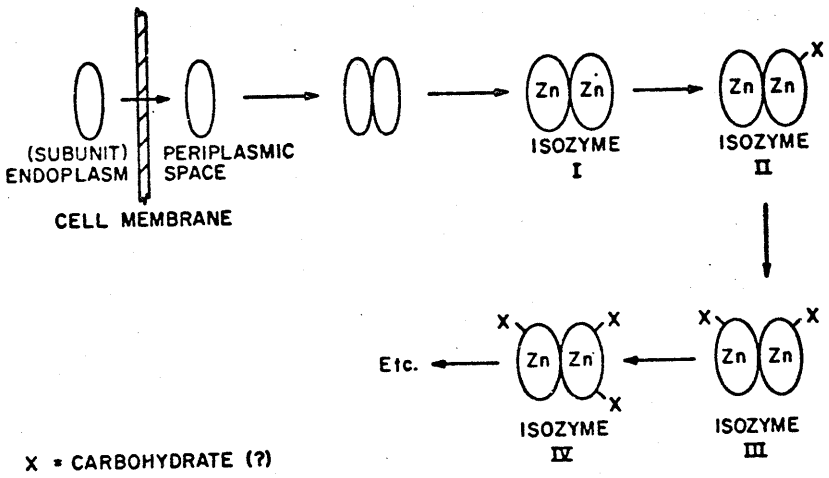
cellulose. They found that pure samples of either isozyme 1 (the isozyme with the least negative charge at pH 7.0 is referred to as isozyme 1) or isozyme 111, after dissociation and reassociation, gave only the original single band on disc-gel electrophoresis. Pure isozyme 11, after dissociation and reassociation, gave three bands corresponding to isozymes 1, 11, and 111. It was also shown that the monomers of isozymes 1 and 111, when mixed before reassociation, gave three bands. This shows that isozyme 11 is composed of one monomer of isozyme 1 and one monomer of isozyme 111.

It has been suggested that the differences in the isozyme composition involve carbohydrate residues (Reid and Wilson, 1971). Diagram 11 represents a possible scheme for isozyme formation (please see next page).

Amano et al, (1970), did some studies on the alkaline phosphatase isoenzymes from cattle serum. Their protein separations were carried out by means of agar, starch and polyacrylamide gel electrophoresis. They observed 5 to 6 isozyme patterns, and these enzymically active bands were recognized to have different activities.

Kang, (1971), studied the alkaline phosphatase isozyme pattern in rats which were subjected to various treatments such as tumor innoculation, liver injuries and ligation of the bile duct, by means of agar-gel electrophoresis, gel filtration on Sephadex G-200, and anion-exchange column chromatography on DEAE-Sephadex A-25. An elevated level of plasma alkaline phosphatase was shown in rats whose livers were experimentally damaged and was most remarkable when the hepatobiliary system was occluded. The alkaline phosphatase activities of plasma and liver, and plasma icterus index increased in parallel in rats with liver tumor meta-

Diagram 11 : This diagram represents a possible scheme for isozyme formation of the E.coli alkaline phosphatase.



stases or with their bile duct ligated, but not in those with their liver injured by CCl_4. By agar-gel electrophoresis four kinds of isozyme pattern of this enzyme were observed. After gel-filtration on Sephadex G-200, rat serum proteins were separated into three peaks and alkaline phosphatase activity was found only in the second peak. The enzyme was further separable into 2 peaks when the second peak eluent from the rat with damaged liver was chromatographed on DEAE-Sephadex A-25. In rats having ligated bile ducts, the acid phosphatase activity of plasma showed no noteworthy changes.

Tan and Aw, (1971), studied the properties of heat-stable alkaline phosphatase isozymes from Hela cells. Electrophoresis on agar-gel revealed 5 isozymes, all of which appeared to be sensitive to enzymatic cleavage by neuraminidase. Isozymes 2 and 4 were heat-stable at 56^{o}C for 2 hours. Magnesium ion stabilized the enzyme against heat denaturation and also aided in overriding substrate inhibition. The heat-stable isoenzymes had a pH optimum of 10.4. They were activated optimally by 10 mM magnesium and inhibited by L-phenylalanine to 20% of their original activity.

(c) The Role of Zinc in the E.coli Alkaline Phosphatase

There has been some uncertainty concerning the metal content of alkaline phosphatase of E.coli and the role of zinc in the catalytic process. Early measurements by Plocke et al (1962) showed that there were two gram atoms per dimer.

A zinc-free inactive apoenzyme was formed by dialysis against 1,10-phenanthroline. Complete activity was restored by zinc; only zinc, cobalt, and possibly mercury produce activity.

Several investigators now find that four zinc ions are bound by the dimer but only two are necessary for activity. Lazdunski et al (1969) showed that the rate of inactivation of the enzyme by EDTA is biphasic, corresponding to two zinc binding sites associated with enzymatic activity. Phosphate decreases the rate of inhibition by EDTA in a manner corresponding to the binding of phosphate with dissociation constants of 1×10^{-6} for the first zinc removal and 6×10^{-6} for the second. They propose that there are four zinc binding sites, of which the strongest and weakest are required for activity. If one site is occupied by zinc and three by Cd^{2+}, there is 11% activity.

Simpson and Vallee (1968) found that when alkaline phosphatase is exposed to 8-hydroxyquinoline-5-sulfonic acid, two zinc atoms are rapidly removed and the enzyme is inactivated to within 10%. The two remaining zinc atoms are removed more slowly, presumably with the loss of the remaining activity. When zinc is added to the apoenzyme the first 2 ions produce 85% activity. Thus it would appear that there are two binding sites that must be occupied by zinc ions for activity (Simpson and Vallee, 1968).

Reynolds and Schlesinger (1969) differed from others in their finding that the enzymic activity of alkaline phosphatase increases linearly with the number of zinc ions bound up to four. Spectrophotometric titrations show that approximately 6 tyrosine residues are not exposed to solvent in the case of the dimer containing three Zn^{2+}. They also found that at pH values between 7.0 and 8.0 and a zinc concentration greater than 10^{-5} M, alkaline phosphatase rapidly and reversibly forms a tetramer, as shown by osmotic pressure and sedimentation studies. At pH 8.0 and an equilibrium concentration of zinc equal to 10^{-4}M, they found 16 Z^{2+} per tetramer.

In summary, all workers except Reynolds and Schlesinger (1969) found that only two zincs are necessary for activity. Some of the dis - agreement in the metal binding work may arise from uncertainties as to whether binding was controlled by kinetics or thermodynamics. To illustrate what is meant, consider the addition of one zinc ion to the apoenzyme. Is the site where the zinc ion is bound the most stable site or the one most accessible? Evidently this must depend on the time, and whether or not the concentration of zinc is controlled by a chelating agent.

(d) The Role of Divalent Cations in the Micrococcus sodonensis enzyme.

The orthophosphate repressible extracellular alkaline phosphatase has been isolated from cultures of M.sodonensis by Glew and Heath (1970).

The secretion of alkaline phosphatase by M.sodonensis has been studied by observing the production and accumulation of extracellular enzyme by log phase cells which have been resuspended in fresh growth medium. The accumulation of extracellular alkaline phosphatase activity is the result of a selective permeation process and is totally dependent upon the pre - sence of divalent cation. Although calcium is an actual component of the enzyme and is required for both catalytic activity and enzyme stability, magnesium appears to be the divalent cation required for the synthesis or release (or both) of alkaline phosphatase from the cell.

It should be obvious by this time that a great deal of research can be done with a homogeneous enzyme preparation, especially research on the structure - function relationship of the enzyme. The present work was an attempt to purify to homogeneity the constitutive alkaline phosphatase from N.crassa and to study some of its physical and chemical properties.

MATERIALS AND METHODS

Growth of Neurospora crassa strain M 16

N.crassa was grown and harvested according to the method of Davis (1970).

Growth of Neurospora crassa strain 533

N.crassa was grown in Vogel's medium (Vogel, 1956) with P_i as the limiting factor to induce the repressible alkaline phosphatase. The mycelium was harvested according to the method of Nyc et al (1966).

Lyophilized Mycelium of Neurospora crassa strain 533

Lyophilyzed mycelium of N.crassa was used in the purification procedures (Davis,1970). This mycelium was a gift from Dr. F. W. J. Davis who started the work on the constitutive alkaline phosphatase from N.crassa.

Enzyme Assay

The constitutive alkaline phosphatase was assayed according to the method of Davis (1970) using a Gilford 2400 Spectrophotometer equipped with a recorder.

The repressible alkaline phosphatase was assayed with the assay mix of Nyc et al (1966) but the reaction was followed on the Gilford 2400 Spectrophotometer instead of the incubation procedure described by Nyc et al (1966).

Protein Determination

Protein was determined according to the method of Lowry et al (1951). Bovine serum albumin (crystalline grade) was used as the standard protein.

Analytical Polyacrylamide Gel Electrophoresis

Analytical polyacrylamide gel electrophoresis was performed according to the method of Davis (1964) with slight modification. No spacer or sample gel was used. Instead, the protein sample was mixed with 50% glycerol in bromphenol blue tracking dye and layered on top of the re - solving gel. Electrode buffer was then layered on top of this. This method has been widely used by Weber and Osborn (1969), and in our depart - ment also. The pH of the resolving gel was 8.9. The electrode buffer was Tris - glycine at pH 8.2. The anode was at the bottom.

SDS - Polyacrylamide Gel Electrophoresis.

SDS - polyacrylamide gel electrophoresis was carried out according to the method of Weber and Osborn (1969).

Dimethyl Suberimidate Method of Molecular Weight Determination

This procedure was carried out according to the method of Davies and Stark (1970). Glyceraldehyde - 3 - phosphate dehydrogenase from rabbit muscle was used as the standard oligomeric protein.

Amino Acid Composition

The amino acid composition determination was carried out according to the method of Moore, Spackman and Stein (1958). The sample was hydro - lyzed in 6 N HCl under nitrogen for 24 hours. The instrument used was the Beckman Automatic Amino Acid Analyzer equipped with an Infotronic Integrator.

Calcium Phosphate Gel

Calcium phosphate gel was prepared according to the method of Keilin and Hartree (1938). The time of preparation of this gel was July, 1970.

Sedimentation Analysis of Native Enzyme

Purified alkaline phosphatase, 4 mg/ml, was subjected to sedimentation analysis in a Spinco Model E Analytical Ultracentrifuge equipped with Schlieren optics. The temperature of the rotor chamber was 20°C and the rotor speed was 60,000 rpm. The type of cell used was a double sector cell.

Sedimentation Analysis of SDS - treated Purified Alkaline Phosphatase

Purified alkaline phosphatase, 5 mg/ml, was treated with 1% SDS, 1% 2 - mercaptoethanol, 0.01 M potassium phosphate, adjusted to pH 7.0 and incubated for 3 hours at 37°C. This preparation was then subjected to sedimentation analysis on a Spinco Model E Analytical Ultracentrifuge equipped with Schlieren optics. The temperature of the rotor chamber was 20°C and the rotor speed was 60,000 rpm. The type of cell used was a double sector cell.

Low Voltage Filter Paper Electrophoresis

Low voltage filter paper electrophoresis was carried out according to the method of Shaw as outlined in his text on Electrophoresis (Shaw, 1969). A voltage gradient of 5 volts per cm was used. The filter paper strips were 5 cm wide and 21 cm long. The Shandon Electrophoresis tank produced by COLAB (Consolidated Laboratories Limited, Toronto, Canada) was used. All runs were carried out at 0 to 4°C.

Glucose Stain

The stain for locating glucose on filter paper chromatograms was made as follows:

(1) Diphenylamine (0.15 g) was dissolved in 20 - 25 ml of ethyl acetate from a total of 100 ml.

(11) 0.80 ml aniline, the remaining ethyl acetate and 11.0 ml of

80% (w/v) H_3PO_4 were added.

(111) Chromatograms were dipped into this solution, dried at room temperature, and finally heated in an oven at 95°C to 100°C until a colored spot appeared (Sherma and Zweig, 1971).

Protein Stain

The stain for locating protein on the chromatogram was made as follows: (1) Stain: 0.10 gram bromphenol blue, 50.0 grams $ZnSO_4$ and 50 ml of glacial acetic acid were mixed and brought up to one liter with distilled water.

The procedures for staining and destaining the chromatograms were as follows:

(1) The chromatograms were dipped into the stain for 16 hours (usually overnight).

(11) After staining, the chromatograms were washed as follows:

(a) 2% glacial acetic acid for 5 minutes

(b) 2% glacial acetic acid for 5 minutes

(c) 2% glacial acetic acid for 10 minutes

(d) 10% glacial acetic acid containing 2% sodium acetate for 2 minutes.

(e) The chromatograms were first dried by blotting off excess liquid, then heated in an oven at 95°C to 100°C until dried (Audubert and de Mende, 1959).

Chemicals

All organic chemicals used during the investigation were purchased from the Sigma Chemical Company. Other chemicals were Reagent Grade, either

from the Fisher Scientific Company or the Baker Chemical Company.

R E S U L T S

Purification of Alkaline Phosphatase using Calcium Phosphate Gel

The purification of an enzyme preparation as at the end of step V (Davis, 1970) was attempted using calcium phosphate gel prepared as stated in the materials and methods.

In initial experiments it was found that the enzyme which binds to the gel was eluted with 0.10 M potassium phosphate at alkaline pH more than at acidic pH. However, buffer A (0.02 M Tris, 0.004 M $MgCl_2$, 0.0001 M $ZnCl_2$) pH 7.7 did not elute the enzyme, while 0.01 M phosphate buffer was not strong enough to elute the enzyme. It was also noticed that the enzyme did not bind significantly to the gel when the protein to gel ratio did not exceed 1:1 (dry weight : dry weight).

Taking these facts into consideration the following purification procedure was devised, the first two steps of which were to bind impurities without significantly binding the enzyme itself:

Step 1

A 7.0 ml sample of a protein solution (step V, Davis, 1970) containing 8,100 enzyme units per ml, a protein concentration of 1.30 mg per ml, and a specific activity of 6,230 enzyme units per mg protein, was mixed with calcium phosphate gel in a 1:1 ratio (dry weight : dry weight). The mixture was stirred for 10 minutes at room temperature, and then centrifuged at 14,000 x g for 20 minutes at 0 to 4°C.

After centrifugation, the supernatant contained 6,600 enzyme units per ml, protein concentration of 662.5 ug per ml, and a specific activity of 9,970 enzyme units per mg protein.

The precipitate was washed with H_2O and eluted with 0.10 M phosphate buffer pH 8.2. The elution showed negligible activity.

Step 11

The supernatant from step 1 was mixed with calcium phosphate gel with an enzyme to gel ratio of 2:1 (dry weight : dry weight). The mixture was stirred for 10 minutes, then centrifuged at 14,000 x g for 20 minutes at 0 to 4°C.

After centrifugation, the supernatant contained 6,000 enzyme units per ml, a protein concentration of 600 ug per ml and a specific activity of 10,000 enzyme units per mg protein.

The precipitate, treated as in step 1, contained negligible activity.

Step 111

The supernatant from step 11 was mixed with calcium phosphate with an enzyme to gel ratio of 2:3 (dry weight : dry weight). The mixture was treated as in step 11.

After centrifugation, the supernatant contained 2,700 enzyme units per ml, a protein concentration of 400 ug per ml and a specific activity of 6,750 enzyme units per mg protein.

The precipitate was washed with water, and eluted with 3.0 ml of 0.10 M of phosphate buffer pH 8.2. It contained 6,600 enzyme units per ml, a protein concentration of 166.6 ug per ml and a specific activity of 38,823 enzyme units per mg protein.

Step 1V

The supernatant from step 111 was mixed with calcium phosphate

gel with an enzyme to gel ratio of 2 : 5 (dry weight:dry weight). The mixture was treated as in previous steps.

After centrifugation, the supernatant contained 18.0 enzyme units per ml, a protein concentration of 300 ug per ml, and a specific activity of 60 enzyme units per mg protein.

The eluted precipitate contained 2,580 enzyme units per ml, a protein concentration of 125 ug per ml, and a specific activity of 20,640 enzyme units per mg protein.

Step lll showed a six - fold purification and step lV showed a three - fold purification. The purified enzyme was run on 7.5% polyacrylamide gels. The gels showed 3 major protein bands (Fig. 1.A.). Gels stained with PNPP substrate showed a yellow color in the region of the 3 protein bands, but the color seemed to diffuse out quickly.

The purified enzyme sample from step lV was left standing for 6 months at 0 to 4^{o}C. When 7.5% gels were run one protein band appeared where the three protein bands previously appeared.

Judging from the intensity of the protein stain on the gels, the enzyme seemed to be about 80% pure. There were a few other minor protein bands (Fig.1.B.).

It is obvious that this procedure is long and tedious especially when the protein concentration has to be accurately determined before the next step is to be proceeded with. This procedure also resulted in only a small recovery of protein and was,therefore,abandoned as a purification procedure. A more reproducible and less tedious method was sought. Sephadex seemed to be the obvious choice.

FIG. 1.A. Diagrammatic representation of the three protein bands seen on polyacrylamide gels immediately after calcium phosphate gel purification.

FIG. 1.B. Polyacrylamide gel (7.5%) of the enzyme purified by calcium phosphate gel. The enzyme was left standing for six months. The pH of the resolving gel was 8.9. The duration of the run was about 1½ hours with the anode at the bottom. The amount of protein applied to the gel was 24 ug.

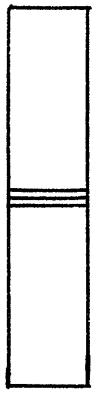


FIG. I.A.

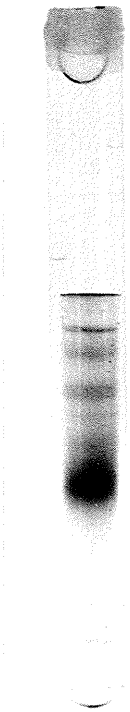


FIG. I.B.

Purification of Alkaline Phosphatase using Sephadex Separation

In the purification procedures using Sephadex, column diameter, column length, Sephadex mesh size, flow rate of the column, pH and buffer changes, and fraction volume collected, were parameters tested to obtain the best resolution between the contaminating proteins and the enzyme protein, and hopefully to obtain a single enzyme protein band on polyacrylamide gel.

It was found that for the 1.5 cm diameter column, whether 30 cm or 90 cm long, the maximum sample volume to be applied for best resolution was 1.0 ml with a protein concentration up to 9.0 mg per ml. No difference in resolution and no advantage resulted from using a 90 cm long column over a 30 cm long column keeping the flow rate the same for both columns (Figs. 2 A, and 2 B). No difference in resolution was found using different mesh sizes such as G - 100, G - 200 when the flow rate was 6.5 ml per hour for all the mesh sizes (Figs. 2; 3A; 4). But a difference in resolution was found depending on the flow rate. When the flow rate was 3.5 ml per hour the resolution between the contaminating protein peak and the enzyme peak was clearer than when the flow rate was 6.5 ml per hour, or 10 ml per hour (Figs. 3A;B; 4; 5). It was also found that more enzyme units could be recovered when the flow rate was slower, 3.5 ml per hour, than when it was 6.5 ml per hour (Figs. 3 and 4). It was found that the recovery of enzyme units for the different mesh sizes were as follows: G - 200 > G - 150 > G - 100 when the flow rate was 6.5 ml per hour (Figs. 2; 3A; 4). The fraction volume that seemed safest to collect was 1.0 ml or less.

The following purifications using Sephadex provide proof for the statements made above:

(a) Sephadex G - 100

Sephadex G - 100 was equilibrated on a 1.5 cm x 90 cm column with buffer A, pH 7.7 at 0 to 4°C. Then 1.0 ml of a protein sample containing 39,000 enzyme units per ml, a protein concentration of 8.5 mg per ml, and a specific activity of 4,588 enzyme units per mg protein, was applied to the column. The flow rate of the column was adjusted to about 6.5 ml per hour and 1.0 ml fractions were collected. The most active fractions contained 0.167 mg protein per ml, 2,034 enzyme units per ml, and a specific activity of 12,449 enzyme units per mg protein. The enzyme units recovered from the fractions that showed activity totalled 14,650. An elution profile is shown in Fig. 2.B.

Polyacrylamide gels of the pooled, most active, fractions showed two major protein bands very close together and one minor protein band. (Fig. 6). Gels stained with PNPP substrate showed activity in the region of the three protein bands. The yellow color seemed to diffuse quickly.

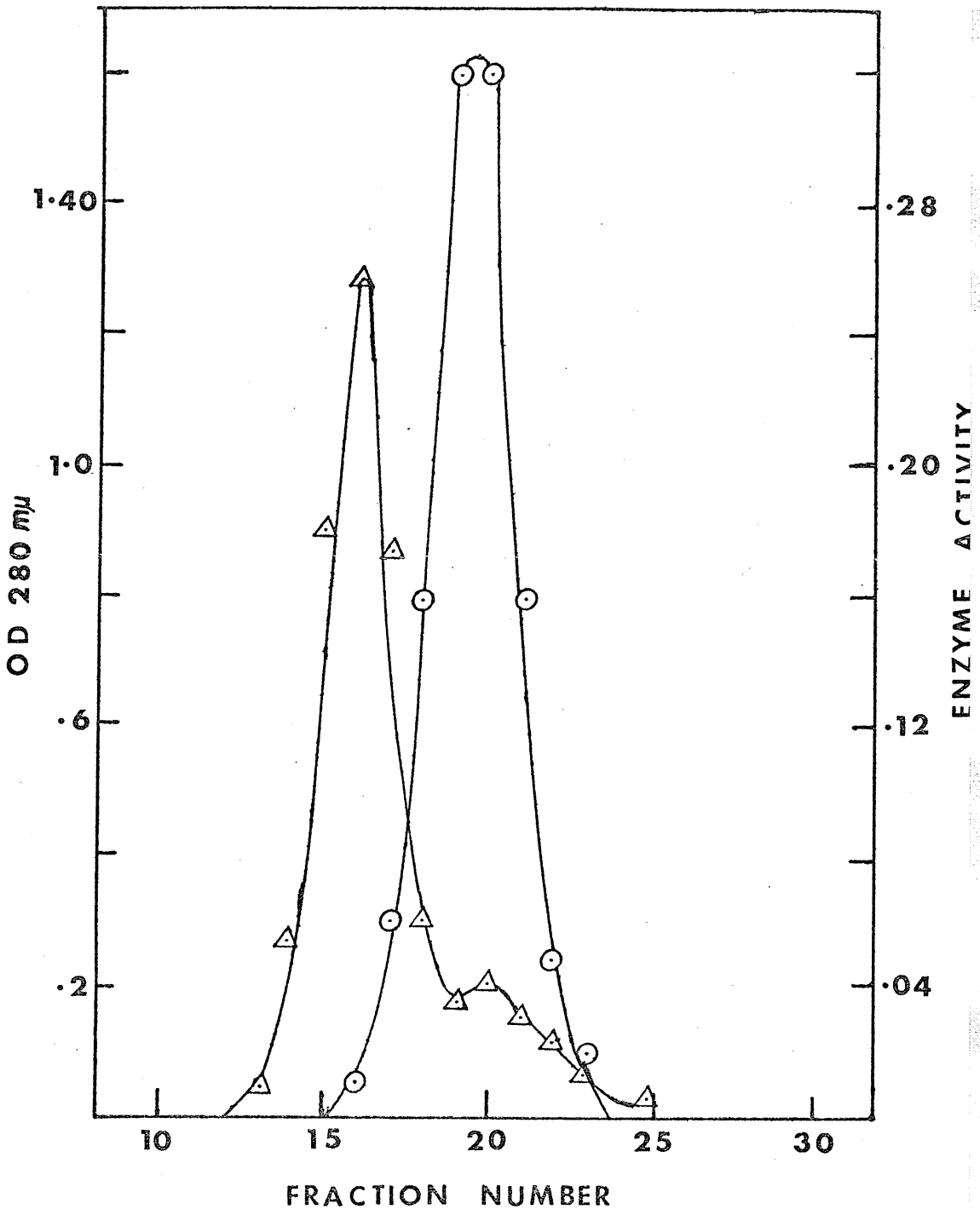
(b) Sephadex G - 150

Sephadex G - 150 was equilibrated with buffer A, pH 7.7, on a 1.5 x 30 cm column at 0 to 4°C. Then 1.0 ml of a protein solution con - taining 26,000 enzyme units per ml, a protein concentration of 5.6 mg per ml, and a specific activity of 4,642 enzyme units per mg protein was applied to the column. The flow rate of the column was 6.5 ml per hour, and 0.50 ml fractions were collected. The pooled, most active, fractions contained 0.140 mg protein per ml, 1,643 enzyme units per ml, and a specific activity of 11,736. The total enzyme units recovered was 13,250. An elution profile is shown in Fig.3.A.

FIG. 2. A. Sephadex G - 100 elution profile from a 1.5 x 30cm column equilibrated with buffer A, pH 7.7. The flow rate of the column was 6.5 ml/hour.

△——△ - Protein Absorbance (O D 280 mμ).

⊙——⊙ - Enzyme Activity (OD 410 mμ).


1·40
1·0
·6
·2
OD 280 mμ
·28
·20
·12
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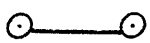
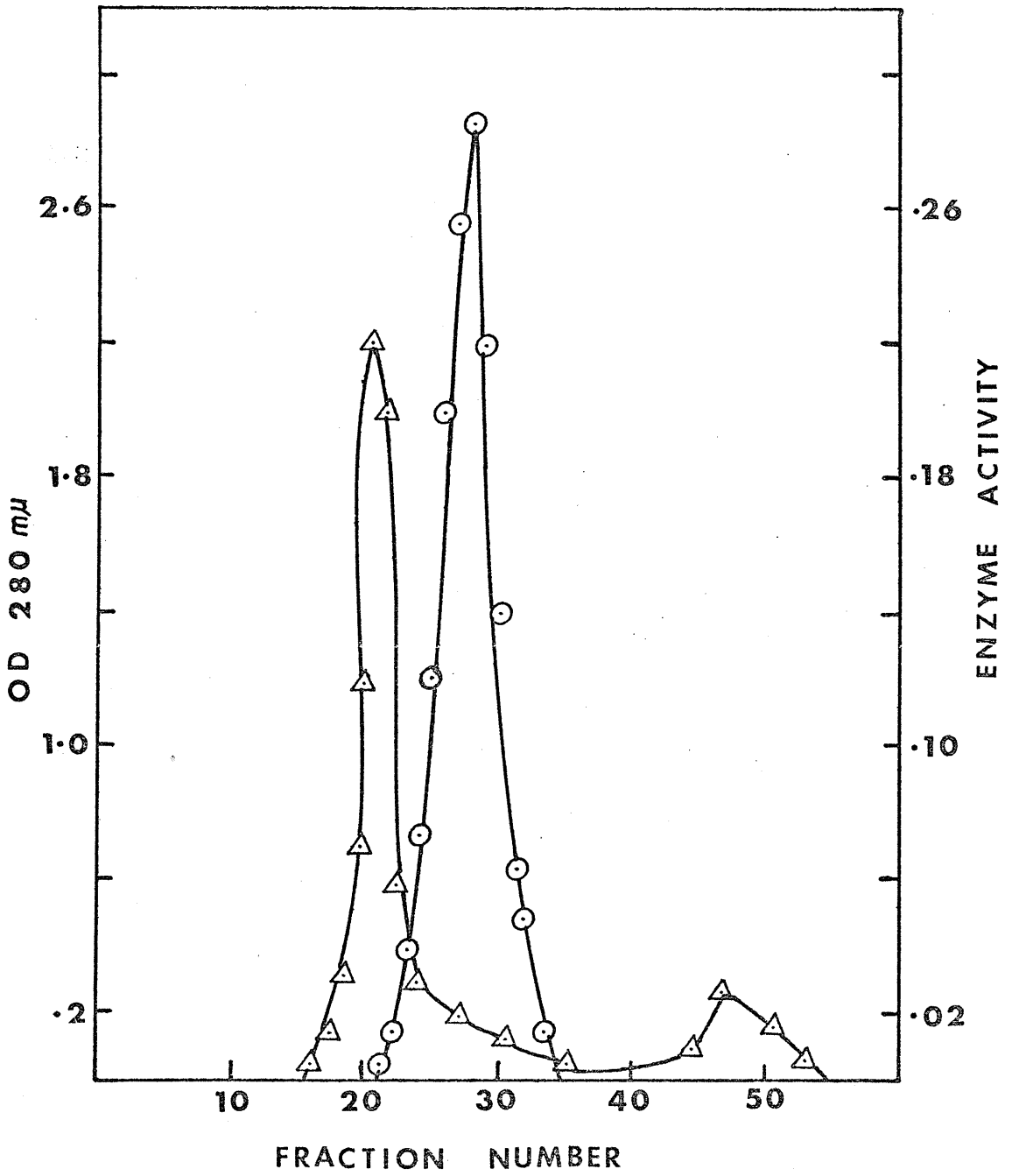
ENZYME ACTIVITY
10
15
20
25
30
FRACTION NUMBER

FIG. 2.B. Sephadex G - 100 elution profile from a 1.5 x 90 cm column equilibrated with buffer A, pH 7.7. The flow rate of the column was 6.5 ml/hour.

 - Protein Absorbance (OD 280 mμ).

 - Enzyme Activity (OD 410 mμ).


2·6
1·8
1·0
·2
OD 280 mμ
·26
·18
·10
·02
ENZYME ACTIVITY
10
20
30
40
50
FRACTION NUMBER

Polyacrylamide gels (7.5%) showed the same 3 protein bands as the ones after the G - 100 elution (Fig. 7).

(c) Sephadex G - 200

Sephadex G - 200 was equilibrated with buffer A, pH 7.7, on a 1.5 x 30 cm column at 0 to 4°C. Then 1.0 ml of a protein solution containing 39,000 enzyme units per ml, a protein concentration of 8.5 mg per ml and a specific activity of 4,588 enzyme units per mg protein was applied to the column. The flow rate was adjusted to 6.5 ml per hour and 1.0 ml fractions were collected. The pooled,most active,fractions contained 0.130 mg protein per ml, 2,225 enzyme units per ml and a specific activity of 17,116. The total enzyme units recovered was 24,200. An elution profile is shown in Fig. 4.

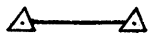
Polyacrylamide gels (7.5%) showed the same 3 protein bands as seen after G - 100 and G - 150 elutions (Fig.8).

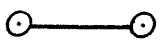
(d) Sephadex G - 150

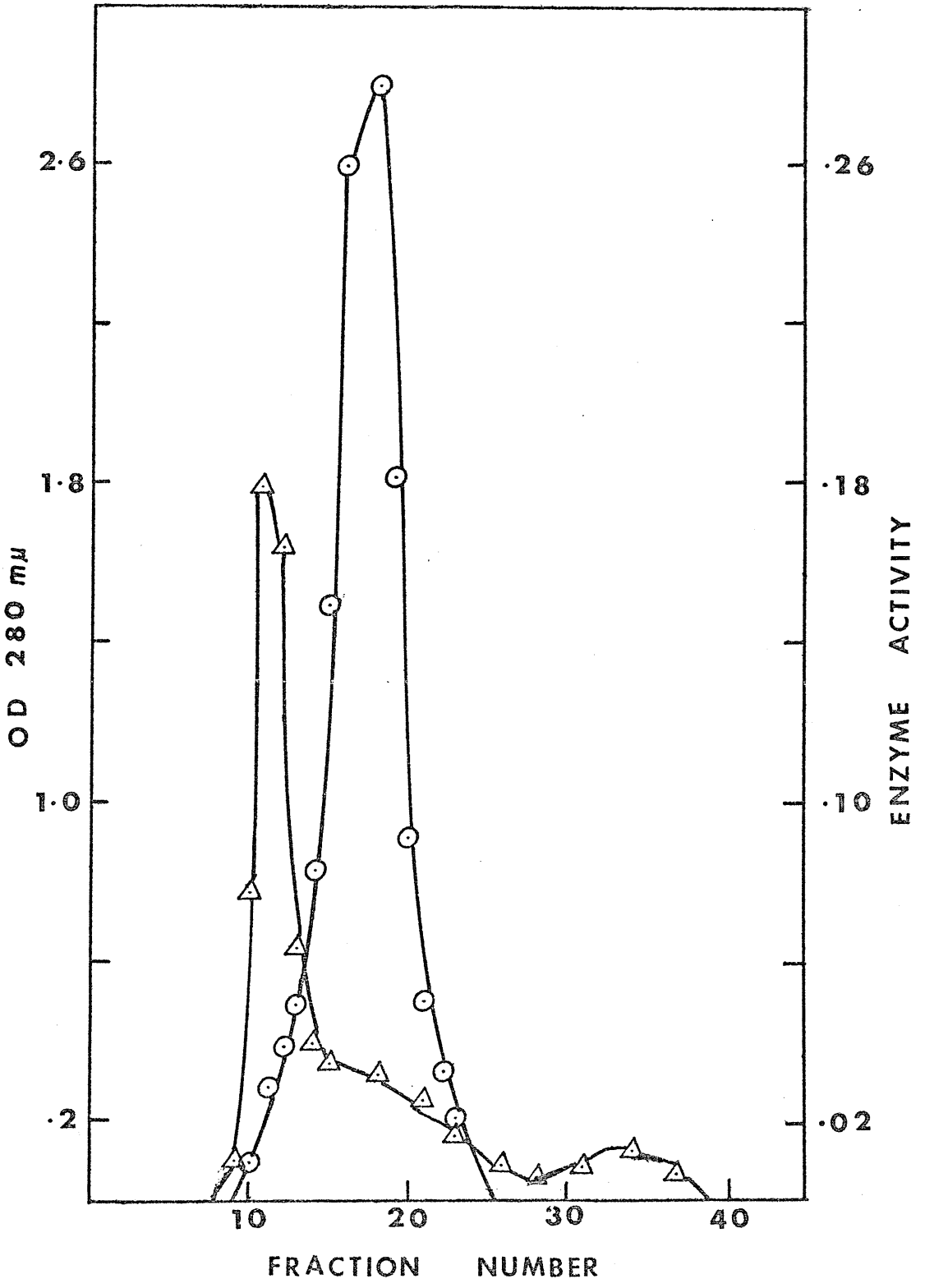
At this point it was decided to change the flow rate of the column to see whether one enzyme protein band could be obtained seeing that the different Sephadex mesh sizes gave the same elution profile and the same 3 protein bands on gels when the flow rate was 6.5 ml per hour.

Therefore, Sephadex G - 150 was equilibrated with buffer A, pH 7.7, on a 1.5 x 30 cm column at 0 to 4°C. Then 1.0 ml of an enzyme preparation containing 26,600 enzyme units per ml, a protein concentration of 6.5 mg per ml and a specific activity of 4,090 enzyme units per mg protein was applied to the column. The flow rate was adjusted to 3.5 ml per hour

FIG. 3.A. Sephadex G - 150 elution profile from a 1.5 x 30 cm column equilibrated with buffer A, pH 7.7. The flow rate of the column was 6.5 ml/hour.

 - Protein Absorbance (OD 280 mμ).

 - Enzyme Activity (OD 410 mμ).


2·6
1·8
1·0
·2
OD 280 mμ
·26
·18
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·02
ENZYME ACTIVITY
10
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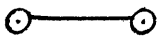
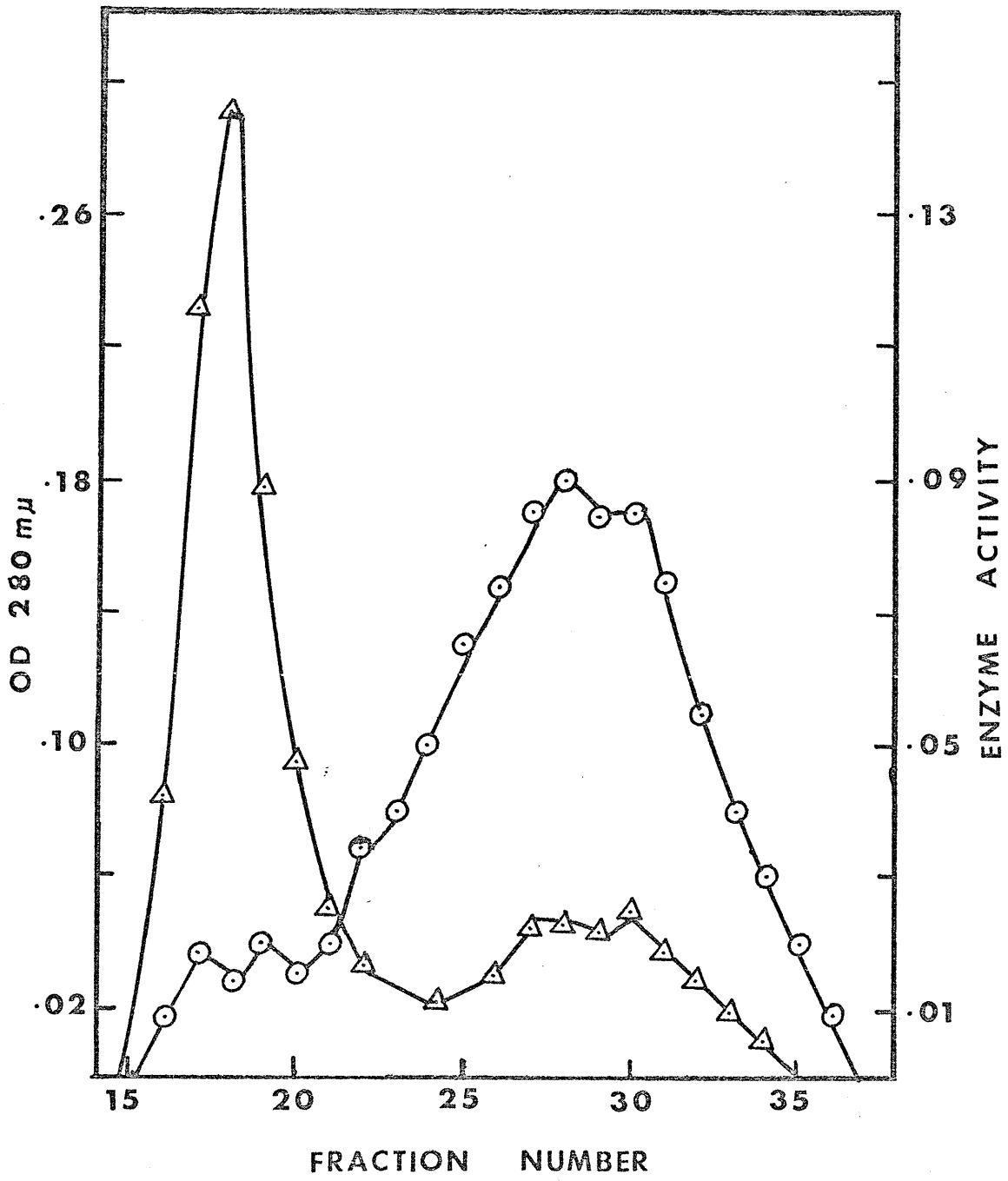
30
40
FRACTION NUMBER

FIG. 3.B. Sephadex G-150 elution profile from a 1.5 x 30 cm column equilibrated with buffer A, pH 7.7. The flow rate of the column was 3.5 ml/hour.

 - Protein Absorbance (OD 280 mμ)

 - Enzyme Activity (OD 410 mμ).


·26
·18
·10
·02
OD 280 mμ
·13
·09
·05
·01
ENZYME ACTIVITY
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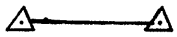
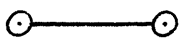
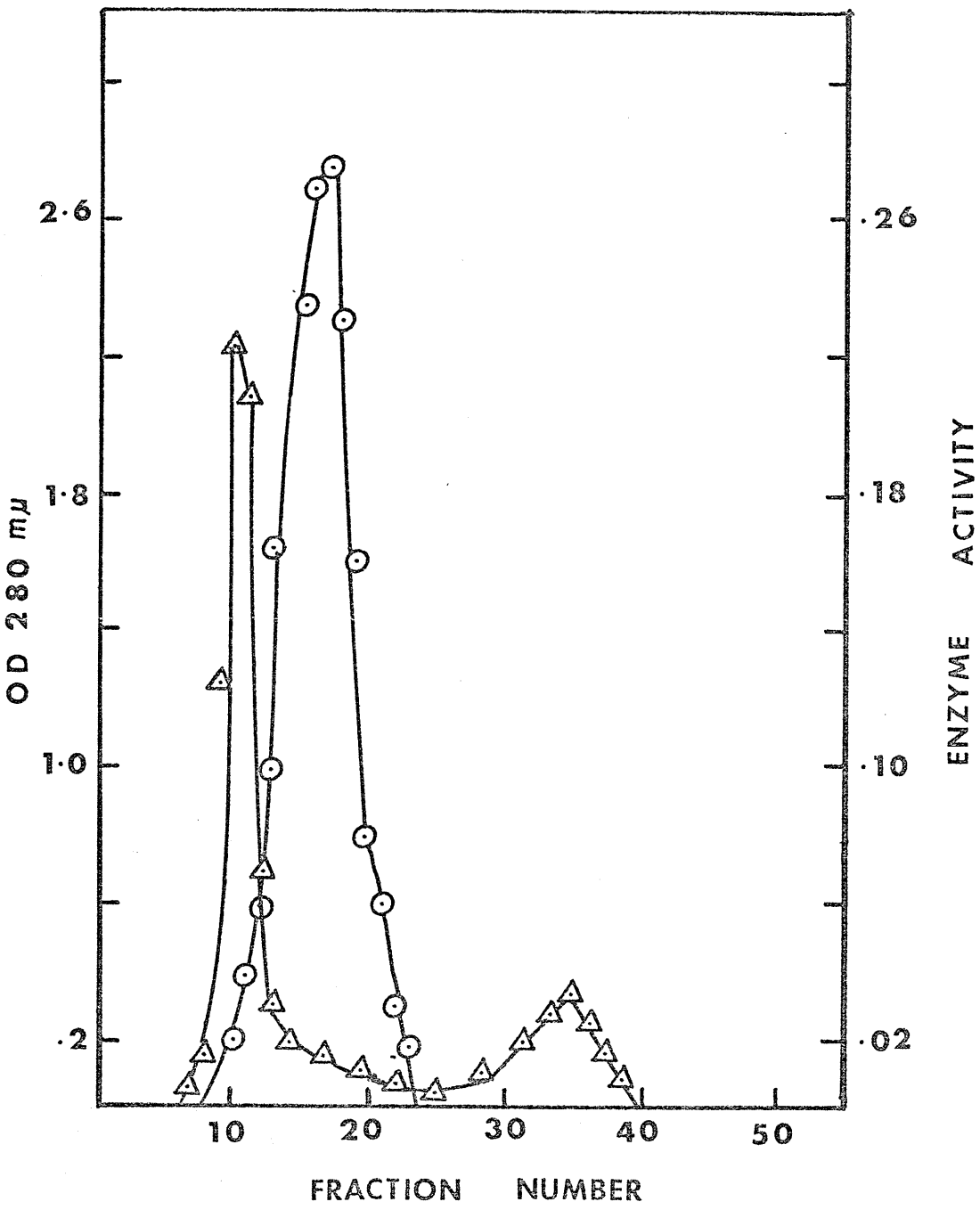
20
25
30
35
FRACTION NUMBER

FIG. 4. Sephadex G - 200 elution profile from a 1.5 x 30 cm column equilibrated with buffer A, pH 7.7. The flow rate of the column was 6.5 ml/hour.

 - Protein Absorbance (280 mμ).

 - Enzyme Activity (OD 410 mμ).


2·6
1·8
1·0
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OD 280 mμ
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ENZYME ACTIVITY
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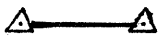
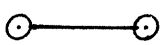
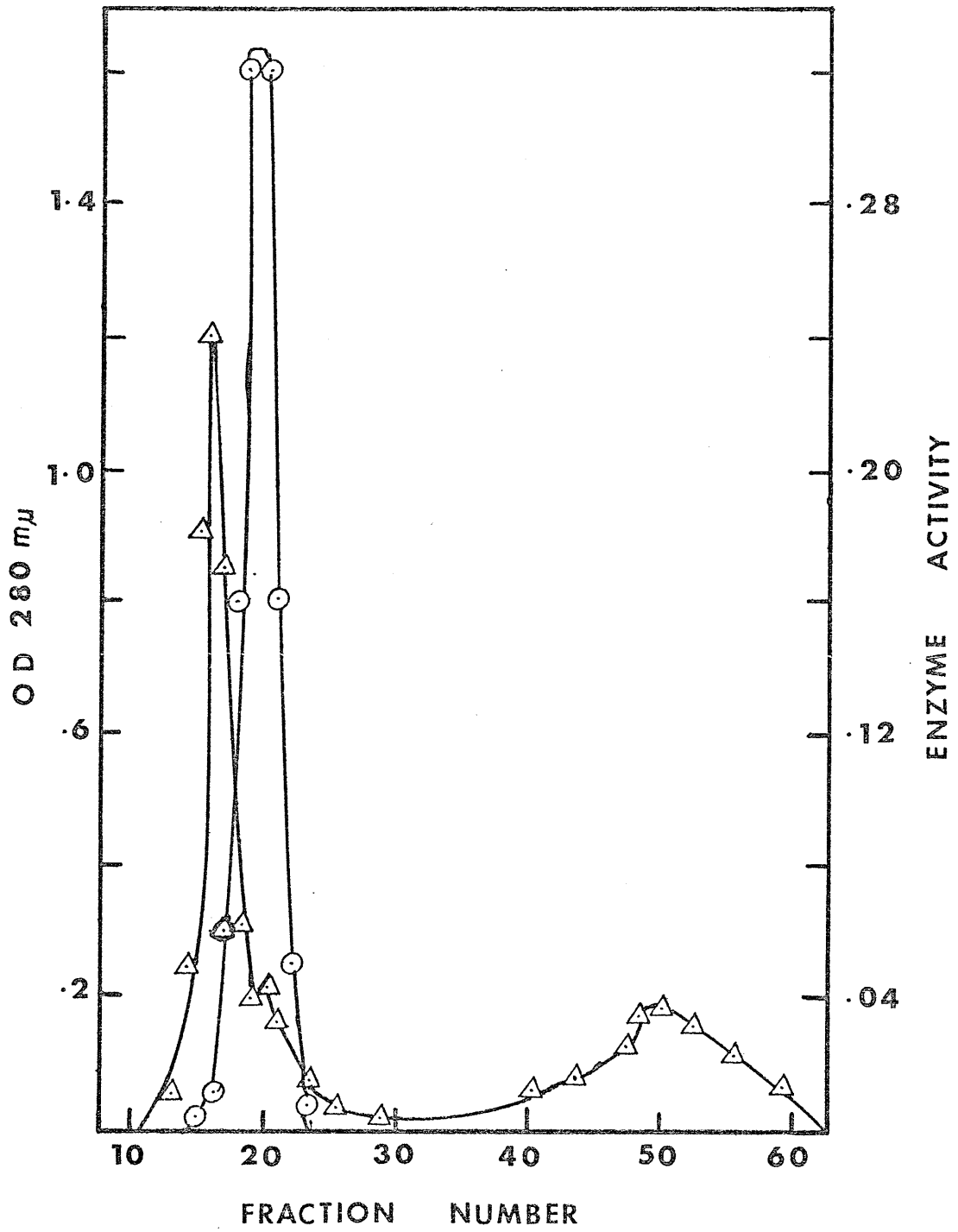
FRACTION NUMBER

FIG. 5. Sephadex G - 200 elution profile from a 1.5 x 30 cm column equilibrated with buffer A, pH 7.7. The flow rate of the column was 10 ml/hour.

 - Protein Absorbance (OD 280 mμ).

 - Enzyme Activity (OD 410 mμ).


1·4
1·0
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OD 280 mμ
·28
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ENZYME ACTIVITY
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60
FRACTION NUMBER

and 1.0 ml fractions were collected. The pooled,most active, fractions contained 0.130 mg protein per ml, 1,628 enzyme units per ml and a specific activity of 12,461. The total enzyme units recovered was 17,500. An elution profile is shown in Fig.3.B.

Polyacrylamide gels (7.5%) showed the same 3 protein bands as in previous purifications.

(e) Sephadex G - 200

At this point it was decided to use a larger diameter column, a 2.5 cm x 40 cm column, and to apply a larger sample of 2.0 ml but to collect the same 1.0 ml fractions to see whether one enzyme protein band would be obtained.

Therefore, Sephadex G-200 was equilibrated with buffer A, pH 7.7, on a 2.5 x 40 cm column. The flow rate was 3.5 ml per hour. The sample applied contained 24,000 enzyme units per ml,a protein concentration of 6.0 mg per ml and a specific activity of 4,000 enzyme units per mg protein.

The pooled, most active, fractions contained a protein concentration of 0.140 mg per ml, 5,050 enzyme units per ml and a specific activity of 36,071. The total enzyme units recovered was 41,000. An elution profile is shown in Fig.9.

Polyacrylamide gels (7.5%) showed 3 major protein bands, two of which were close together and slower moving (Fig.10). The two slower bands were active on gels with the substrate PNPP

The protein sample above applied to the column was in phosphate buffer, pH 6.4. The column above, however, had been equilibrated with buffer A, pH 7.7. It was thought at this point that the column should be equilibrated

FIG. 6. Polyacrylamide gel (7.5%) of the enzyme eluted from a Sephadex G - 100 column (1.5 x 30 cm) with buffer A, pH 7.7. The pH of the resolving gel was 8.9. Duration of the run was $1\frac{1}{2}$ hours with the anode at the bottom.

FIG. 7. Polyacrylamide gel (7.5%) of the enzyme eluted from a Sephadex G - 150 column (1.5 x 30 cm) with buffer A, pH 7.7. Conditions were as described in Fig. 6.

FIG. 8. Polyacrylamide gel (7.5%) of the enzyme eluted from a Sephadex G - 200 column (1.5 x 30 cm) with buffer A, pH 7.7. Conditions were as described in Fig. 6.

The amount of protein run on the above gels ranged from 20 to 30 µg.

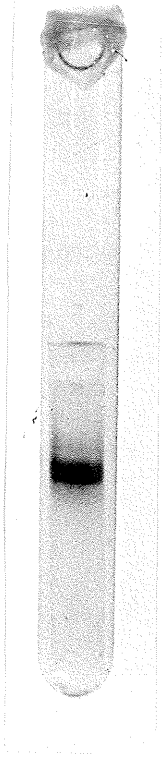


FIG. 6

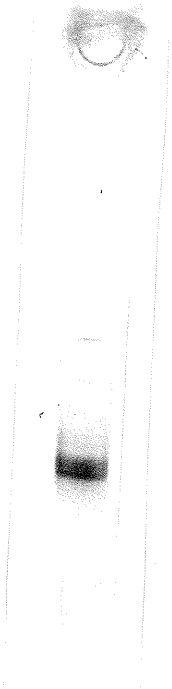


FIG. 7

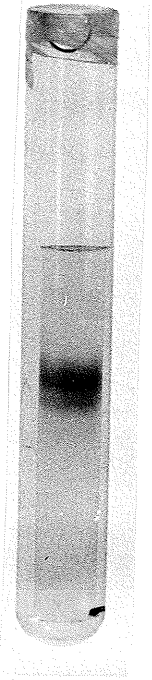
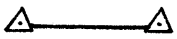
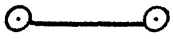
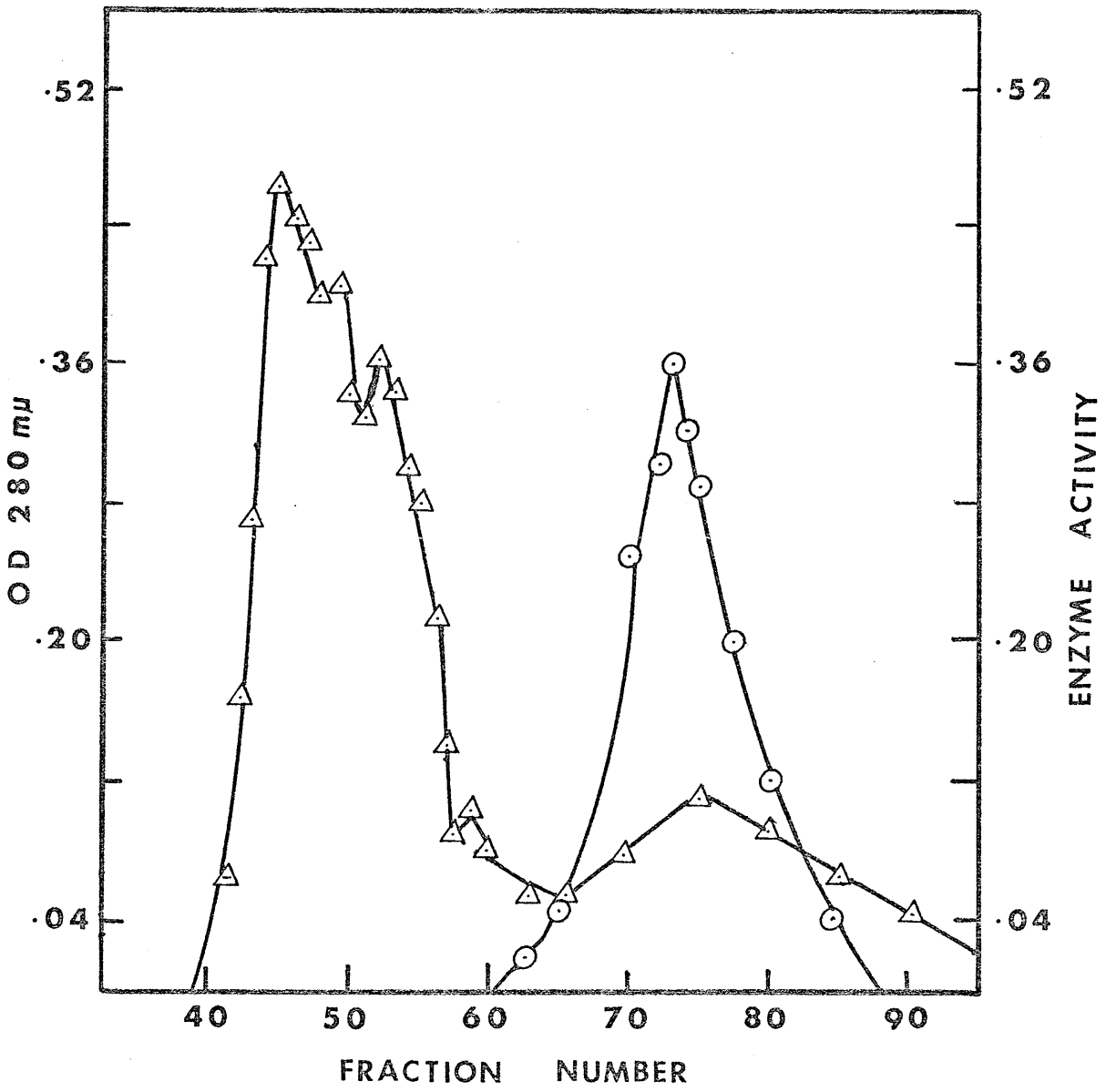


FIG. 8

FIG. 9. Sephadex G - 200 elution profile from a 2.5 x 40 cm column equilibrated with buffer A, pH 7.7. The flow rate of the column was 3.5 ml/hour.

 - Protein Absorbance (OD 280 mμ).

 - Enzyme Activity (OD 410 mμ).


·52
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OD 280 mμ
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ENZYME ACTIVITY
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80
90
FRACTION NUMBER

with 0.01 M phosphate buffer pH 6.4 to see whether elution with the same buffer the enzyme was stored in would result in a single band of protein. Therefore 2.0 ml of the same sample as above was applied to the column and the flow rate was adjusted to 3.5 ml per hour. After the most active fractions were pooled, they contained 2,850 enzyme units per ml, a protein concentration of 0.120 mg per ml and a specific activity of 23,417 enzyme units per mg protein. The total enzyme units recovered was about 30,000. Polyacrylamide gels showed 3 major protein bands but these were more evenly spaced on the gel (Fig. 11). This decrease in specific activity as compared to elution with buffer A could be a result of inhibition by P_i or conversely, the high specific activity observed with buffer A could be a result of activation by Zn ions or Mg^{2+} ions or both Zn and Mg ions.

Since one protein band could not be obtained after using 0.01M phosphate buffer at pH 6.4, the same buffer was tried at pH 7.7, alkaline pH. No difference in the results were seen. On 7.5% polyacrylamide gel the 3 major protein bands appeared, but, in addition to the 3 major bands there were some faint bands between these 3 major bands (Fig. 12). A control gel of the sample that was applied to the column was run. This control was in phosphate buffer pH 6.4. The concentration of the control applied to the gel was much higher than that of the purified enzyme. Yet the faint bands discernable after the G - 200 at pH 7.7 elution were not discernable on the control gel.

Since one protein band could not be seen on polyacrylamide gel, it was decided to see whether any of the following:

(a) the bromphenol blue tracking dye

FIG. 10. Polyacrylamide gel (7.5%) of the enzyme eluted from a Sephadex column (2.5 x 40 cm) with buffer A, pH 7.7. The pH of the resolving gel was 8.9. Duration of the run was 1½ hours with the anode at the bottom.

FIG. 11. Polyacrylamide gel (7.5%) of the enzyme eluted from a Sephadex column (2.5 x 40 cm) with 0.01 M phosphate buffer, pH 6.4. Conditions were as described in Fig. 10.

FIG. 12. Polyacrylamide gel (7.5%) of the enzyme eluted from a Sephadex column (2.5 x 40 cm) with 0.01 M phpsphate buffer, pH 7.7. Conditions were as described in Fig. 10.

The amount of protein run on the above gels ranged from 25 to 30 µg per gel.

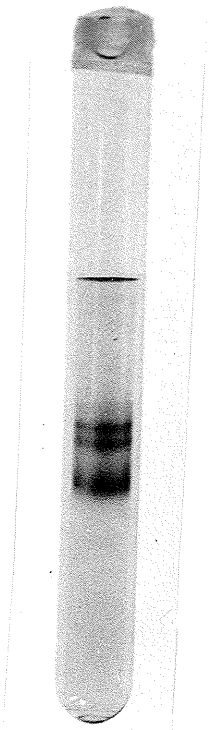


FIG. 10

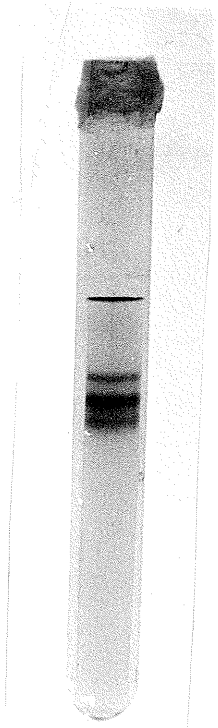


FIG. 11

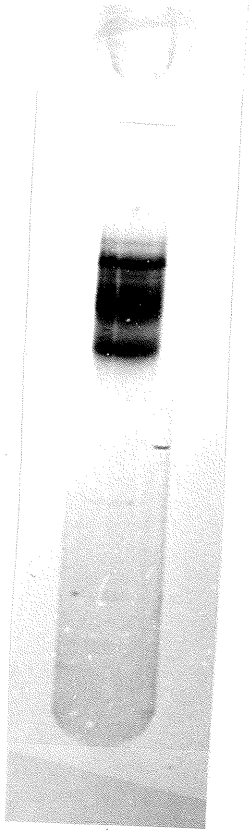


FIG. 12

(b) the excess ammonium persulfate

(c) or both (a) and (b),

were responsible for splitting an otherwise single protein band into the 3 bands. Therefore, the following experiment was carried out:

(1) The first gel was run with purified protein plus tracking dye.

(2) The second gel was run with purified protein but no tracking dye.

(3) The third and fourth gels were pre-run for about 1 hour to rid them of any excess ammonium persulfate. Then the third gel was run with purified protein plus tracking dye. The fourth gel was run with purified protein and no tracking dye.

However, after staining and destaining, the three major protein bands appeared as before.

At this point it was thought that the inactive fast moving protein band might be inactive enzyme. If this was the case then by dissociating these 3 protein bands into subunits, these subunits should appear as one band on SDS - polyacrylamide gels if they were identical.

SDS - Polyacrylamide gel Electrophoresis of Purified Enzyme

The purified protein was run on 7.5% SDS - gels according to the method of Weber and Osborn (1969). The three protein bands seen on analytical polyacrylamide gels were reduced tooone band on SDS - gels. It was therefore, concluded that the 3 protein bands seen on gels after Sephadex G - 200 were probably polymers of the same species, that is, they were all enzyme bands (Fig. 13).

Homogeneity of Native Enzyme by Ultracentrifugation

It was decided to check the homogeneity of the native enzyme on the

FIG. 13. SDS - polyacrylamide gel (7.5%) of purified alkaline phosphatase. Procedures were as described in the materials and methods. Duration of the run was about 4½ hours with the anode at the bottom. The gels were run at 8 mA per tube. The amount of protein run was 130 µg.

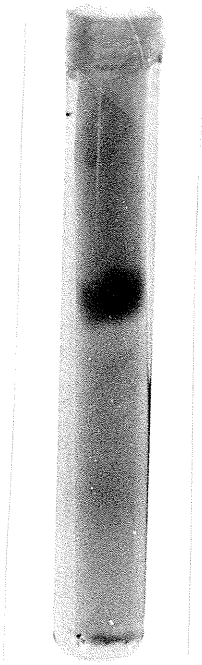


FIG. 13

FIG. 14. Model E run of the native alkaline phosphatase after Sephadex G - 200 purification. The enzyme was in 0.01 M phosphate buffer, pH 6.4. The concentration of protein was 4 mg/ml. The rotor speed was 60,000 rpm. Pictures were taken at 8 minutes intervals. This picture was taken 35 minutes after the rotor reached full speed.

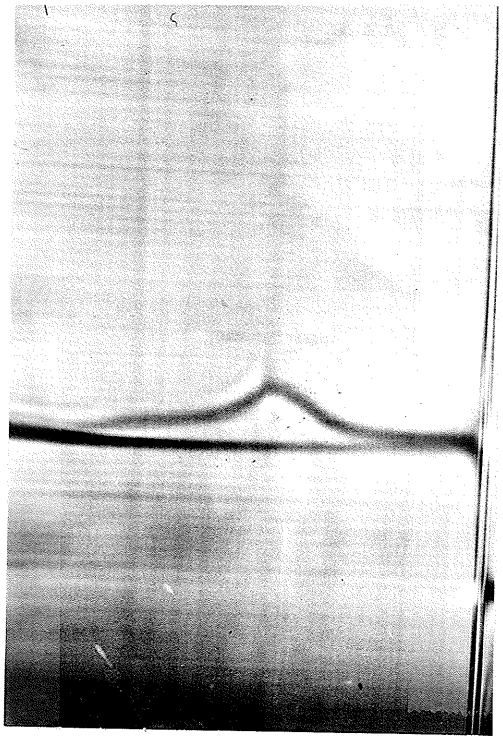


FIG. 14

Model E Analytical Ultracentrifuge, to see whether 3 peaks or one peak would appear. The enzyme preparation was in 0.01 M phosphate buffer pH 6.4 and at a concentration of 4 mg per ml. The run was allowed to proceed for about seventy five minutes after the rotor reached full speed and throughout that time one peak appeared (Fig. 14). The rotor speed was 60,000 rpm. Therefore, the enzyme preparation after elution from Sephadex G - 200 with 0.01 M phosphate buffer seemed to be homogeneous as judged on the Model E Ultracentrifuge. A sedimentation constant of 6.1 S was calculated for the enzyme preparation (Fig. 15).

Homogeneity of SDS - treated Enzyme by Ultracentrifugation

Purified enzyme by Sephadex G - 200, 5 mg per ml, was treated with 1.0% SDS, 1.0% 2-mercaptoethanol, 0.01 M potassium phosphate, adjusted to pH 7.0, incubated for 3 hours at 37°C in a water bath, and finally run on the Model E Ultracentrifuge at 60,000 rpm for 100 minutes after the rotor reached full speed. One peak appeared throughout the run (Fig. 16). Therefore, the SDS - treated enzyme seemed to be homogeneous on the Model E.

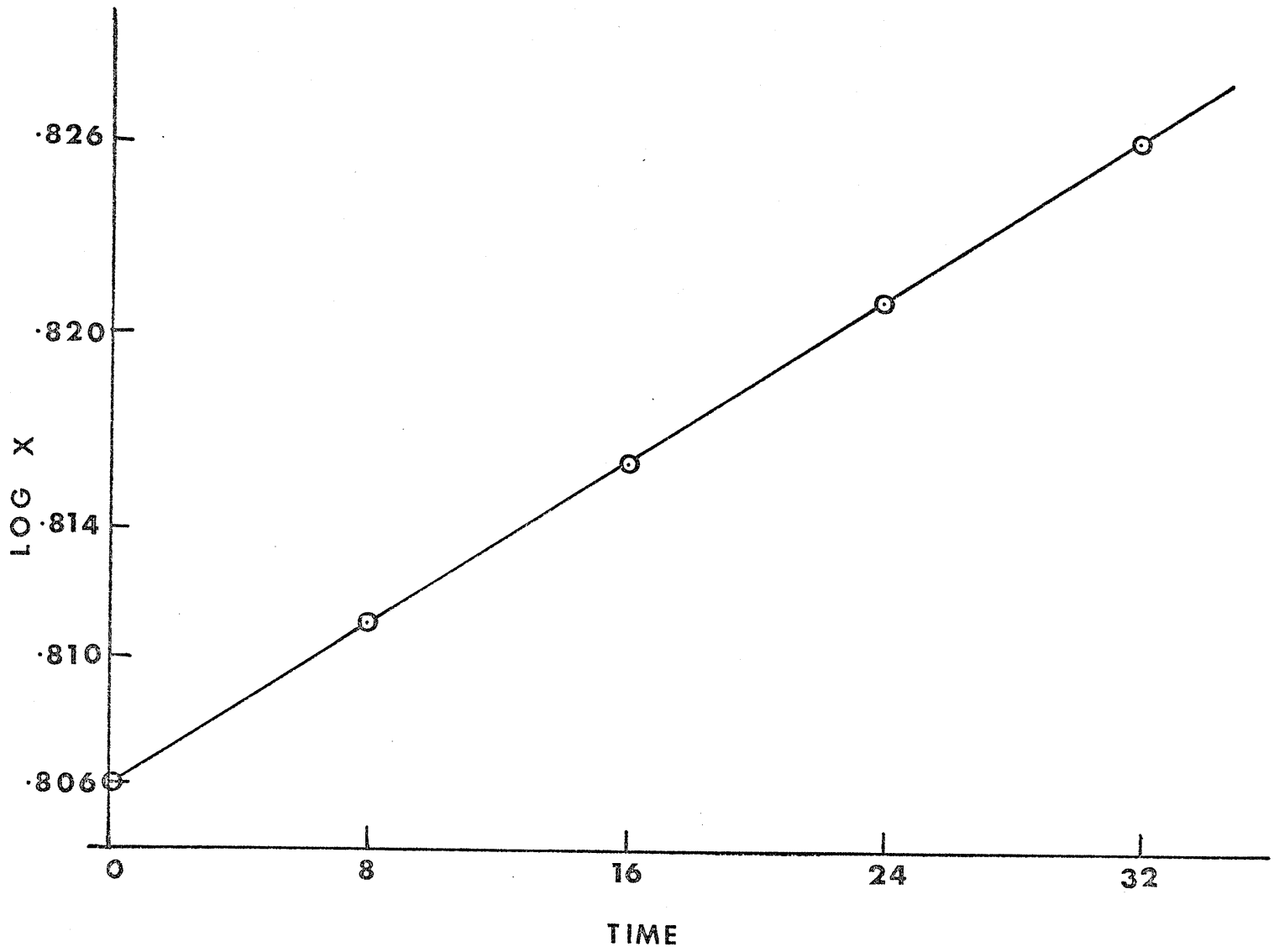
Overall Purification of Alkaline Phosphatase

From now on enzyme was purified by gel filtration on Sephadex G-200 equilibrated either with buffer A or 0.01 M phosphate buffer on a 2.5 cm x 40 cm column. Table 1 shows the overall purification (step 1 to V are those of Davis, 1970).

Minimum Molecular Weight Determination by SDS - Polyacrylamide Gel Electrophoresis

SDS - polyacrylamide gel electrophoresis was used to find the minimum

FIG. 15. Shows plot of Log x (ordinate) vs Time (abscissa) of the sedimentation of the native alkaline phosphatase on the Model E Analytical Ultracentrifuge (x is the distance of boundary to axis of rotation). Pictures were taken at 8 minutes intervals.


·826
·820
·814
·810
·806
LOG X
0
8
16
24
32
TIME

molecular weight of the enzyme according to the method of Weber and Osborn (1969).

(a) The minimum molecular weight by the dimethyl suberimidate method was found to be 30,000 $\pm$ 2,000 (Fig. 17).

(b) The minimum molecular weight of the enzyme using the standard proteins Bovine serum albumin, Trypsin, Lysozyme and alpha-Chymotrypsin, was found to be 28,000 (Fig. 18).

Amino Acid Composition

1.60 mg of the purified enzyme, which had been treated with SDS and run on the Model E Ultracentrifuge, was dialyzed against distilled water overnight. The amino acid composition of this sample was determined according to the method of Moore et al, (1958). Hydrolysis of the sample was carried out with 6 N HCl under nitrogen for 24 hours. The cysteine and tryptophane content of the enzyme were not determined. The amino acid composition in mole percent is shown in Table 11.

Enzyme Preparation left standing for two months

An interesting thing happened during the course of this investigation as follows:

An enzyme preparation was inadvertently left standing for 2 months after dialysis against buffer A (end of step 1V of Davis's purification procedure). After this period step V (Davis, 1970) was proceeded with. However, very little precipitate formed upon $ZnCl_2$ precipitation, almost undiscernable. Nevertheless, the preparation was centrifuged at 14,000 x g for 20 minutes at 0 to 4°C. The tiny speck of precipitate was dissolved in 0,01 M phosphate buffer pH 6.4 and recentrifuged to get rid of any

FIG. 16. Model E run of SDS - treated alkaline phosphatase after Sephadex G - 200 purification. Procedures were as described in the materials and methods. The rotor speed was 60,000 rpm. Pictures were taken at 16 minutes intervals. This picture was taken 95 minutes after the rotor reached full speed.

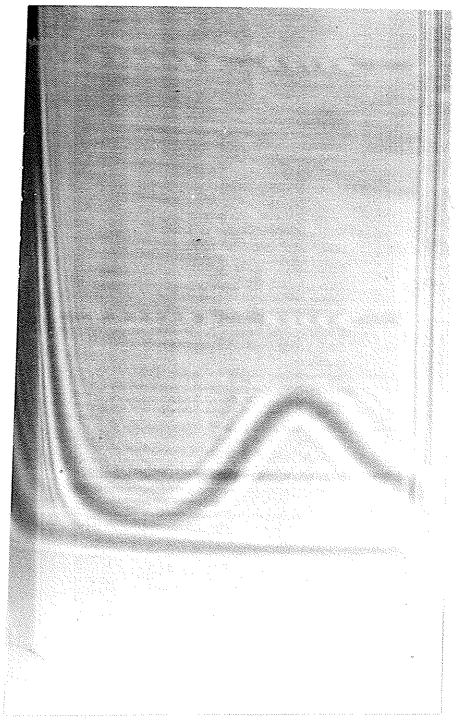


FIG. 16

TABLE I

OVERALL PURIFICATION OF ALKALINE PHOSPHATASE

Step	Description of Method	Volume (ml)	Enzyme Units	Protein Concentration (mg)	Specific Activity (enzyme units per mg protein).
1	Preparation of crude extract.	900	383,000	11,070	35
11	DEAE cellulose batch purification	1925	167,000	1,330	126
111	Heat denaturation	1915	147,000	955	154
1V	Ammonium sulfate fractionations	10.5	82,000	80	1,025
V	Zinc chloride precipitation	2.3	80,000	23.8	3,361
V1	Sephadex G - 200	2.0	48,000-72,000	2.0	24,000 - 36,000

FIG. 17. Shows SDS - polyacrylamide gel electrophoresis of alkaline phosphatase, and glyceraldehyde - 3 - phosphate dehydrogenase from rabbit muscle. Procedures were as described in the materials and methods. The composition of the gel was 7.5%. The duration of the run was $4\frac{1}{2}$ hours with the anode at the bottom.

A - Tetramer of Gl - 3 - P Dehase (mol. wt. 144,000).

B - Trimer of Gl - 3 - P Dehase (mol. wt 108,000).

C - Dimer of Gl - 3 - P Dehase (mol. wt. 72,000).

D - Monomer of Gl - 3 - P Dehase (mol. wt. 36,000).

E - Monomer of Alkaline Phosphatase (mol. wt. 30,000).

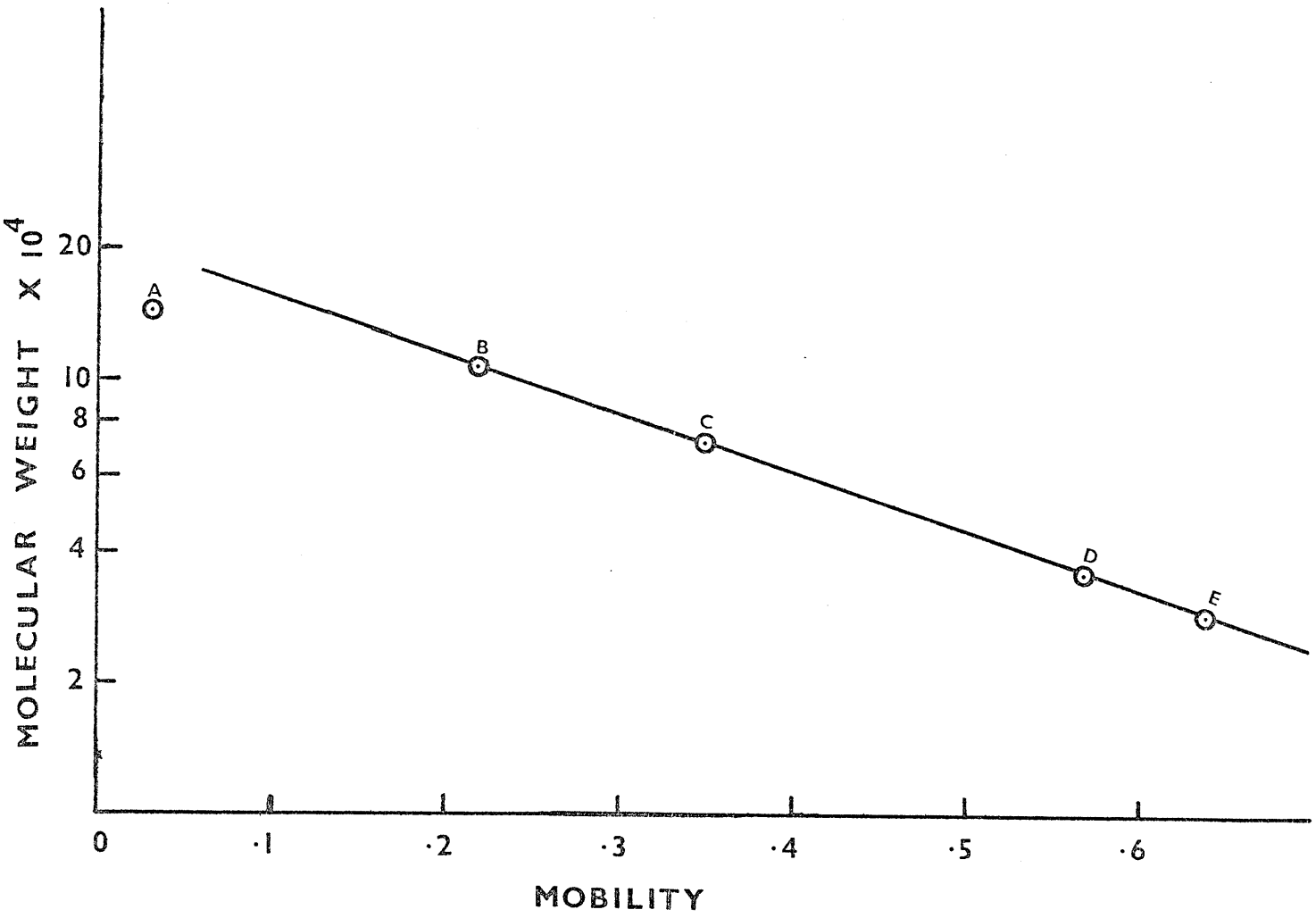

MOLECULAR WEIGHT X 10^4
20
10
8
6
4
2
A
B
C
D
E
0
·1
·2
·3
·4
·5
·6
MOBILITY

FIG. 18. Shows SDS - polyacrylamide gel electrophoresis of alkaline phosphatase and standard proteins. Procedures were as described in the materials and methods. The composition of the gel was 7.5%. The duration of the run was $3\frac{1}{2}$ hours at 8 mA per tube with the anode at the bottom.

A - BSA (subunit mol. wt. 68,000).

B - Alkaline Phosphatase (subunit mol. wt. 28,000).

C - Trypsin (subunit mol. wt. 23,300).

D - Lysozyme (subunit mol. wt. 14,300).

E - Alpha-Chymotrypsin (subunit mol. wt. 11,000).

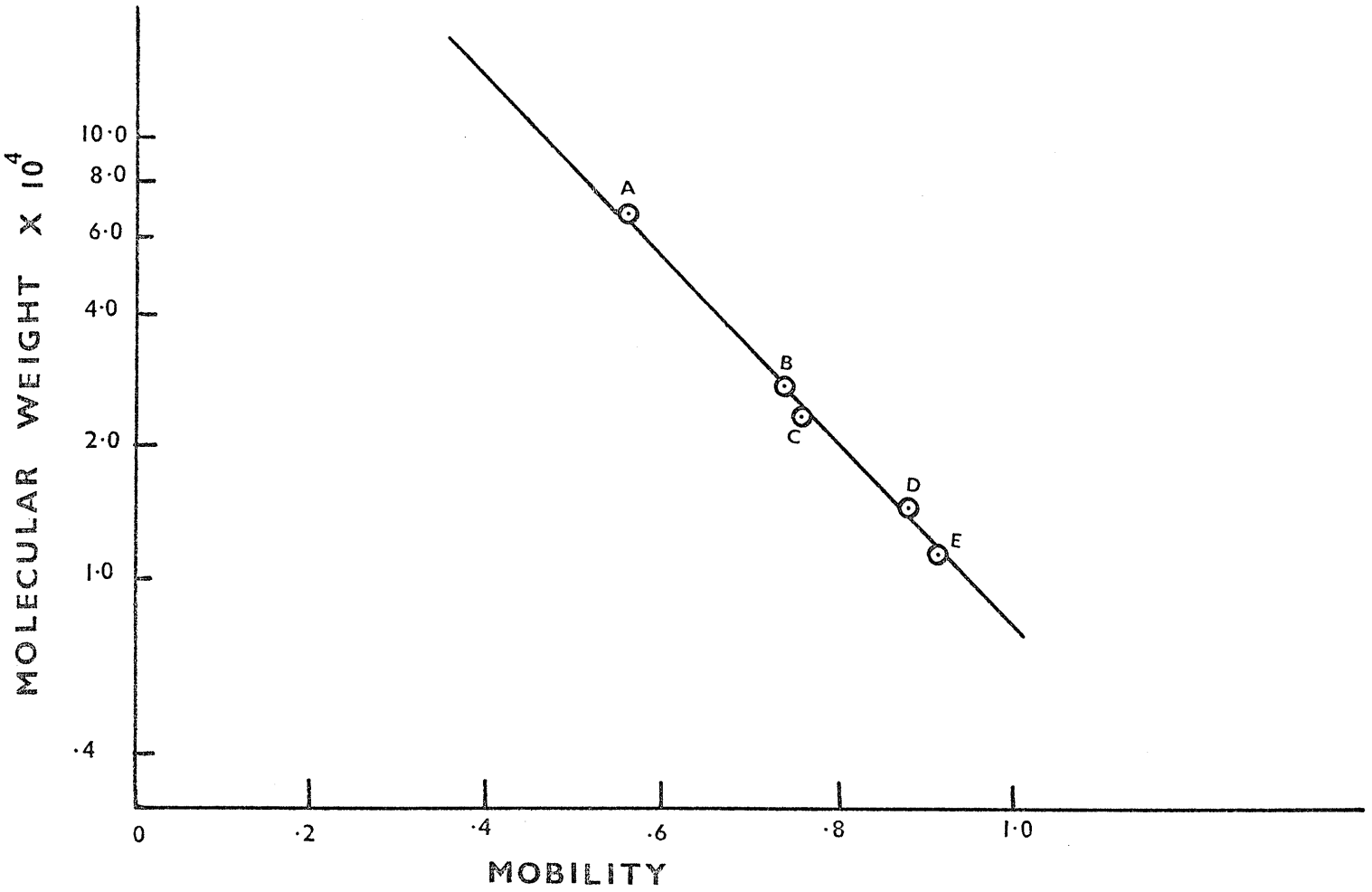

MOLECULAR WEIGHT X 10^4
10·0
8·0
6·0
4·0
2·0
1·0
·4
A
B
C
D
E
0
·2
·4
·6
·8
1·0
MOBILITY

FIG. 19. Polyacrylamide gel (7.5%) of the enzyme left standing for 6 months in buffer A, pH 7.7. The pH of the resolving gel was 8.9. Duration of the run was 1½ hours with the anode at the bottom. The amount of protein run on the gel was 30 µg.

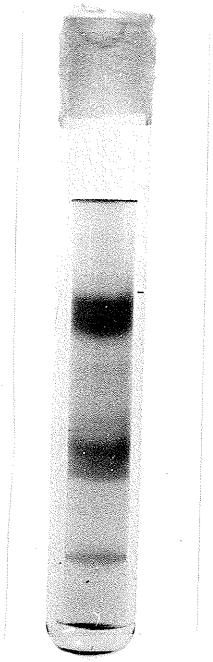


FIG. 19

FIG. 20. Polyacrylamide gel (7.5%) of the enzyme preparation immediately before Sephadex purification. The pH of the resolving gel was 8.9. Duration of the run was $1\frac{1}{2}$ hours with the anode at the bottom. The amount of protein run on the gel was 75 µg.

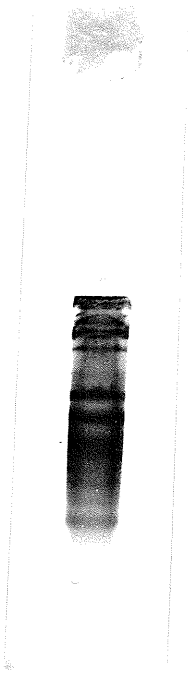


FIG. 20

undissolved material. This eluted sample was run on 7.5% gels and what was seen is shown in Fig 19. Three major protein bands appeared and no other discernable protein bands. All 3 protein bands were active enzyme bands, active with PNPP substrate. The two slower moving bands on the gel resembled the two bands seen after the elution of enzyme from G - 200 with buffer A, pH 7.7. What is usually seen after step V is shown by the gel in Fig. 20. The preparation at the end of step lV is usually precipitated immediately after dialysis against buffer A.

Isoelectric Point

The isoelectric point of the enzyme preparation immediately before Sephadex G - 200 was found to be at pH 4.15 using 0.10 N sodium acetate buffer. The method used was Low Voltage Paper Electrophoresis: Whatman #1 filter paper strips, 5 cm wide and 21 cm long, were divided into two halves by drawing a line with pencil at the 2.5 cm mark along the length of the paper. The samples, enzyme preparation and glucose, were applied at the 9.0 cm mark on opposite sides of the dividing line. The run was allowed to continue for 3 hours. After 3 hours a visible precipitate at pH 4.15 could be seen on the filter paper strips where the enzyme preparation was applied. The strips were taken out and cut along the dividing line. The enzyme half was stained either with protein stain or substrate, and the glucose half was stained with the glucose stain. Runs were done at pH 3.5, 4.0, 4.15, 4.5, 5.0, 5.5, 6.0, 7.0, 8.0, Below pH 5.0 the enzyme was not active as judged by the absence of the yellow color when substrate was added. Yellow color appeared above pH 5.0.

The migration distance from the origin of sample application of the

TABLE III

ISOELECTRIC POINT DETERMINATION

pH	Chromatogram number	Glucose migration (cm)	Protein migration (cm)
3.85	1	(+) 0.90	(+) 0.70
	2	(+) 1.05	(+) 0.50
4.15	1	(+) 0.80	(+) 0.80
	2	(+) 0.70	(+) 0.70
4.15	1	(+) 0.80	(+) 0.80
	2	(+) 0.80	(+) 0.80
5.00	1	(+) 0.50	(+) 0.20
6.00	1	(+) 0.90	(-) 0.50

protein and glucose is given in Table 111 at various pH values. (+) means migration towards the positive electrode from the point of application of the sample and (-) means migration towards the negative electrode. At pH 4.15 both glucose and the protein spot had the same migration distance. Therefore, it was concluded that the isoelectric point of the protein preparation was at this pH, 4.15.

Comparison of Relative Mobilities on Polyacrylamide gels of the Repressible Alkaline Phosphatase and the Constitutive Alkaline Phosphatase from Neurospora crassa

N. crassa was grown in Vogels medium (Vogel, 1956) with P_i as the limiting factor to induce the repressible alkaline phosphatase. The constitutive enzyme from strain 533 was obtained from the lyophilized mycelium (materials and methods). The mutant strain, M 16, was grown in P_i rich Vogel's medium (1956) to repress the repressible alkaline phosphatase.

Crude extracts were prepared as follows:

(1) Crude extract of the repressible enzyme was prepared according to the method of Nyc et al, (1966).

(11) Crude extracts of the constitutive enzyme from strain 533 and mutant strain, M 16, were prepared according to the method of Davis (1970).

(111) 5% polyacrylamide gels were run with these crude extracts and were stained as follows:

(a) The repressible enzyme was stained with the assay mix prepared according to the method of Kadner et al, (1968). One band of enzyme activity appeared on the gel with a relative mobility (R_m) of 9.80×10^{-2}, as compared with the tracking dye.

(b) Another gel with the repressible enzyme was stained with assay mix prepared according to the method of Davis, (1970). Two bands of enzyme activity appeared: The repressible enzyme with a R_m of 1.01×10^{-1} and the constitutive enzyme with R_m of 7.7×10^{-2}.

(c) A gel run with the constitutive enzyme was stained with the repressible assay mix which inhibits the contitutive enzyme. No activity appeared.

(d) Another gel run with the constitutive enzyme was stained with the assay mix of Davis, (1970). One band of enzyme activity appeared with a R_m of 7.8×10^{-2}.

(e) A gel run with the crude extract of the mutant strain, M 16, showed no repressible enzyme activity. Another gel showed the constitutive enzyme which had a R_m of 7.8×10^{-2}.

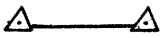
Therefore, the mobility of the constitutive enzyme differs from that of the repressible enzyme. Also the mobility of the constitutive enzyme is not affected whether the organism is grown in a phosphate limiting medium or in a phosphate rich medium. It should be noted that the minor activity (Davis, 1970) was not observed here perhaps because the gels were 5% in composition. This pore size may not be small enough to resolve both activities. Or perhaps the minor activity is absent when the organism is grown under phosphate limiting conditions.

Rechromatography Experiment

This experiment was done to see whether one protein band would appear on the polyacrylamide gel after re chromatography on Sephadex G - 200:

(1) Enzyme was applied to a 1.5 x 30 cm column packed with Sephadex G - 200 and equilibrated with 0.01 M PO_4 buffer pH 6.4. The flow rate was

FIG. 21. Sephadex G - 200 elution profile from a 1.5 x 30 cm column equilibrated with 0.01 M phosphate buffer, pH 6.4. The flow rate of the column was 4.0 ml/hour.

 - Protein Absorbance (OD 280 mμ).

 - Enzyme Activity (OD 410 mμ).

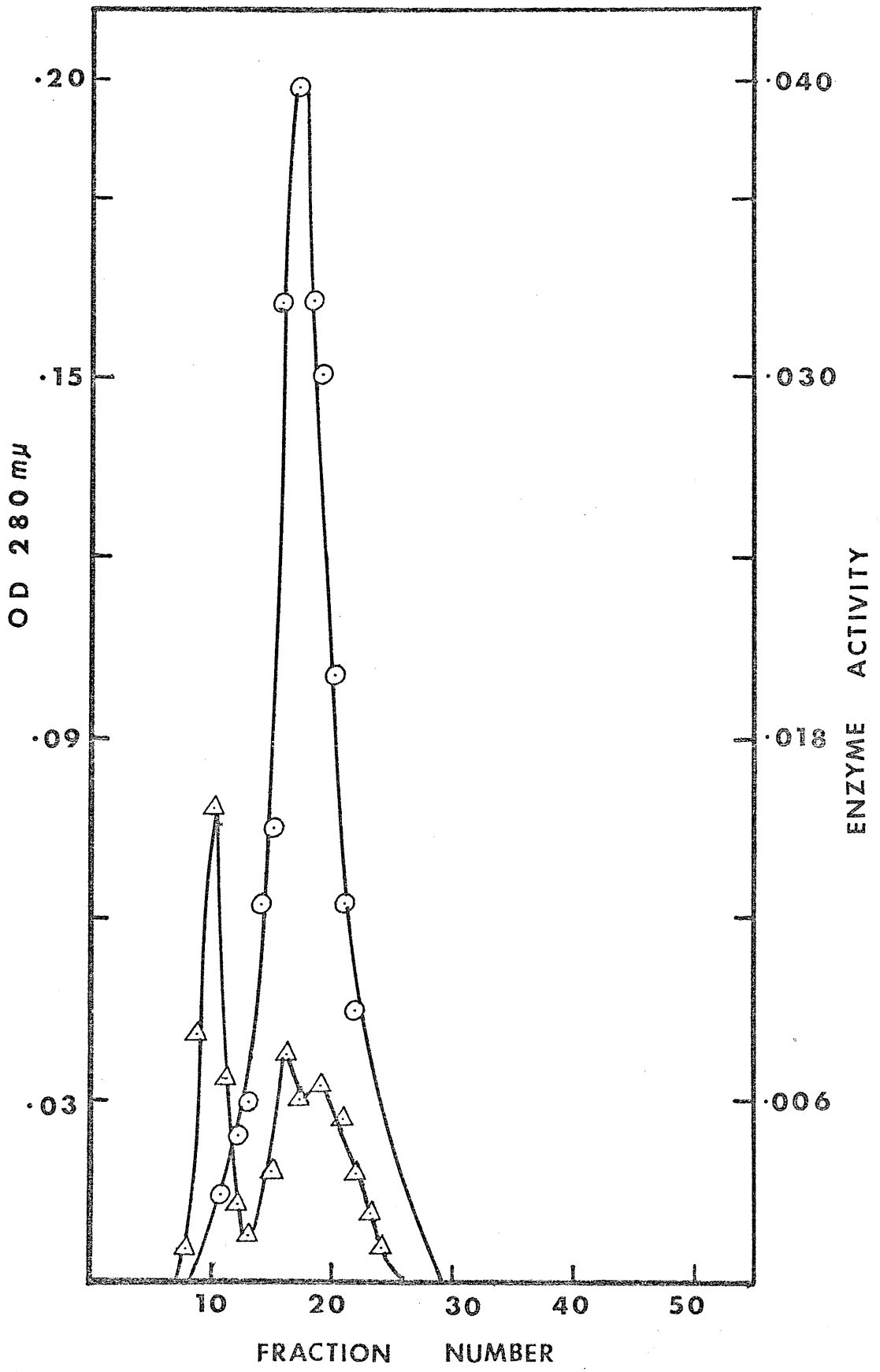
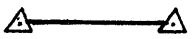
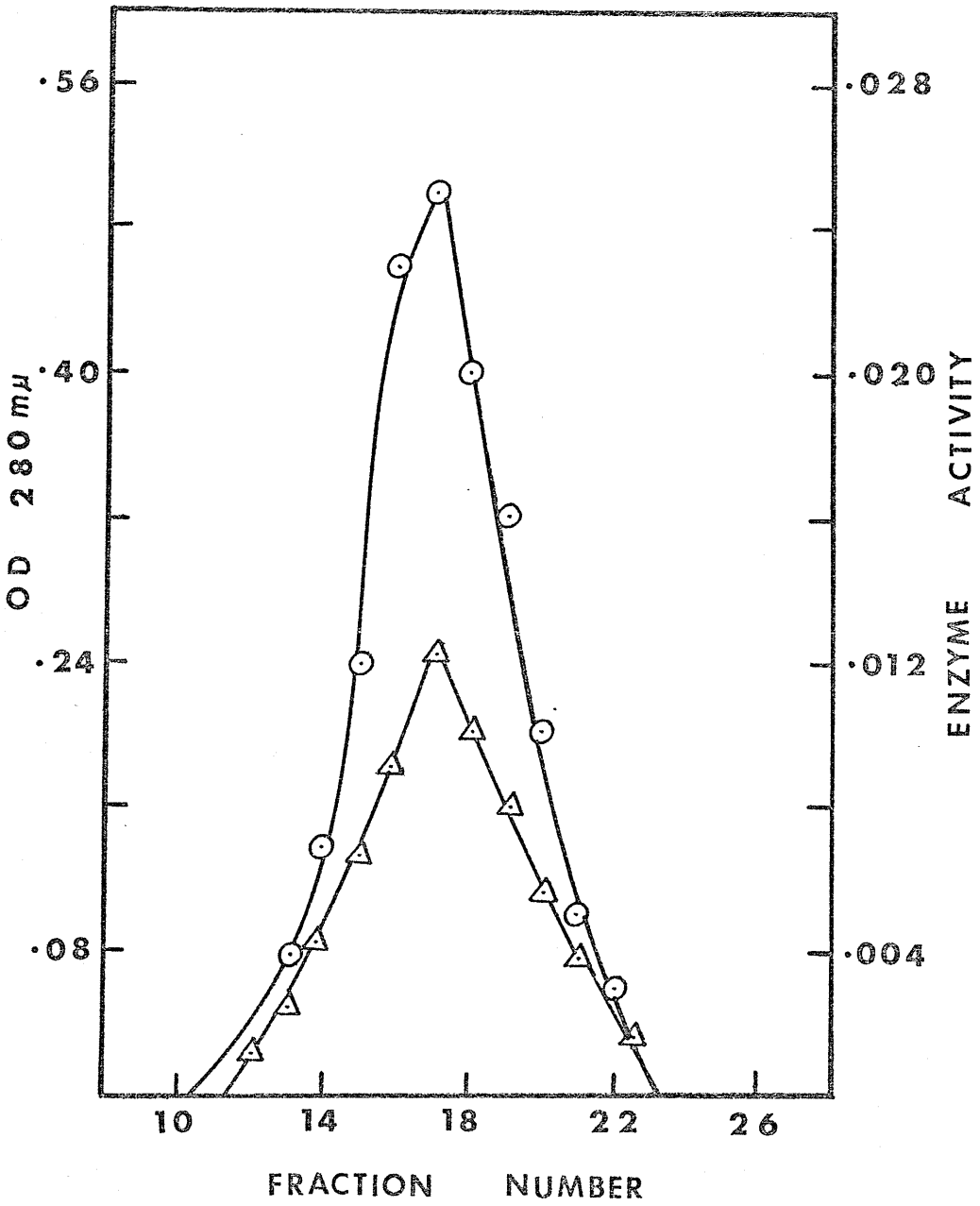

·20
·15
·09
·03
OD 280 mμ
·040
·030
·018
·006
ENZYME ACTIVITY
10
20
30
40
50
FRACTION NUMBER

FIG. 22. Elution profile of enzyme rechromatographed on a Sephadex G - 200 column (1.5 x 30 cm) equilibrated with Tris buffer pH 7.0. The flow rate of the column was 4.0 ml/hour.

 - Protein Absorbance (OD 280 mμ).

 - Enzyme Activity (OD 410 mμ).


·56
·40
·24
·08
OD 280 mμ
·028
·020
·012
·004
ENZYME ACTIVITY
10
14
18
22
26
FRACTION NUMBER

about 4 ml/hour. An elution profile is shown in Fig. 21.

On polyacrylamide gels (7.5%) 3 protein bands appeared as before.

(11) The samples from (1) above were pooled, their volume reduced by dialysis against air and then dialyzed overnight against 0.02 M Tris - HCl pH 7.0. At the same time the Sephadex G - 200 column was equilibrated with the same Tris buffer, pH 7.0. The sample was applied after dialysis to the column and the flow rate was adjusted to about 4 ml/hr. An elution profile is shown in Fig. 22.

On 7.5% gels the 3 protein bands still appeared.

DISCUSSION OF RESULTS

The constitutive alkaline phosphatase from Neurospora crassa has been purified to homogeneity as judged by SDS - Polyacrylamide gel electrophoresis, sedimentation velocity of the native enzyme, and sedimentation velocity of SDS - treated enzyme. On analytical polyacrylamide gels after Sephadex G - 200 purification, 3 protein bands appeared, two of which were active with the substrate PNPP. The minor activity of Davis (1970) was not present on gels after Sephadex purification.

These 3 bands of protein were seen on polyacrylamide gels whether the initial purification was by calcium phosphate gel or by Sephadex. The first thought that comes to mind when one sees more than one major protein band on polyacrylamide gel after purifying an enzyme through many steps is that the preparation is still impure. But purity, or to use a less rigid and widely accepted scientific term, homogeneity, does not mean that a single band of protein has to be seen on polyacrylamide gels. There are certainly many factors of which we are unaware that can cause the appearance of several bands of protein on polyacrylamide gels. Therefore, other methods must be used to test for homogeneity e.g. SDS - polyacrylamide gel electrophoresis, sedimentation velocity analysis on the Model E Analytical Ultracentrifuge, pH changes and rechromatography experiments. A case in point is the E. coli alkaline phosphatase. On polyacrylamide gels from 3 to 5 active protein bands are seen. However, when the preparation is subjected to sedimentation velocity analysis on the Model E Ultracentrifuge one enzyme peak appears (Reid and Wilson, 1971). The preparation is, therefore, said to be homogeneous. Therefore,

the phenomenon observed here for the Neurospora crassa alkaline phosphatase is not new.

The enzyme is easily dissociated into subunits by SDS. It would seem that the subunits are held together by weak bonds since the concentration of SDS used is approximately 0.01 to 0.05 M which is very weak when compared to the concentration of some other dissociating agents such as 6 M guanidine hydrochloride or 8 M urea. Perhaps these weak bonds are easily broken. This may explain the presence on polyacrylamide gels of the inactive protein band seen after Sephadex purification.. This inactive protein band may also explain, in part, the decrease in the number of enzyme units after Sephadex purification.

The sedimentation constant of the native enzyme is probably an average value of the three species seen on polyacrylamide gel after Sephadex G - 200 (assuming that the gel itself is not responsible for the appearance of these 3 protein bands). The E. coli enzyme has a molecular weight of 86,000 and a sedimentation constant of 6.0 at pH 8.0 (Reid and Wilson, 1971). The molecular weight of the purified N. crassa enzyme could have been found on a sucrose density gradient with standard proteins, an experiment which could have given some information as to whether there were three species, and whether the molecular weight was an average one. Davis, (1970) observed a molecular weight of 89,000 when peak 1 and peak 11 enzyme were mixed and ran on a 4% to 20% sucrose density gradient. This was an average molecular weight of peak 1 and peak 11 enzyme of a 50% pure enzyme preparation.

Davis, (1970) found that the molecular weight of the native enzyme purified by Cellogel Electrophoresis was in the region of 112,000 to

120,000. The molecular weight of the subunits by SDS was found here to be in the region of 28,000 to 30,000. This would mean that the native enzyme is a tetramer. However, the following should be noted: in preliminary experiments Davies and Stark (1970) showed that apparent molecular weights of cross-linked species on SDS - polyacrylamide gels are actually slightly higher than the values expected from the molecular weight of the protomers. It has also been reported (Rivers and Impiombato, 1968) that the binding of sodium dodecyl sulfate to several proteins is reduced by the presence of disulfides; cross-links introduced by amidination may also reduce binding of SDS and, consequently, decrease migration. Therefore, this method probably gave a slightly higher molecular weight of the subunit of N. crassa.

The difference in the position and the number of protein bands observed on polyacrylamide gels when the enzyme was eluted from the Sephadex column with buffer A, pH 7.7, with 0.01 M phosphate buffer pH 6.4, or with 0.01 M phosphate buffer pH 7.7, may be due to a zinc ion effect, a phosphate ion effect, a pH effect, or a combination of effects. For the E. coli enzyme, both a zinc ion effect and a pH effect is necessary for tetramer formation (Schlesinger, 1968). The E. coli enzyme is said to exist as a dimer (Reid and Wilson, 1971), with a subunit molecular weight of 43,000. Since the molecular weight of the subunit of the N. crassa enzyme is much smaller than that of the E. coli enzyme, it may just be possible that many more aggregates can be formed. Perhaps movement down a Sephadex column is also necessary for this aggregation to take place.

It was also interesting to note that the 2 slower moving protein bands seen on gels (Fig. 19) of the enzyme preparation that was left standing for 2 months resembled very closely the 2 slower moving bands

seen on gels after elution from the Sephadex column equilibrated with buffer A, pH 7.7 (Fig. 10). This enzyme preparation also showed the minor activity of Davis (1970). Therefore, there are actually three activities before Sephadex, two of which belong to the 2 slower moving bands which are so close together that they would appear as one activity when stained with PNPP substrate and give the impression that the yellow color diffuses when actually there are two activities giving the impression of diffusion. Therefore, the postulation of Davis (1970) that there actually might be 3 activities is, indeed, true.

The isoelectric point of the N.crassa enzyme preparation immediately before Sephadex purification was found to be at pH 4.15 using 0.10 N sodium acetate buffer. The enzyme, however, is inactive at this pH. Since the isoelectric point is at pH 4.15 we would expect the enzyme to be highly acidic. The amino acid composition of the enzyme shows that the enzyme is indeed acidic. The isoelectric point of the E.coli enzyme was found to be at pH 4.5 (Reid and Wilson, 1971). Also the isoelectric point of alkaline phosphatase from Aspergillus nidulans is reported to be at pH 4.0 by cellulose acetate electrophoresis and 3.6 by starch-gel electrophoresis (Dorn, 1968). Isoelectric precipitation can be a useful tool in the purification of an enzyme. However, in the cases cited here the isoelectric point was not used in purification procedures.

The amino acid composition shows aspartic acid, glutamic acid, glycine and alanine to be in the highest amounts. Leucine and lysine appear in appreciable amounts also. Methionine, however, is present in the lowest amount. Tryptophane and cysteine were not determined. Perhaps, the relative amounts of the different amino acids present in a molecule can influence

the conformational changes that take place. Perhaps pH changes may enhance or reduce these conformational changes. This might explain the different aggregation states seen (assuming these are aggregates) on gels after elution of the enzyme from Sephadex with phosphate buffer at pH 6.4 and pH 7.7. However, it can also be argued that these faint bands are im-- purities. If they are impurities they certainly did not show up as a separate peak when this sample was treated with SDS and run on the Model E (Fig. 16). And as mentioned earlier, a control gel did not reveal these faint protein bands. However, before any definite conclusion can be drawn about this whole subject much research is needed.

The rechromatography experiment showed the three protein bands on polyacrylamide gels as before. The elution profile of Fig. 21 was the first chromatography on Sephadex G - 200 with 0.01 M phosphate buffer, pH 6.4. Fig. 22 shows the rechromatography of the most active fractions from Fig. 21 on Sephadex G - 200 with Tris buffer at pH 7.0. After rechromatography only one protein peak appeared which corresponded to the enzyme peak.

One obvious question that comes to mind is why does Neurospora crassa need such a multiplicity of alkaline phosphatases? The constitutive enzyme is always present even when the organism is grown under phosphate limiting conditions. Therefore, why the need for the enzyme that we call the repressible alkaline phosphatase? The answer to this question is still unknown. Only much more research can add light to this intriguing question. It would be interesting, however, to grow the Neurospora crassa strain 533 in a phosphate limiting medium, isolate the constitutive enzyme and see whether there is any differerence between this constitutive enzyme and that isolated from the same strain grown in a phosphate rich medium. Also

the constitutive enzyme isolated from the organism grown under P_i limiting conditions can be compared to the repressible alkaline phosphate.

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