Approximately 90% of washed cells of PS 55 were lysostaphin-sensitive. Acid phosphatase was localized by controlled lysis of spheroplasts that had been stabilized in 30% polyethylene glycol. Half the enzyme was removed with lysostaphin-solubilized material (cell wall). Following lysis of spheroplasts, the remaining half of enzymatic activity (48%) was associated with the cytoplasmic membrane.

The initial step of the purification procedure for acid phosphatase was elution of the enzyme. Maximal elution of the loosely bound fraction, presumably from the surface of cells, occurred in the alkaline pH range. From log-phase cells, elution was maximally effected with buffered 1.0 M KCl (pH 8.5). The eluted fraction was dialyzed twice and passed through two cycles of molecular sieving (Sephadex G-100). Specific activity of the purified product was 2350 which represented a 300-fold purification. Approximately 17% of the initial activity (loosely and firmly bound) was recovered and the 280/260 ratio was 1.72. The final product was free of other enzymatic activities initially present (coagulase, lipase, and deoxyribonuclease).

Purified acid phosphatase appeared homogeneous after gel filtration, starch-block electrophoresis, and analytical ultracentrifugation. Maximal enzymatic activity occurred at pH 5.2 between 45 and 50 C, but the enzyme was most stable in the alkaline pH range (8.5, 9.5) at temperatures below 50 C. Iodoacetate and EDTA were effective inhibitors

while mercaptoethanol and Cu^{++} proved to be stimulators. The purified enzyme which appeared basic in nature at pH 8.0, was most active against the substrates p-nitrophenyl phosphate and glyceraldehyde 3-phosphate. K_m for the former substrate was 4.5×10^{-4} M and the energy of activation for the hydrolytic cleavage of the same substrate was 19.5 Kcal/mole. Approximations of the molecular weight made by gel filtration and ultracentrifugation were 54,000 and 53,000 respectively.

PURIFICATION, CHARACTERIZATION, AND LOCALIZATION OF STAPHYLOCOCCAL ACID PHOSPHATASE

By

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This thesis is affectionately dedicated to my wife who provided the necessary stimulus and purpose for this achievement.

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INTRODUCTION

Production of acid phosphatase by the pathogen Staphylococcus aureus is one of many biological properties expressed by this organism. Like the production of coagulase, hemolysin, and toxins, acid phosphatase has sometimes been closely associated with virulence of the staphylococci for a given host (Elek, 1959). It is desirable to study those characteristics of pathogenic bacteria that are often linked to toxicity and virulence or some factor which is relevant to certain disease manifestations. Investigations of some enzyme systems have often proved fruitful in this regard (Nygren, Hoborn, and Wahlen, 1966).

Though phosphatase activity of S. aureus has often been reported, the enzyme has not been studied in great detail. Since epidemic strains produce a significant amount of the enzyme, there is need to elucidate some basic properties of the enzyme. This investigation was undertaken to purify, characterize, and localize staphylococcal acid phosphatase to permit insight into the biological function of this enzyme.

REVIEW OF LITERATURE

Phosphatases in Bacteria

Acid phosphatase activity has been demonstrated in many types of bacterial cells, yet the enzyme has no known role in normal bacterial intermediary metabolism (Fraenkel and Horecker, 1965, Hofsten, 1961). Elek (1959) stated that there were two phosphatase activities of *Staphylococcus aureus*, one optimal at pH 6 and the other at pH 10.

When 400 strains of *S. aureus* were screened for phosphatase activity, approximately 90% of the strains were both coagulase- and phosphatase-positive (Gupta and Charavarte, 1954). The authors noted some correlation between coagulase and phosphatase activities in *S. aureus*. In fact, Elek (1959) suggested that phosphatase activity, like coagulase and hemolysin activities, may be closely associated with virulence of the staphylococci for a given host. Cannon and Hawn (1963) observed acid phosphatase activity in all strains of the main phage groups of *S. aureus* derived from clinically confined patients and from healthy carriers. Enzymatic activity which was measured at pH 6.0 paralleled relative cell number. Comparing some enzymatic activities of certain strains of *S. aureus* and *S. albus*, Dominguez and Regueiro (1966) found that the former possessed dehydrogenase, lecithinase, caseinase, coagulase, phosphatase,

and deoxyribonuclease activities, but S. albus was deficient in these same activities. Strains of S. aureus were phosphatase-rich and pyrophosphatase-poor, while those of S. albus demonstrated the reverse. However, S. albus produced twice as much catalase as S. aureus.

Recently, acid and alkaline phosphatase activities of S. aureus were attributed to two different enzymes (Shah and Blobel, 1967). The authors described the formation of a repressible (by inorganic phosphorus) alkaline phosphatase when 18 strains were grown in 0.35% acid-hydrolyzed casein. Alkaline phosphatase was measured at pH 9.1 and acid phosphatase at pH 5.6. The cells demonstrated no detectable alkaline phosphatase activity until the phosphate level in the medium was below 1 ug/ml and the rate of growth decreased. However, acid phosphatase production was not influenced by the different levels of inorganic phosphorus in the medium; thus, acid phosphatase was not repressible under these conditions. A conflicting report by Kuo and Blumenthal (1961) asserted that inorganic phosphate had no inhibitory effect on the synthesis of either acid or alkaline phosphatase in any of the 17 coagulase-negative and 3 coagulase-positive strains of S. aureus which they studied. Abramson (1967) reported the presence of another phosphatase in S. aureus which was active at pH 7.2. He separated this esterase activity from other staphylococcal enzymes by gel filtration and by agar-gel electrophoresis.

Though the acid phosphatase activity of S. aureus has often been reported in the literature, this writer is aware of only one report (Barnes and Morris, 1957) in which the kinetics of enzyme production and activity were studied. Even this report suffers since all investigations were performed on enzyme preparations consisting of whole cells.

Pyrophosphatase activity has also been observed in some strains of S. aureus (Swartz and Merselis, 1962). Enzymatic activity was observed only in disrupted cells, not in the culture medium or whole cells. The enzyme was heat activated and was active against the pyrophosphate bond in nicotinamide adenine dinucleotide (NAD) at pH 9.5. However, it was unable to cleave the pyrophosphate of nicotinamide adenine dinucleotide phosphate (NADP). Nygren, Hoborn, and Wahlen (1966) observed a phospholipase activity of S. aureus which hydrolyzed the substrate lecithin.

Phosphatase activity has been found in numerous microorganisms, and there is notably extensive literature on the study of these enzymes in Escherichia coli. Torriani (1960) observed that neither acid nor alkaline phosphatase in E. coli was secreted into the culture medium. However, the enzymes were freely accessible in vivo to the substrates present in the surrounding medium. She demonstrated that whereas acid phosphatase was a constitutive enzyme, alkaline phosphatase was formed only when inorganic phosphorus in the medium became limiting.

Pradhan, Rege, and Sreenivasan (1962) attributed the repression of alkaline phosphatase by inorganic phosphorus to negative feed-back control. This mechanism was an important factor in the economy of organic phosphate reserves in the organism. Hofsten (1961) noted when succinic acid or glycerol served as carbon source for growth of E. coli, high levels of acid phosphatase were effected; although carbohydrates had a repressive effect, inorganic phosphorus in the growth medium had no adverse effect. Recently, Dvorak, Brockman, and Heppel (1967) reported that acid hexose phosphatase in E. coli was subject to catabolite repression, and enzymatic activity was significantly reduced by glucose, glucose 6-phosphate, glycerol, and glycerophosphate.

The phosphatase activity of other enteric bacteria has also been reported (Voros et al., 1961). Friedberg and Avigad (1967) noted the formation by Pseudomonas fluorescens of alkaline phosphatase activity which was induced by limiting quantities of inorganic phosphorus in the growth medium. Substrate specificity of the enzyme was similar to that of E. coli, but the enzyme was more sensitive to EDTA inhibition and was not inhibited by cyanide and various mercaptans. In Salmonella typhimurium, an organism more closely related to E. coli, three different phosphatases were observed in cell-free extracts. The criteria for differentiation of these enzymes were: pH optimum for activity; differences in inhibitory action by inorganic phosphorus and fluoride; and variations in the hydrolytic rates of several phosphorylated

substrates (Carrillo-Castaneda and Ortega, 1967). The acid phosphatases (pH 4.0 and 5.5) were similar to the acid phosphatase of E. coli. However, the alkaline phosphatase was quite different since it was non-repressible by inorganic phosphate, dependent upon the carbon source used by growing cells, and unstable to heating.

Efforts to relate the virulent nature of S. aureus to its enzymatic properties and phage-typing pattern have been made (DeWaart et al., 1963). Phage group I demonstrated high values for oxygen uptake and phosphatase activity, but low values for extracellular protein (Solomon and San Clemente, 1963). In all strains tested, coagulase activity, phosphatase activity, and mannitol fermentation occurred together or not at all. However, Pan and Blumenthal (1961) found no correlation between acid phosphatase activity and coagulase production by this organism. Nonetheless, they did imply that strains susceptible to group I phages produced more acid phosphatase than those belonging to the other phage groups. Interestingly, almost half the strains isolated in hospitals belonged to phage group I, and a very high percentage of these strains were antibiotic resistant. Two years later, the same investigators (Blumenthal and Pan, 1963) studying numerous strains of S. aureus belonging to phage group I, found that a larger percentage of cells lysed by phage 80 produced more acid phosphatase than strains not lysed by the same phage. Fodor, Rozgonyi, and Csepke (1963) noted that phage-propagating strain 80/81, which is regarded

as the most widely spread epidemic type, had characteristically low coagulase and hyaluronidase activities, but high phosphatase activity. In fact, phage group I strains on the whole demonstrated high phosphatase activity and low coagulase and hyaluronidase activities. On the other hand, strains belonging to phage group II showed high coagulase and hyaluronidase activities, but moderate phosphatase activity. Phage group III strains produced small amounts of phosphatase and moderate amounts of coagulase and hyaluronidase.

Elution of Enzymes from Microorganisms

Schwimmer and Pardee (1953) proposed two general methods for mildly extracting enzymes from bacteria: treatment with a dilute salt solution and selective extraction with a solution in which few enzymes are soluble. In fact, Neu and Heppel (1965) noted that a good first step in an enzyme purification procedure is the selective removal of enzymatic protein from the cells. Certain enzymes of E. coli were released by suspending the cells in cold water, following treatment with a solution of tris (hydroxymethyl) aminomethane (Tris) and ethylenediaminetetraacetic acid (EDTA) in hypertonic sucrose. "Osmotic shock" was the name given to this procedure. This sudden osmotic transition released the following enzymes: acid phosphatase, alkaline phosphatase, a cyclic phosphodiesterase, and 5'-nucleotidase. These were not extracellular enzymes since they were not found in the

medium during any phase of the growth cycle. Nossal and Heppel (1966) reported that during this elution procedure, only 4% of the total cellular protein was released. Later, Heppel (1967) noted that the enzymes which were released by "osmotic shock" were confined to a region between the bacterial cell wall and the cytoplasmic membrane. Dvorak et al. (1967) took advantage of the selective release of enzymes and used the procedure as an initial step in purifying acid phosphatase of E. coli.

Coles and Gross (1967) observed the liberation of penicillinase from S. aureus with various anions, including polyanions. They attributed this release of enzyme to an ionic exchange between penicillinase and anions. In fact, Cutinelli and Galdiero (1967) observed the ionic-binding properties of the surface of S. aureus. They demonstrated that divalent cations were bound more readily than monovalent ions and concluded that the cell wall of S. aureus behaved like a weak ion-exchange resin. In our laboratory, elution of acid phosphatase from S. aureus was a function of pH and ionic strength, and we concluded that the association of the enzyme with the cell surface resulted from electrostatic interaction (Malveaux and San Clemente, 1967). Takeda and Tsugita (1967), also employing ionic elution, effected the release of alkaline phosphatase with Mg^{++} from cellular debris of Bacillus subtilis. This step alone led to a very substantial increase in the specific activity of the preparation. They noted further that high salt concentration was required for dissolution of the eluted enzyme.

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In an extensive study of the release of acid phosphatase from yeast cells, Weimberg and Orton (1964) employed various methods for elution of the enzyme. Such procedures included sonic oscillation, grinding with alumina and glass beads, protoplast formation, treatment with mercaptans, and ionic elution with KCl. Acid phosphatase was eluted from resting cells of Saccharomyces mellis by 0.5 M KCl, but the enzyme was not eluted at higher salt concentrations (2.0 M) unless a thiol was included (Weimberg and Orton, 1965). The same investigators (Weimberg and Orton, 1966) later reported that from cells of Saccharomyces fragilis the enzyme was dissociated with KCl and mercaptoethanol. However, conditions for quantitative elution of the enzyme from this organism were not found. Maximal enzymatic release occurred during the stationary phase of growth, and a relatively inactive form of the enzyme was liberated.

Purification and Properties of Bacterial Phosphatases

The problems encountered in purifying phosphatases are similar to those experienced in most procedures for protein purification. There is no ideal method of protein purification; however, there are certain fundamental principles upon which practically all fractionation methods are based (Clark, 1964). Some factors which must be carefully considered include solubility properties, adsorptive properties, heat denaturation, and protein-nucleic acid complexes. The most important characteristics of a protein are the number and

arrangement of the molecular charges which are subsequently dependent upon pH, temperature, composition of solvent, and the character of the proteins present (Green and Hughes, 1955). Moreover, successful purification of a protein is often dependent on the rapidity of the different fractionation steps (Bjork, 1961). Chromatographic procedures are notably convenient, especially if the protein to be purified is eluted at an early stage of the experiment.

Some bacterial acid phosphatases have eluded purification and subsequent characterization. Hofsten and Porath (1962) reported that precipitation procedures were of limited efficiency for purification of acid phosphatase produced by E. coli. Not only were their fractionations incomplete, but the precipitated material resisted dissolution in water or weak buffer. The enzyme also appeared extremely sensitive to surface inactivation, and it was unstable in dilute solutions. Nevertheless, the acid phosphatase of E. coli was partially purified by first being passed through a DEAE-Sephadex column equilibrated at pH 8.2. Further purification was achieved by means of SE-Sephadex, zone electrophoresis, and gel filtration through Sephadex G-75. The product was purified 200-fold, and it exhibited a low specific activity and a low turnover number because of partial denaturation during the extraction and purification procedures. When the enzyme was characterized by ultracentrifugation, rapid polymerization occurred. By amino acid analysis the minimal molecular weight was estimated to be 13,000, and only one

enzyme peak was observed after starch-gel electrophoresis. The enzyme showed remarkable stability in 1 M acetic acid, but it was denatured in the presence of neutral salts. Dvorak et al. (1967) observed two acid phosphatase fractions which they isolated from the "osmotic-shock" fluid of E. coli. The two enzymatic activities included an acid hexose phosphatase and a nonspecific acid phosphatase. The latter enzyme hydrolyzed p-nitrophenyl phosphate more readily than any other substrate tested. It exhibited a pH optimum for activity near pH 5, and no striking effects by metal ions were observed. The enzyme resisted purification and was inhibited by 1×10^{-2} M EDTA. On the other hand, the acid hexose phosphatase was purified 870-fold by DEAE-cellulose and hydroxylapatite chromatography. Although ribose 5-phosphate was hydrolyzed at a substantial rate, the enzyme displayed a striking specificity for hexose phosphate esters. The pH optimum of activity was between pH 5.5 and 6.0, and the K_m for glucose 6-phosphate was 3.3×10^{-4} M. Metal ions had no effect on enzymatic activity, and the enzyme was fully active in the presence of 1×10^{-2} M EDTA. Purified product was homogenous on disc-gel electrophoresis, and the overall yield was 33%.

The alkaline phosphatase of E. coli has been studied more extensively and has been shown to be a zinc-containing enzyme (Plocke and Vallee, 1962). It was inhibited by EDTA and its activity was restored by addition of Zn^{++}. Cd^{++}, Co^{++}, Pb^{++}, and Cu^{++} inhibited the enzyme by displacement

of the native Zn^{++}. Reynolds and Schlesinger (1967) demonstrated that the alkaline phosphatase of E. coli consisted of two protein subunits which existed as extended coils in aqueous solution at pH 2, as globular species at pH 4, and as compact macromolecules at pH 6 to 8. This last conformational state had the same helical configuration as the native enzyme which required Zn^{++} for its dimerization and activity.

Two phosphodiesterases of B. subtilis which had their maximal activity at pH 7.3 were purified and characterized (Taniguchi and Tsugita, 1966). Both enzymes were activated by Co^{++} and inactivated by inorganic phosphorus. The enzymes, however, were structurally unrelated. Takeda and Tsugita (1967) purified an additional phosphatase from the same organism. This enzyme was optimally active at pH 10.5, and its activity which was inhibited by EDTA was restored far more effectively by Co^{++} than by Zn^{++}. Thus, the enzyme differed considerably from alkaline phosphatase of E. coli.

The alkaline phosphatase of Pseudomonas fluorescens demonstrated a substrate specificity similar to that of the alkaline phosphatase of E. coli (Friedberg and Avigad, 1967). However, the enzymes were different since the former was several hundred-fold more sensitive to EDTA and was not inhibited by cyanide or various mercaptans.

Localization of Enzymes in Microorganisms

The integral relationship between cytology and enzymatic functions has proved to be a meaningful one in many types of

cells (Marr, 1960). Most of the studies involving localization of enzymes in living systems has been done in large animal cells. As Marr (1960) pointed out, due to the small size of bacterial cells, localization of enzymes in these cells has required some ingenuity in biochemical techniques.

Pollock (1962) placed the enzymes of bacteria into two main categories: extracellular enzymes which are found in the culture medium and cell-bound enzymes which are either intracellular or surface bound. He stated further that an enzyme may be partially cell-bound and partially extracellular for the same cell; but production of extracellular enzymes is largely confined to gram-positive organisms. Salton (1967) noted that bacterial membranes contain a wide variety of enzymatically active proteins or enzyme complexes. Pollock (1962) reported that formation of exoenzymes frequently occurred at the surface of the cell, outside the cell membrane. In this case the last step in protein synthesis took place at the cell surface. His proposed mechanism for such an hypothesis was that amino acids had to reach a specific enzyme-forming template situated on the cell membrane either by diffusion from inside the cell or by direct contact from the medium. How the mRNA reached this cytological area was not disclosed. Another mechanism of enzyme release suggested by the same investigator was an enzymatic process in which cell-bound "autolysin" was implicated in the liberation of exocellular enzymes. In this case the "autolysin", possessing lysozyme-like properties, acted upon the cell surface and

released enzymatic proteins. Rogers (1956) had earlier suggested that extracellular proteins of S. aureus might be extruded as "capsular-like" material that dissolved from the cell surface into the medium.

Marr (1960) suggested two general methods for localizing enzymes in bacteria. These included direct cytochemistry which had limited use for bacteria and analytical morphology which involved cellular disruption. The first of these methods has coupled electron microscopy with differential staining (Voelz, 1964, Done et al., 1965, and Baillie et al., 1967). Indirect approaches to enzyme localization have also proved beneficial. As described previously, Neu and Heppel (1964) liberated certain enzymes from E. coli by a wash procedure which they called "osmotic shock". This result led them to believe the eluted enzymes were located at the cell surface. Shugart and Beck (1966) found a similar location for a proteinase which was liberated from the surface of Streptococcus faecalis by sonic treatment of the cells. Murti (1960) emphasized that controlled lysis by the combined action of metal-complexing agents and lysozyme could yield preparations suitable for localization and fractionation of enzymes. Following protoplast formation of the cells, the osmotically fragile bodies were lysed, and the lysate fractions prepared by differential centrifugation were analyzed for enzymatic activity. By this method, three major fractions were obtained: cell wall and/or lysozyme-solubilized material; "protoplast" membrane; and intracellular contents.

The method has found application for localizing both enzymes and certain pigments (Bose and Gest, 1965, Raza Nasir and Murti, 1965, and Salton and Ethisham-Ud-Din, 1965).

As indicated above, the method of localizing enzymes in bacteria as described by Murti (1960) was dependent upon the formation of protoplasts. Mandelstam and Strominger (1961) and Virgilio et al. (1966) noted that the cell wall of S. aureus was insensitive to lysozyme. Recently, however, many enzymes have been demonstrated to lyse viable cells of S. aureus (Burke and Pattee, 1967). These enzymes have been extracted from many microbial sources, including Pseudomonas (Burke and Pattee, 1967), Aeromonas (Coles and Gilbo, (1967), Streptomyces (Ghuysen et al., 1965), Flavobacterium (Kato et al., 1962), Chalaropsis (Hash, 1963), and even Staphylococcus (Schindler and Schuhardt, 1964).

Mitchell and Moyle (1957) were the first to note protoplast formation of S. aureus. The osmotically fragile bodies formed by an autolysin system were permeable to glycerol, but not to NaCl or sucrose. Hash, Wishnick, and Miller (1964) described a procedure for protoplast formation of S. aureus using a fungal N-acetylhexosaminidase. The protoplasts were stabilized in 0.5 M sucrose and were free of amino sugars and rigid cell walls. Recently, Schuhardt et al. (1967) reported that protoplasts of S. aureus were effected with 30% NaCl and lysostaphin. The enzyme lysostaphin was described first by Schindler and Schuhardt (1964), purified and characterized in part by the same investigators

(Schindler and Schuhardt, 1965), and characterized further by Browder et al. (1965). The staphylolytic agent was described as a peptidase which liberated N-terminal glycine and alanine from the cell wall of S. aureus. Lysostaphin displayed maximal activity at pH 7.5 and had a molecular weight of 30,000.

Apparently, the enzymes of bacteria, as those of higher cells, are confined to discrete compartments at the subcellular level (Murti, 1960). Cedar and Schwartz (1967) noted that all of the bacterial enzymes so far reported to be situated in the periplasmic region were degradative; otherwise, such enzymes could cause cellular destruction. Malamy and Horecker (1961) introduced the term "periplasm" to describe the vague region between the cytoplasmic membrane and cell wall of bacterial cells. They concluded that the alkaline phosphatase of E. coli existed outside the cell membrane since the enzyme was liberated quantitatively into the surrounding medium when the cells were converted to protoplasts. Hofsten (1961) noted a similar location for the acid phosphatase of the same organism. Later, Neu and Heppel (1964) came to the same conclusion since most of the enzyme was liberated during "osmotic shock." More recently, Kushnarev and Smirnova (1966), using the electron microscope, reported that alkaline phosphatase of E. coli B was located in the exterior layer of the cell wall. Kidwai and Murti (1965) noted that other enzymes were present in the plasma membrane of E. coli. By controlled lysis following

spheroplast formation, they obtained succinic dehydrogenase, lactic dehydrogenase, and cytochrome b_5 oxidoreductase in the membrane fraction; but the enzymes could not be solubilized by conventional methods. In addition, Cedar and Schwartz (1967) were able to detect asparaginase activity in the periplasmic region of E. coli. Contrasted to the alkaline phosphatase of E. coli, Takeda and Tsugita (1967) found that the same enzyme in B. subtilis is more firmly bound to cellular fragments. When these cells were disrupted with lysozyme and then centrifuged, almost all alkaline phosphatase activity was found in the cell debris fraction.

The adenosine triphosphatase activity of Mycoplasma was found tightly bound to the membrane fraction by various investigators using different methods (Pollack, Razin, and Cleverdon, 1965, Rottem and Razin, 1966, and Munkres and Wachtel, 1967). The same enzyme was present in the cytoplasmic membrane of Streptococcus faecalis. Adenosine triphosphatase was associated with the membrane via linkages with Mg^{++}, or similar cations, and enzyme elution depended on the removal of binding cations.

Phosphatase activity of yeast cells has also been localized in numerous laboratories. Since ionic compounds were required for enzyme elution, Weimberg and Orton (1965) concluded that acid phosphatase of Saccharomyces mellis was tightly bound to the cell wall by electrostatic interaction. They also noted that sulfide bonds were involved in some manner in the association. In Baker's yeast, acid phosphatase

was located at the cell surface and two alkaline phosphatases were located wholly inside the cell membrane (Suomalainen, Linko, and Oura, 1960 and Suomalainen, Nurminen, and Oura, 1967). Studying the same organism, Tonino and Steyn-Parve (1963) made similar observations.

Mitchell and Moyle (1956) noted that part of the protein of the plasma membrane of S. aureus consisted of enzymes, the active centers of which were probably in contact with the surrounding growth medium. At least 90% of the cytochrome system of this organism was associated with the membrane. Other activities found in the plasma membrane included succinic dehydrogenase, lactic dehydrogenase, malic dehydrogenase, formic dehydrogenase, α-glycerophosphate dehydrogenase, and glucose 6-phosphate dehydrogenase. Most of the acid phosphatase and a small amount of glucose 6-phosphatase were also membrane-associated (Mitchell, 1959). Elek (1959) implied that phosphatase of S. aureus was an intracellular enzyme and was not detectable in the growth medium.

Studying the coagulase activity of S. aureus, Dutie (1954) described two types, one free in the culture medium and the other bound to the cells. The free, but not bound coagulase, was destroyed at 56 C after 4 min, and the enzymes were found to be antigenically distinct. On the other hand, Coles and Gross (1967) found free and bound penicillinase activity of S. aureus attributable to the same enzyme. The active protein was ionically bound to the cell wall and could be liberated by organic anions. However, release was not due

solely to ionic elution, but rather to anionic enzyme activation. These investigators concluded penicillinase was probably liberated from mesosomes.

Role of Phosphatase in Microorganisms

Phosphatases are essential to microorganisms because they hydrolyze phosphate esters or can transfer phosphates from one organic group to another (Kuenzler and Perras, 1965). Production of phosphatases acting on extracellular substrates may give a competitive advantage to such cells. Heppel (1967) pointed out that the proteins at the cell surface may bind and transport substances in the medium across the cell membrane.

Since alkaline phosphatase was repressed by inorganic phosphate in the medium, Hofsten (1961) stated that the role of the enzyme was to cleave various phosphate esters when the cells were starved of inorganic phosphate at a neutral or alkaline pH. On the other hand, acid phosphatase was present in the cells under all conditions of growth and was probably used for growth on hexose phosphates at an acid pH. The possibility of a more anabolic function for the enzyme was not eliminated. In a later publication, Hofsten and Porath (1962) suggested additionally that acid phosphatase may regulate permeability of the cell. Fraenkel and Horecker (1965) stated that the function of acid phosphatase in *E. coli* was unknown. However, they suggested it might be involved in providing inorganic phosphorus and degrading

exogenous nucleic acids. The enzyme was not assigned a role in known intermediary metabolism, and there was no clear example of a phosphorylated compound whose utilization required acid phosphatase. Dvorak et al. (1967) reported that acid phosphatase on the surface of E. coli probably actively degraded organic phosphates in the medium.

In the case of yeast cells, Kuo and Blumenthal (1961) reported that acid phosphatase regulated the level of inorganic phosphorus in the cell. However, Pauwels (1964) demonstrated that in yeast cells the enzyme had no direct function in phosphate absorption. He asserted that glycolysis was the principal process to which phosphate uptake was coupled, and if phosphatase played a role in phosphate absorption, the enzyme was not limiting in the overall process.

Abrams (1965) clearly demonstrated that membrane-localized adenosine triphosphatase in Streptococcus faecalis regulated permeability of the plasma membrane to extracellular solutes. Adenosine triphosphate, generated during glycolysis, interacted with the membrane-bound enzyme and subsequently induced conformational changes advantageous to increased permeability.

Kedzia et al. (1966) implied that acid phosphatase of S. aureus is related to "some regulatory mechanisms for inorganic phosphate pool concentration" inside the cell. They stated further that the enzyme probably played an important role in the penetration of certain phosphorylated compounds into the cell. Mitchell (1957) described a

"translocase" mechanism for moving inorganic phosphate across the plasma membrane of S. aureus.

The enzymatic activities of certain bacteria have in some cases been related to the organism's ability to cause disease (Nygren et al., 1966). Virulence of S. aureus and coagulase activity have been shown to be related (Elek, 1959). Similarly, the production of acid phosphatase by this organism has sometimes been linked with virulence in a given host. Kedzia et al. (1966) found that strains of S. aureus producing septecemia, pyemia, and boils possessed 3 to 5 times more phosphatase activity than organisms found in healthy carriers. Likewise, Fodor et al. (1963) observed phosphatase activity was especially high in phage group I which contained most virulent organisms. They pointed out, however, phosphatase production could not be regarded as a sole indicator of virulence since virulence was the end-product of many metabolic processes; and thus, the disease-causing characteristics of the organism could not be attributed to the activity of only one enzyme.

MATERIALS AND METHODS

Cultures and Their Maintenance

To screen for acid phosphatase activity, a total of 30 phage-propagating strains of Staphylococcus aureus were used, 25 from the International-Blair series (Blair and Carr, 1960) and 5 from the Seto-Wilson group (Seto and Wilson, 1958). Propagating strain (PS) 3A was used during the early studies on production and elution of enzyme, and PS 55 was used in subsequent experiments. Stock cultures were maintained on Trypticase Soy Agar[1] slants at 4 C and were transferred every six weeks.

Production of Acid Phosphatase

Media. Acid phosphatase screening was performed on cultures grown in Brain Heart Infusion[2]. Cells for studying kinetics of enzyme production were cultivated in Trypticase Soy Broth[1] and in the casein acid-hydrolysate medium of Stutzenberger, San Clemente, and Vadehra (1966) with the following modifications: 0.05% glycerophosphate was substituted for the inorganic phosphate salts, and 0.05 M tris

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[1]Baltimore Biological Laboratories, Baltimore, Md.

[2]Difco Laboratories, Detroit, Mich.

(hydroxymethyl) aminomethane (Tris)-chloride (pH 7.5) was used to buffer the medium. Cells for the remaining studies were cultivated routinely in Trypticase Soy Broth.

Cultural conditions. Tube cultures containing 15 ml of Brain Heart Infusion were used to screen acid phosphatase activity. For other experiments, 3% log-phase shake cultures served as inocula and were added to either Trypticase Soy Broth or the casein acid-hydrolysate medium. Test cultures were shaken at 37 C on a rotary shaker at approximately 120 cycles/min.

Quantitative and Qualitative Methods

Acid phosphatase activity was measured routinely by a method modified from Barnes and Morris (1957) using p-nitrophenyl phosphate, disodium salt[3] as substrate buffered at pH 5.2 with acetate. When adequate controls were used, it was not necessary to eliminate residual color (due to the presence of p-nitrophenol) by means of HCl. In studies of enzyme kinetics and utilization of different substrates, the reaction was stopped with 1 ml of 10% trichloroacetic acid (TCA), and liberated orthophosphate was measured colorimetrically according to the method of Fiske and SubbaRow (1925).

Protein determinations were made according to the method of Lowry, et al. (1951), using crystallized, bovine Fraction V albumin as protein standard. When salt concentrations

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[3]Mann Research Laboratories, Inc., New York.

prohibited use of this colorimetric technique, protein measurements were made spectrophotometrically according to the method of Warburg and Christian (1957). Residual glucose in the growth medium during acid phosphatase production in the semidefined medium was measured by the method of Noelting and Bernfeld (1948).

Numerous techniques were employed to detect the presence or absence of associated staphylococcal products during the purification of acid phosphatase. Coagulase was measured by the titer method of Tager and Hales (1948) at 37 C. Deoxyribonuclease activity was analyzed by placing 0.2 ml of each purification fraction inside separate wells cut into DNase Test Agar[2]. Treated plates of agar were incubated at 37 C for 12 hr and then flooded with 1 N HCl. Hemolysins were detected by streaking 0.2 ml of each purification fraction onto sheep blood and human blood agar plates. The plates were incubated for 8 hr at 37 C and observed for hemolysin activity. Lipase activity was assayed according to the method of Nachlas and Seligman (1949) using p-nitrophenyl palmitate[4] as substrate. The reaction occurred in veronal buffer (pH 7.4) at 40 C. Following a 1 hr incubation period, samples were assayed for chromogenic product. Fibrinolysin activity was analyzed after 12 hr at 37 C on fibrin plates which were made by adding citrated bovine fibrinogen[4] to warm agar. Presence of carbohydrate was detected by the

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[4]Sigma Chemical Co., St. Louis, Mo.

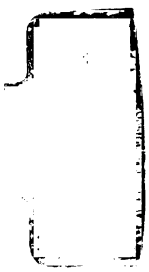
Molish test (Clark, 1964) and of nucleic acid by the method of Warburg and Christian (1957).

Acid Phosphatase Screening

Acid phosphatase screening was performed on 24-hr cultures (stationary) of S. aureus grown in Brain Heart Infusion at 37 C. Aliquots of each culture were adjusted to an optical density of 0.5, and enzyme activity was measured in the presence of 0.01% thimerosal. The remaining cells were harvested by centrifugation. Depending upon the ease of dissociation from the cells, three forms of acid phosphatase were identified. Acid phosphatase detached from the cells and dissolved in the cell-free supernatant fluid was designated "free" acid phosphatase. The sedimented cells were washed six times by resuspension in 0.15 M NaCl solution and subsequent recentrifugation. The total enzyme in the combined washings was termed "loosely bound." Enzyme activity in the sixth washing was not measurable. The designation "firmly bound" was reserved for residual activity still associated with the cells after the salt-washing series.

Enzyme Production in Undefined (Trypticase Soy Broth) and Semidefined Media

The rate of whole culture and free acid phosphatase production and cell density (read at 625 mu) were determined during shake cultivation at 37 C in a one-liter quantity of cells cultured in Trypticase Soy Broth. These same parameters



were measured in cultures grown in the casein acid-hydrolysate medium with and without added glycerophosphate. In the latter case, the rate of glucose utilization was also determined.

Localization of Acid Phosphatase

Effect of lysostaphin[5] on S. aureus PS 55. Early stationary-phase cells were harvested by centrifugation and washed once in 10 ml of 0.05 M Tris-chloride (pH 7.5) buffer containing 0.15 M NaCl. The cells were resuspended in the wash solution to effect an absorbancy of 1.0 (10% light transmission) at 610 mu in the Spectronic 20 colorimeter[6]. Cells were stored in crushed ice until ready for use. Stock solutions of lysostaphin which were prepared daily consisted of 5 units/ml of chilled 0.05 M Tris-chloride buffer (pH 7.5) containing 0.15 M NaCl. A series of lysostaphin solutions were then prepared in triplicate (optically matched tubes) from the stock solution to contain 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, and 0.6 unit/tube. Chilled buffer was then added to bring all tubes to a final volume of 5 ml. Control tubes containing no lysostaphin were included. To each tube there was added 1 ml of the cell suspension prepared above, and the absorbancy (O.D.) was read at zero time. All assay tubes were incubated in a 37 C water bath for exactly 10 min and then chilled quickly in an ice-water bath to stop the reaction.

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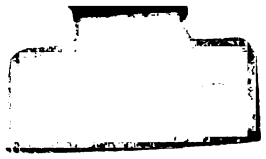
[5]Mead Johnson and Company, Evansville, Ind.

[6]Bausch and Lomb Optical Company, Rochester, N. Y.



Optical density at 610 mu was read and percent reduction in absorbancy for each tube was determined.

Polyethylene glycol (PEG)[7] as stabilizing agent of spheroplasts. The stability of S. aureus PS 55 in the presence of lysostaphin was tested with polyethylene glycol (Carbowax 4000) and without the stabilizing agent. Assay and control tubes were prepared in triplicate. The stabilized system contained 0.5 ml of the cell suspension, 0.4 ml lysostaphin (10 units/ml), 1.1 ml Tris-NaCl (pH 7.5), and 2.0 ml PEG. Final concentration of PEG ranged from 7.5% to 40%. In the lytic system, PEG was replaced by buffer-salt solution. To test the effect of PEG alone on cells, tubes were prepared to contain 0.5 ml cells, 2.0 ml PEG, and 1.5 ml Tris-NaCl. Another set of control tubes containing 0.5 ml cells and 3.5 ml buffer-salt solution was included to observe the effect of Tris-NaCl alone on the cells. Blank tubes contained the stabilizing agent whenever necessary. Each tube was incubated in a 37 C water bath and absorbancy was measured at 610 mu at given intervals of time.

Evidence for formation of spheroplasts. Two different methods were employed to show that the spheroplasts were osmotically fragile. In the first method, ultraviolet-absorption spectra (220-310 mu) of supernatant fluids (7000 x g for 20 min) of intact cells, spheroplasts stabilized in 30% PEG, lysed cells, and lysed spheroplasts were determined in a

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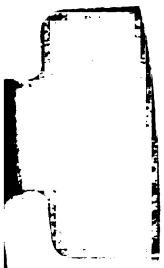
[7]Union Carbide, New York.



Beckman Spectrophotometer (Model DU)[8]. In the second method, spheroplasts and intact cells were harvested by centrifugation at 7000 x g for 20 min and were resuspended in an equal volume of distilled water. The extent of lysis was noted by determining absorbancy at 610 mu before and after the samples were suspended in water.

Localization of the enzyme by an indirect method. Early stationary-phase cells of S. aureus PS 55 were converted to spheroplasts as described above. The osmotically fragile bodies were removed by centrifugation (7000 x g for 10 min) and were disrupted by resuspension in distilled water. Insoluble material was then concentrated by centrifugation at 20,000 x g for 20 min. Intact cells which were suspended in 30% PEG or Tris-NaCl (pH 7.5) and which were not treated with lysostaphin served as controls. Three cellular fractions were assayed for activity: lysostaphin-solubilized material and/or material eluted from intact cells; intracellular contents of spheroplasts or wash solutions of intact cells; and intact cells or membrane fractions of lysed spheroplasts.

Purification of Acid Phosphatase

Crude enzyme was prepared in cells of S. aureus PS 55 cultured in 16 liters of Trypticase Soy Broth at 37 C on a rotary shaker for 16-20 hr. Enzymatic activity of the whole culture was determined by assaying an appropriately diluted

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[8]Beckman Instruments, Inc., Fullerton, Calif.

aliquot of the culture. To determine protein content of the cells, a 100-ml aliquot of culture was centrifuged, and cellular protein was extracted with 10 ml of 2 N NaOH according to the method of Mitruka et al. (1967). Insoluble material was removed by centrifugation and protein content of the supernatant fluid was determined by the method of Lowry et al. (1951). Dry weight measurements of the culture were also made.

Elution of loosely bound phosphatase. To determine the optimal pH range of elution, three samples (20 ml each) of actively dividing cells grown in Trypticase Soy Broth were centrifuged and resuspended in equal volumes of 0.1 M buffer solutions at pH 5.3 (acetate), 7.0, and 8.5 (Tris-chloride). The suspensions at each pH value were incubated at 37 C and at 25 C for 2 hr, and the cells were harvested by centrifugation. Cells were washed with 0.15 M NaCl and resuspended in the same salt solution, and all fractions were assayed for enzymatic activity.

The effect of ionic strength on elution of acid phosphatase from cells was also determined. Washed log-phase cells grown in Trypticase Soy Broth and stationary-phase cells grown in the casein acid-hydrolysate medium were suspended in solutions varying from 0.1 to 2.0 M KCl at pH 7.5 for 60 min at 25 C. The cells were concentrated by centrifugation and resuspended in water after further washings. Cell-free culture medium was assayed for free (extracellular) enzyme. Loosely bound enzymatic activity was determined in the salt

and wash solutions, and the cells suspended in water were analyzed for the firmly bound fraction of acid phosphatase. Dry weight and viable count determinations were made on the whole culture and on the final cell suspensions in water to correct for any loss of cells during the eluting process.

In routine purification experiments cells (PS 55) were harvested from a 16-liter culture at 25 C in a Servall centrifuge (Type SS-1) equipped with the "Szent-Gyorgyi and Blum" continuous flow system[9]. The cells were washed once with about 400 ml of 0.1 M KCl in 0.05 M Tris-chloride (pH 8.5), centrifuged, and resuspended in the same volume of 1.0 M KCl in 0.5 M Tris-chloride (pH 8.5). Elution was effected by gentle agitation on a reciprocal shaker at 25 C for 60 min.

Dialysis of the eluted fraction. The cells were separated from the eluted material by centrifugation. The supernatant fluid then was placed in a cellulose dialyzing membrane[10] (previously boiled for 5 min in distilled water), and it was dialyzed against 10 volumes of 0.01 M Tris-chloride (pH 8.5) for 12 hr at 4 C. The precipitate formed during dialysis (D-I-P) was sedimented in a Sorvall refrigerated centrifuge (Model RC-2)[9] at 4 C, redissolved in 1.0 M KCl-0.5 M Tris-chloride (pH 8.5) solution, and was redialyzed under the conditions described above.

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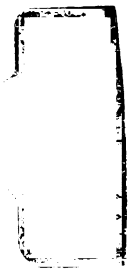
[9]Ivan Sorvall, Inc., Norwalk, Conn.

[10]Arthur H. Thomas Co., Philadelphia, Pa.



Gel filtration. The precipitate formed after the second dialysis (D-II-P) was again dissolved in 1.0 M KCl-0.5 M Tris-chloride solution. This same solvent was used to equilibrate a 2.5 x 38 cm column of Sephadex G-100[11]. Void volume of the chromatographic column was determined with Blue Dextran 2000[11] at 5 C. A small quantity of sucrose was added to the protein sample (4.5 ml) which then was layered on the column with a syringe. The applied sample was eluted with the same solvent, and 4.3 ml fractions were collected on a Model No. 85001 fraction collector[12]. Each tube was scanned at 280 and 260 mu in the Beckman Du spectrophotometer to determine its protein content. These same fractions were individually assayed for acid phosphatase activity using p-nitrophenyl phosphate as substrate. Certain tubes containing maximal enzymatic activity were combined and dialyzed against 0.01 M Tris-chloride (pH 8.5) for 12 hr at 4 C. The precipitated material (S-I-P) was redissolved in the salt-buffer solvent; sucrose was added; and the sample was rechromatographed as previously described. The collected fractions again were assayed for protein and enzymatic activities, and certain tubes with maximal enzymatic activity were combined. All characterizations were performed on this purified product.

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[11]Pharmacia Fine Chemicals, Piscataway, N. J.

[12]California Corp. for Biochemical Research, Los Angeles, Calif.

Characterization of Acid Phosphatase

Homogeneity of purified enzyme. Electrophoretic homogeneity was determined by starch-block electrophoresis. Starch preparation and electrophoresis were performed according to the method of Campbell et al. (1963). Treated starch was poured into a plexiglass template, and excess buffer was removed with absorbent paper. The surface of the starch was leveled, and a sample well was made in the starch block 10 cm from the cathode end.

The plexiglass template containing the starch block then was placed across the bridge gap of a Universal apparatus[13]. Approximately 0.2 ml protein solution (2.3 mg/ml) was placed in the sample well which was promptly filled with starch. A current of 3 mA was applied across the starch block for 4 hr at 4 C. When electrophoresis was completed, the starch block was cut into 1 cm wide segments, each of which was eluted with 2 ml of cold 0.15 M NaCl. Each fraction was analyzed for protein and acid phosphatase.

Purified acid phosphatase was analyzed for homogeneity also in a Spinco model E analytical ultracentrifuge[14] by the sedimentation velocity method at 4 C. The sample (0.4 ml, 280/260 = 1.21) containing 3.5 mg protein/ml, was placed in a single sector S.B. cell[14] along with 0.1 ml solvent (0.6 M KCl in 0.1 M Tris-chloride, pH 8.5). The run was made at

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[13]Shandon Scientific Co., London, England.

[14]Beckman Instruments, Inc., Spinco Division, Palo Alto, Calif.

56,100 rpm for 80 min using an An-D analytical rotor[14]. The bar angle was 65°, and pictures were taken at certain time intervals. Calculations of $S_{20,w}$ were done according to the method of Schachman (1957).

Effect of pH on the activity of acid phosphatase. Enzymatic activity of purified product was analyzed between pH 4 and 10. The following buffers were employed in the assay system: 0.1 M acetate (pH 4.0-5.6); 0.1 M sodium arsenate-HCl (pH 6.5); 0.1 M Tris-chloride (pH 7.3-8.5); and 0.1 M glycine-NaOH (pH 9.4-10.0). All samples were incubated at 37 C for 30 min.

Effect of ionic strength on the activity of acid phosphatase. Water-soluble, purified enzyme was dialyzed against distilled water at 4 C for 48 hr. The effect of both KCl and NaCl on enzymatic activity was measured in a series of assay tubes in which the final salt concentration ranged from 0.01 M to 2.0 M. Buffer concentration in all cases was 1×10^{-3} M acetate. Two separate controls were employed: one at each salt concentration in which the reaction was stopped at zero time, and the other which contained no added salt.

Effect of temperature on the activity of acid phosphatase. Employing the same substrate concentration, the activity of a uniform amount of enzyme was measured at 25, 30, 37, 45, 52, and 60 C. At 4 C and 15 C enzyme was more concentrated, but the final readings were multiplied by appropriate dilution factors so that all results were comparable.

Initial velocity of purified enzyme. In these experiments, the phosphatase reaction at pH 5.2 was stopped by adding 1.0 ml of 10% trichloroacetic acid (TCA), and liberated orthophosphate from the substrate, p-nitrophenyl phosphate, was determined by the colorimetric method of Fiske and SubbaRow (1925). Initial velocities were determined by stopping the reaction with TCA at certain intervals of time and assaying for phosphatase activity. Each set of assay tubes contained a different concentration of substrate, ranging from 5×10^{-4} M to 1×10^{-2} M. Three separate sets of controls were employed. In the first control, enzymatic activity was stopped with TCA at zero time. In another, substrate was deleted from the assay system; and in the third control, the enzyme was omitted. The blank consisted of a mixture of buffer and TCA. The data were plotted by the methods of Lineweaver and Burk (1934) and Hofstee (1959). Determinations of K_m and V_{max} were made as suggested by Dixon and Webb (1964).

Effect of different compounds on acid phosphatase. The relative activity of acid phosphatase was determined in the presence of different compounds. Buffer concentration in all cases was 1×10^{-1} M acetate. In some cases different concentrations of the compounds were employed. In all assay systems, two controls were employed: in one the reaction was stopped at zero time, and in the other inhibitor was omitted.

Effect of different divalent cations on acid phosphatase. Relative activity of the enzyme which had been dialyzed against water was determined in the presence of various divalent cations. Final concentration of metal ions in the assay system was 1×10^{-3} M. The following metal ions (used as the chloride form) were employed: Co^{++}, Mn^{++}, Mg^{++}, Ca^{++}, Zn^{++}, Cu^{++}, Hg^{++}, Pb^{++}, and Ba^{++}. Controls consisted of assay tubes devoid of metal and those in which the reaction was terminated at zero time.

Heat stability of the enzyme. Enzymatic activity was measured in the presence and absence of Cu^{++} (1×10^{-3} M). In either case, the enzyme in 0.01 M Tris was heated to the desired temperature (30, 40, 50, 60, 70, 80, or 100 C), kept at this temperature for 5 min, and quickly cooled in ice water. Treated samples then were assayed at 37 C and pH 5.2. An unheated control permitted calculation of percent residual activity.

Stability of the enzyme under different conditions. Dialyzed enzyme was stored at 25 C in water, 1.0 M acetic acid, 1.0 N NaOH, and in buffer solutions (0.1 M and 1.0 M) at pH 5.2, 7.5, 8.5, and 9.5. At the end of 1 day, 3 days, and 6 days the stored samples were assayed for acid phosphatase activity at 37 C.

Activity of acid phosphatase with various substrates. Hydrolytic activity of acid phosphatase with various phosphorylated esters was determined at pH 5.2 and 37 C. Final concentration of each substrate (dissolved in water) in the

assay tubes was 1 x 10^{-2} M. After 30 min the reaction was terminated with 1 ml of 10% TCA, and liberated orthophosphate was determined colorimetrically by the method of Fiske and SubbaRow (1925). Three separate sets of controls were employed. In the first case, enzymatic activity against each substrate was stopped at zero time by adding TCA. In the second set of controls, substrate was deleted from the assay system; and in the third set, enzyme was omitted. Blank tubes contained a mixture of buffer and TCA. A value of 100 was arbitrarily assigned to the activity of acid phosphatase against p-nitrophenyl phosphate, and relative enzymatic activity against other substrates was designated accordingly.

RESULTS

Acid Phosphatase Screening

All phage-propagating strains of Staphylococcus aureus selected from the International-Blair and Seto-Wilson series showed acid phosphatase production after 24 hr of growth. Subsequently, when all samples were adjusted to an optical density value of 0.5, propagating-strain (PS) 3A surpassed all others in phosphatase activity (Fig. 1). High enzymatic activity was not confined to any particular phage-propagating group. Relative amounts of acid phosphatase in the free, loosely bound, and firmly bound fractions of the phage-propagating strains are shown in Fig. 2. Free phosphatase activity in spent culture medium ranged from 6% in PS 52A/79 to 60% of the total activity in PS 52, 3C, 6 and 73. Enzymatic activity in the loosely bound fraction was relatively higher and ranged from 25% in PS 7 to 82% in PS 70. Other propagating strains demonstrating high activity in this fraction included 3A, 55, 77, and 42D of the International-Blair series and S1 of the bovine-adapted series. In general, minimal acid phosphatase activity was associated with the firmly bound fraction of S. aureus, with no apparent activity in both PS 70 and PS 73 to 46% of the total activity in PS S4.

Fig. 1. Specific acid phosphatase activity of whole cultures of Staphylococcus aureus (samples adjusted to optical density of 0.5) cultivated in Brain Heart Infusion (BHI) for 24 hr under stationary conditions. Cultures were selected from the International-Blair and Seto-Wilson series of phage-propagating strains.

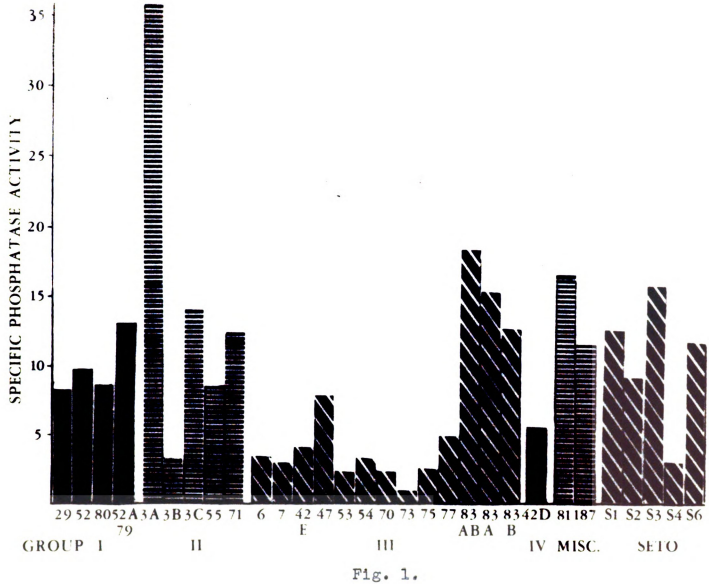
SPECIFIC PHOSPHATASE ACTIVITY
35
30
25
20
15
10
5
29 52 80 52A 3A 3B 3C 55 71 6 7 42E 47 53 54 70 73 75 77 83ABA 83B 83 42D 81 187 S1 S2 S3 S4 S6
79
GROUP I II III IV MISC. SETO

Fig. 1.

Fig. 2. Relative amounts of acid phosphatase in terms of free, loosely bound, and firmly bound fractions taken from BHI cultures of the International-Blair and Seto-Wilson series of phage-propagating strains of Staphylococcus aureus.

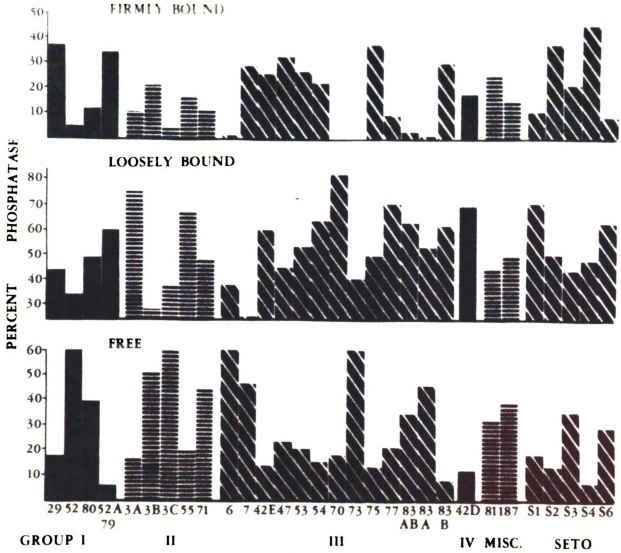
FIRMLY BOUND
LOOSELY BOUND
FREE
PERCENT PHOSPHATASE
50
40
30
20
10
80
70
60
50
40
30
60
50
40
30
20
10
29 52 80 52A 3A 3B 3C 55 71 6 7 42E 47 53 54 70 73 75 77 83A 83B 42D 81 187 S1 S2 S3 S4 S6
79
GROUP I
II
III
IV MISC.
SETO

Fig. 2.

Enzyme Production in Undefined and Semidefined Media

The rate of whole culture acid phosphatase production in Trypticase Soy Broth paralleled cell density of a shake culture of PS 3A (Fig. 3). However, free enzymatic activity was maximally obtained after 10 hr of growth and started to decline 16 hr later. In the casein acid-hydrolysate medium containing glycerophosphate, the rate of whole culture acid phosphatase production also increased with cell number (Fig. 4), but the total amount of phosphatase produced was about one-fifteenth of that elaborated in Trypticase Soy Broth (Fig. 3). However, 18 hr later enzyme production again increased after a period of lag. Free enzyme activity followed essentially the same pattern. In the absence of glycerophosphate, no substantial increase was noted in acid phosphatase production during the stationary phase of the growth curve (Fig. 5). Depletion of glucose coincided with the termination of exponential cell division.

Localization of Acid Phosphatase

Effect of lysostaphin on S. aureus PS 55. Figure 6 illustrates a plot of lysis (percent reduction in absorbancy at 610 mu) of intact cells against lysostaphin concentration. Not more than 90% reduction in absorbancy was noted at any concentration of the lytic agent. As little as 0.05 unit of lysostaphin/ml was sufficient to effect maximal lysis. A unit of lysostaphin (as defined by the commercial supplier)

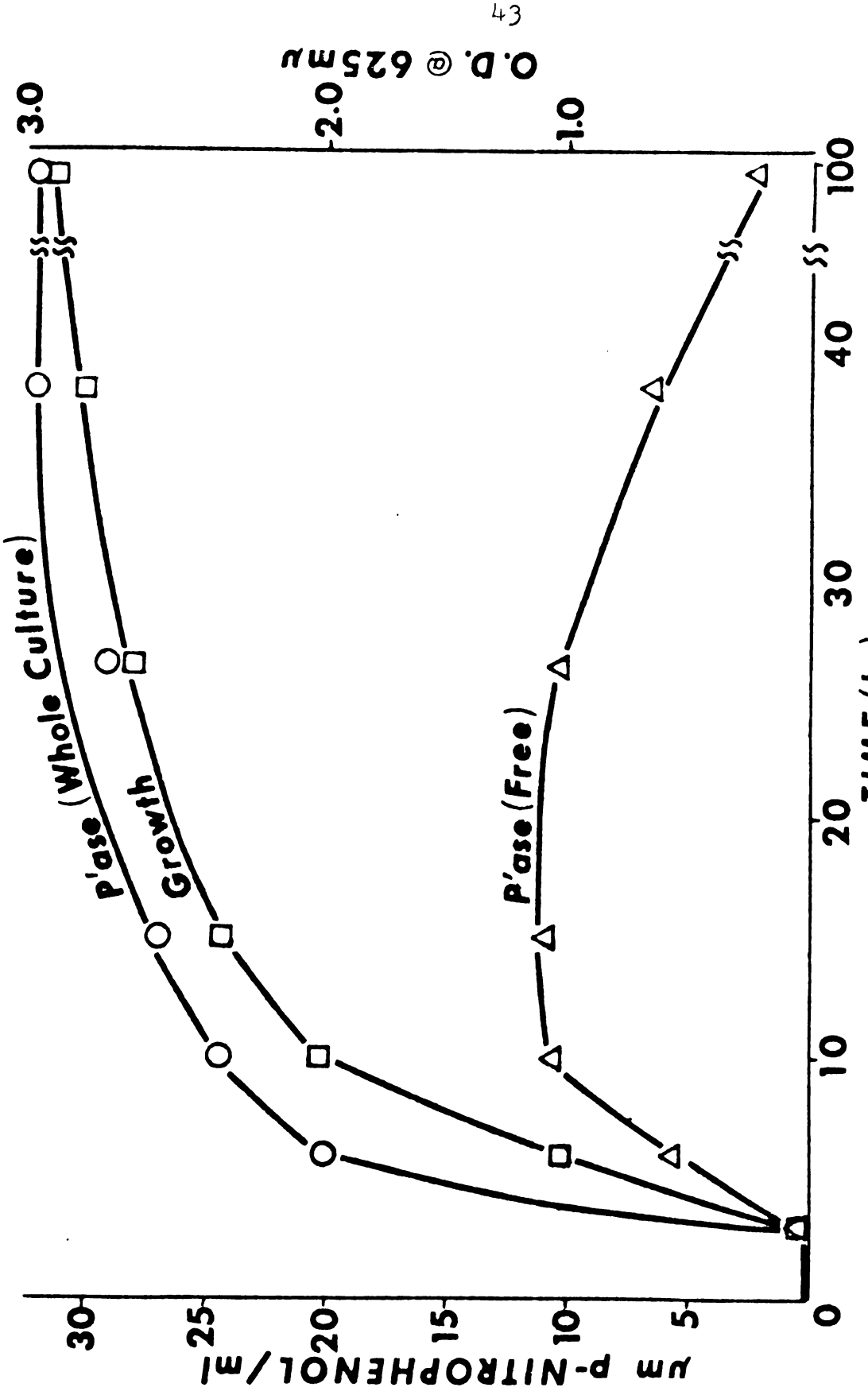

Fig. 3. Rate of acid phosphatase production (whole culture and cell-free supernatant fluid) and cell growth (O.D. at 625 mu) measured in shake cultures (Trypticase Soy Broth) of S. aureus PS 3A at 37 C.

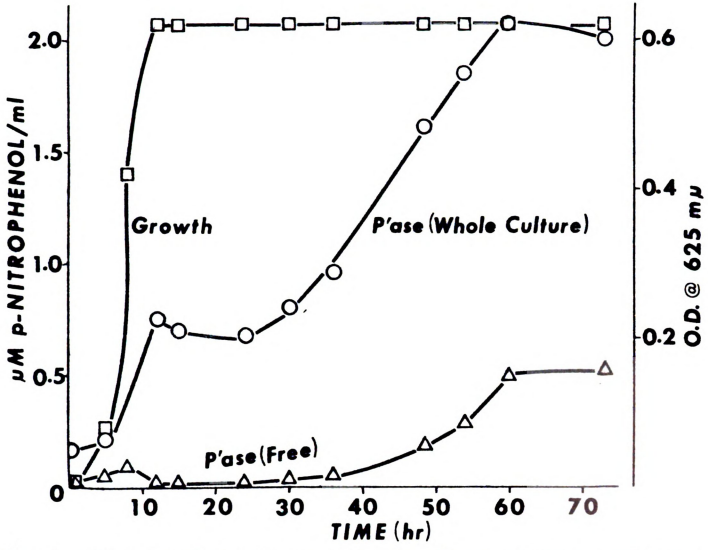

Fig. 4. Rate of acid phosphatase production (whole culture and cell-free supernatant fluid) and cell growth (O.D. at 625 mu) measured in shake cultures (casein acid-hydrolysate medium with glycerophosphate) of S. aureus PS 3A at 37 C.

Fig. 5. Rate of whole culture acid phosphatase production. glucose utilization, and cell growth measured in shake cultures (casein acid-hydrolysate medium without glycerophosphate) of S. aureus PS 3A at 37 C.

μM p-NITROPHENOL/ml and O.D.@ 625mμ
0
0.2
0.4
0.6
Growth
P'ase (Whole Culture)
Glucose
6
12
18
24
30
36
42
48
54
TIME (hr)
RESIDUAL GLUCOSE (mg/ml)
0
0.25
0.50
0.75
1.00

FIG. 5.

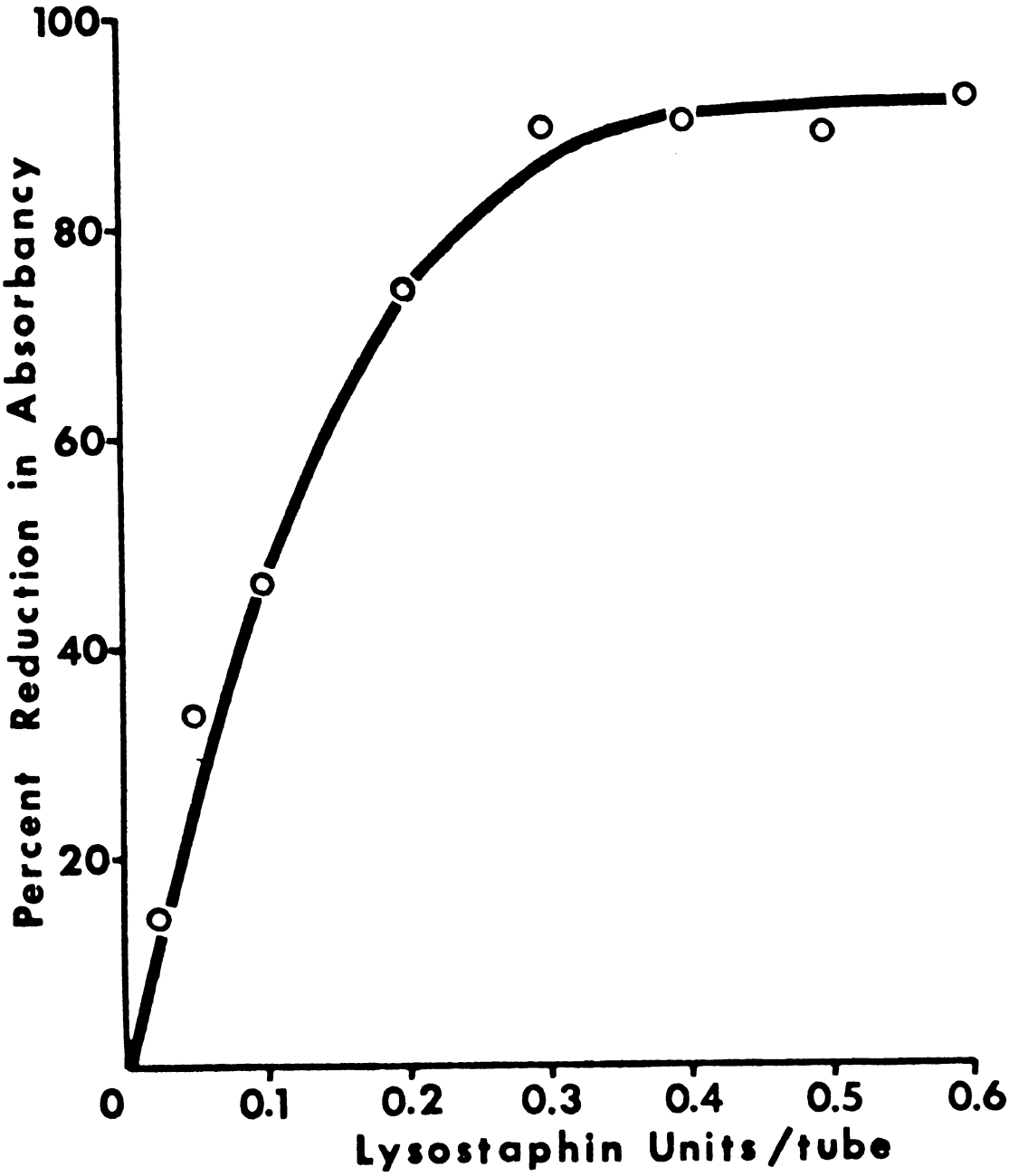

Fig. 6. Effect of lysostaphin on S. aureus PS 55 in 0.05 M Tris-0.15 M NaCl, pH 7.5. Amount of lysis was measured in a 6.0 ml volume at 610 mu following a 10 min exposure of the cells.

is "that quantity of material that produces a 50% reduction in optical density in 10 min at 37 C of a 6.0 ml volume of a standard suspension (O.D. = 0.25) of S. aureus, strain FDA 209P cells, using a Coleman junior spectrophotometer, model 6A, a 620 mu filter, and 18 x 150 mm tubes."

Polyethylene glycol as stabilizing agent of spheroplasts. The ability of polyethylene glycol (PEG) to stabilize spheroplasts of S. aureus PS 55 is illustrated in Fig. 7. As noted earlier, approximately 10% of the cells were lysostaphin-resistant. After 1 hr in 7.5% and 10% PEG, only 7% and 15% respectively of the spheroplasts were stabilized. With 20% PEG about half the spheroplasts remained intact for 1 hr. When PEG concentration was 30%, approximately 70% of the spheroplasts were osmotically stable, and higher concentrations of PEG demonstrated no further stabilizing effect.

Evidence for formation of spheroplasts. Ultraviolet-absorption spectra of supernatant fluids of intact cells, spheroplasts stabilized in 30% PEG, lysed cells, and lysed spheroplasts are shown in Fig. 8. Ultraviolet-absorbing material of stabilized spheroplasts slightly exceeded that of intact cells. However, considerably more material absorbing at 260 mu was apparent in the supernatant fluids of lysed spheroplasts and lysed cells when compared with intact spheroplasts and intact cells.

Table 1 illustrates there was no reduction in turbidity when intact cells in PEG were centrifuged and resuspended in water. Spheroplasts, on the other hand, underwent considerable


Percent Reduction in Absorbancy

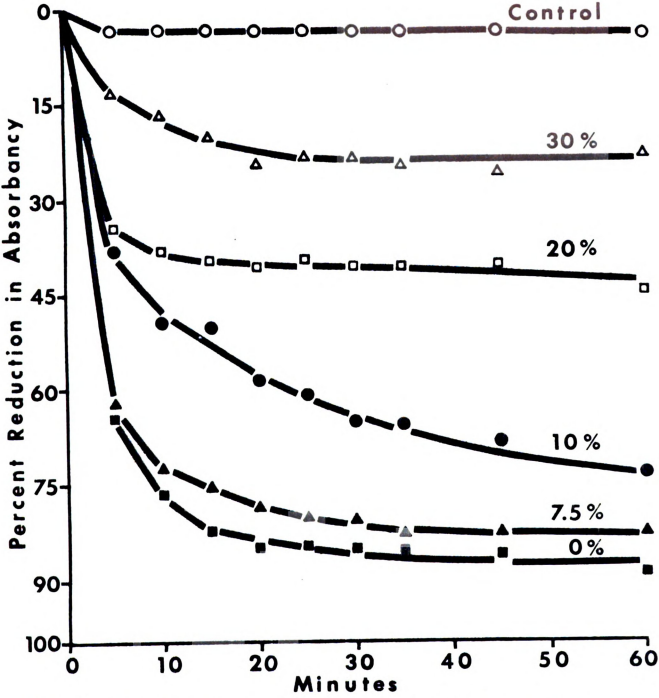

Fig. 7. Rate of lysis of S. aureus PS 55 in the presence of lysostaphin (1.0 unit/ml) at 37 C and different concentrations (w/v) of polyethylene glycol (MW= 3000-3700).

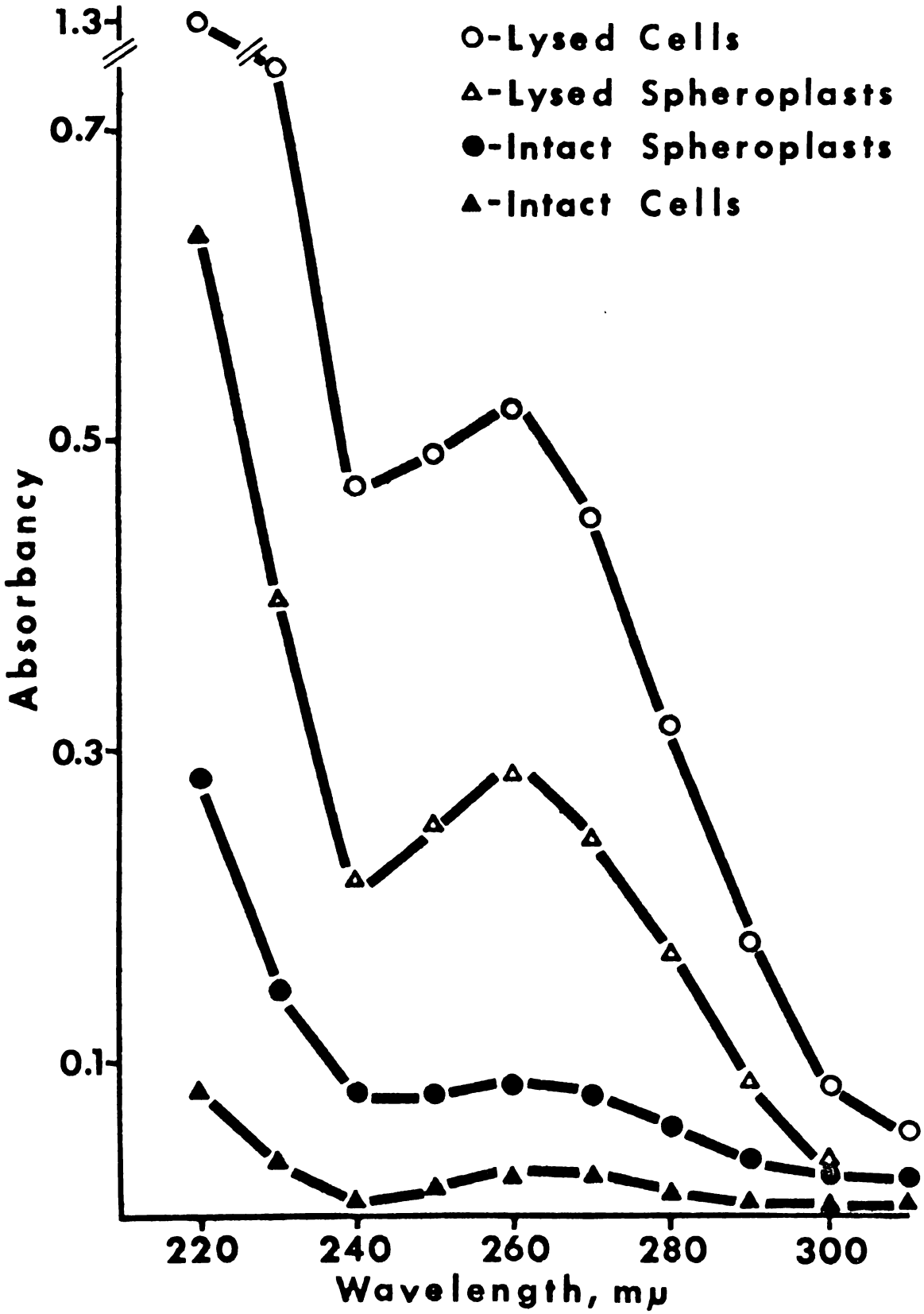

Fig. 8. Ultraviolet-absorption spectra of supernatant fluids of intact cells, intact spheroplasts, lysed cells, and lysed spheroplasts.

Table 1. Absorbancy of intact cells, osmotically protected (30% polyethylene glycol), and unprotected S. aureus PS 55 in the presence of lysostaphin (intact cells were not treated with lysostaphin) before and after resuspension in water following centrifugation at 7000 x g for 10 min.

	Absorbancy at 610 mu		
	Intact cells (30% PEG)	Spheroplasts (PEG + Lysost.)	Lysate (Lysostaphin)
Before	.420	.170	.070
After	.430	.080	———

lysis when treated in the same manner. Absorbancy of spheroplasts resuspended in water was comparable to that of unprotected cells.

Localization of the enzyme by an indirect method. Half the acid phosphatase activity was liberated when the cells were converted to spheroplasts (Fig. 9, Table 2). Following lysis of these spheroplasts, most of the remaining half of activity was associated with the fraction containing the cytoplasmic membrane. In the case of intact cells which were treated with PEG and dilute salt solution, most of the enzymatic activity remained bound to the cells.

Purification of Acid Phosphatase

Phage-propagating strain 3A was initially selected as the source of acid phosphatase because it had the highest enzymatic activity, and most (65%) of this activity was loosely bound (Fig. 1 and 2). During the course of purification studies, however, the major portion (60%) of the total enzyme activity shifted to the free (culture medium) fraction. This shift prompted the selection of another strain, S. aureus PS 55, as the source of loosely bound acid phosphatase. A summary of the stepwise purification procedure for loosely bound acid phosphatase is illustrated in Fig. 10.

Cellular protein (0.39 mg/ml) extracted with 2 N NaOH represented 43% of the dry weight of cells cultured in 16 liters of Trypticase Soy Broth. The whole culture usually

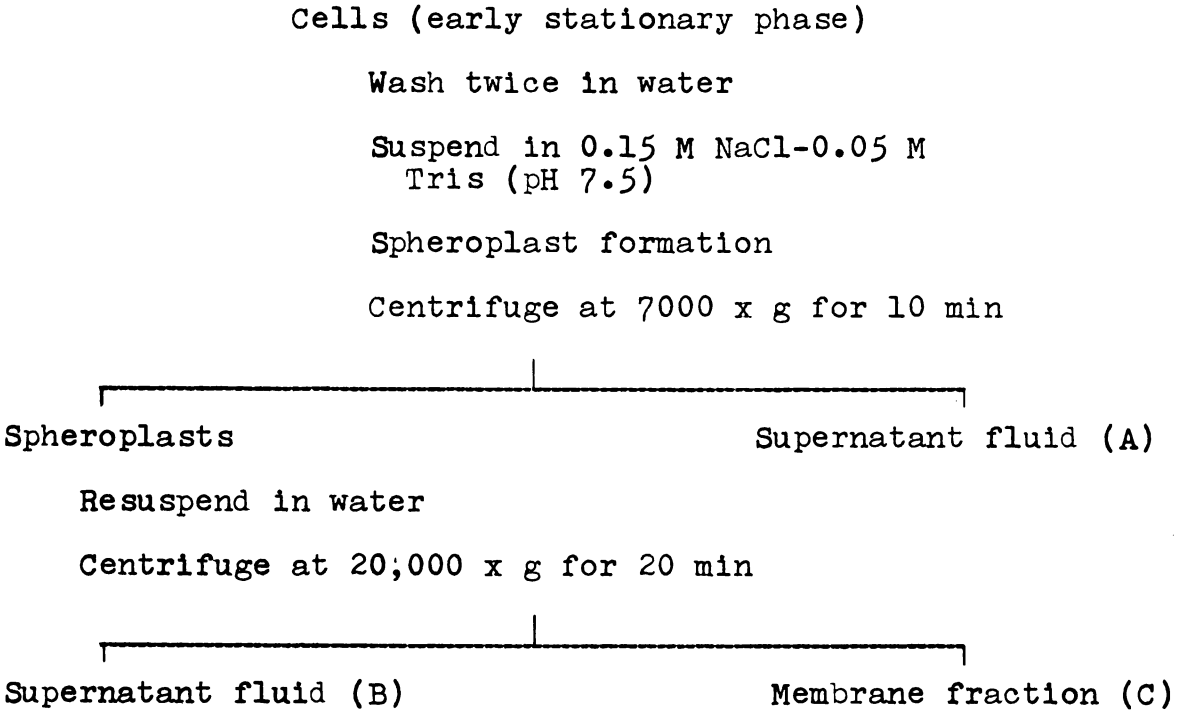

Fig. 9. Flow-sheet diagram for preparing different cellular fractions for the purpose of localizing acid phosphatase in S. aureus PS 55.

Table 2. Percent acid phosphatase activity of lysostaphin-solubilized and/or eluted material (A, see Fig. 9); intracellular contents of spheroplasts or wash solutions of intact cells (B); intact cells or membrane fractions of lysed spheroplasts (C).

	Fraction		
	A	B	C
Spheroplasts	50.0	2.3	47.7
Cells in 30% PEG, pH 7.5	3.1	23.0	54.4
Cells in 0.15 M NaCl, pH 7.5	13.8	2.3	58.1

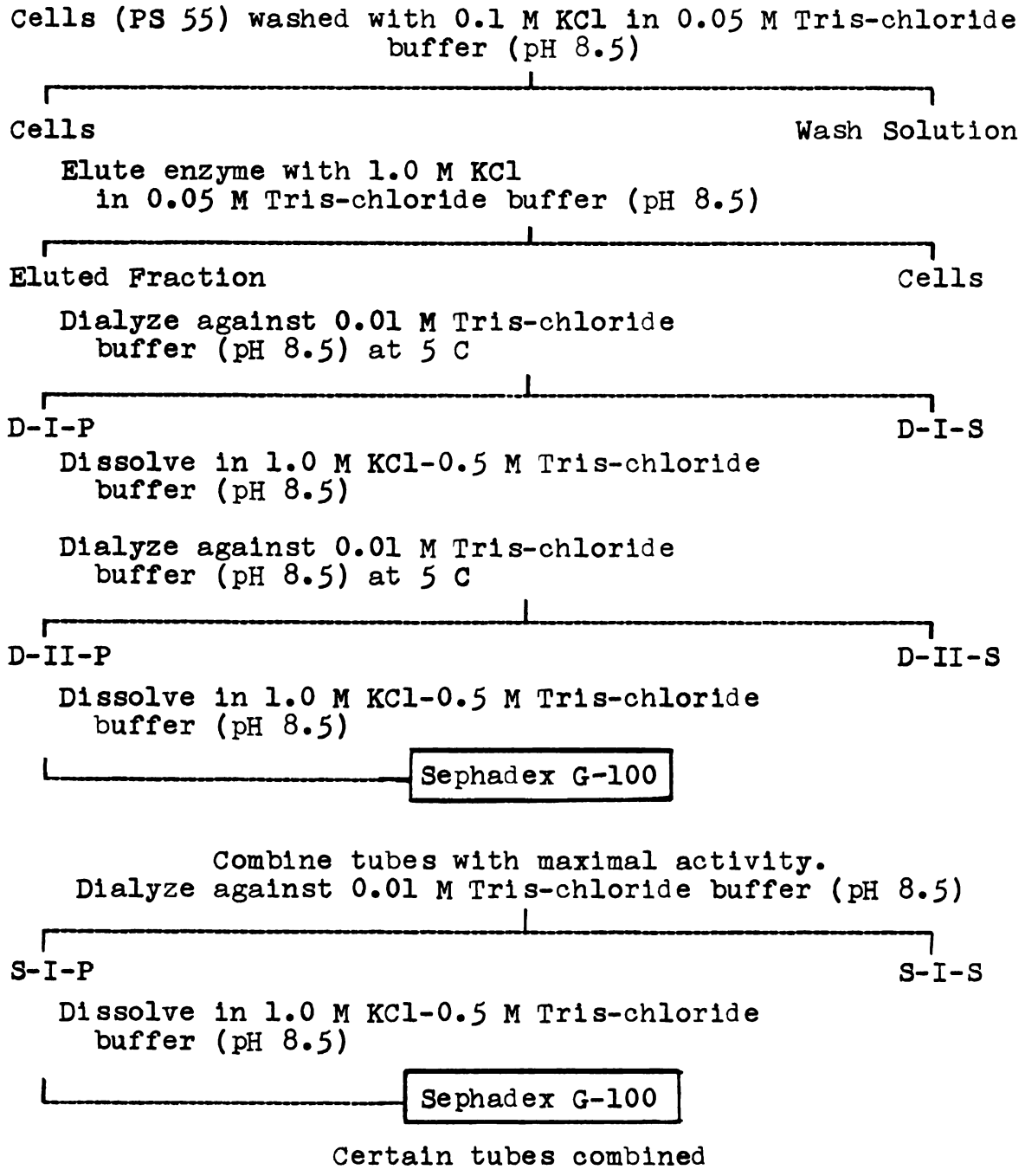

Fig. 10. Flow-sheet diagram for the purification of staphylococcal (PS 55) acid phosphatase.

contained 15-20 units of enzymatic activity/ml, and the cells normally accounted for half this activity.

Elution of loosely bound phosphatase. Initial studies of elution of enzyme were carried out on PS 3A when the organism produced a significant amount of loosely bound enzyme. Maximal enzymatic activity was eluted in the alkaline pH range (Table 3). Acid phosphatase was equally eluted at 25 or 37 C. Of the enzyme associated with actively dividing cells grown in Trypticase Soy Broth, 70% was loosely bound to the cells (Fig. 11). The same figure shows that the enzyme was maximally eluted from the cells with 1.0 M KCl at pH 7.5, and that the amount eluted increased as a function of ionic strength up to 1.0 M. However, the elution pattern of acid phosphatase was different in the case of stationary-phase cells grown in the casein acid-hydrolysate medium (Fig. 12). In this case, relatively higher enzymatic activity was found in the free fraction, and maximal elution occurred at a final concentration of 2.0 M KCl. In routine purification experiments, the elution step (Fig. 10) effected a seven-fold purification of enzyme. The 280/260 ratio of the eluted fraction was 0.71.

Dialysis of the eluted fraction. During dialysis of the eluted fraction against dilute buffer (pH 8.5), the enzyme was precipitated. At salt concentrations less than 0.5 M, solubility of the enzyme rapidly decreased (Fig. 13), and at 0.2 M, more than 90% of the enzymatic activity was precipitated. The fraction D-I-P (Fig. 10) had a specific activity of 1300

Table 3. The pH dependence of acid phosphatase elution from cells cultivated in shake cultures of S. aureus with 0.1 M buffer solutions at 25 and 37 C.

Fraction	Activity[a]					
	Elution pH @ 37 C			Elution pH @ 25 C		
	5.3	7.0	8.5	5.3	7.0	8.5
Eluted	2.82	1.90	8.11	0.10	1.50	9.80
Retained	25.60	32.00	23.00	25.50	31.60	27.60

[a]Expressed as micromoles of p-nitrophenol/ml/30 min at 37 C.

Fig. 11. Relative amounts of free, loosely bound, and firmly bound acid phosphatase in shake cultures (Trypticase Soy Broth). Enzyme elution was effected with different concentrations of KCl at pH 7.5.

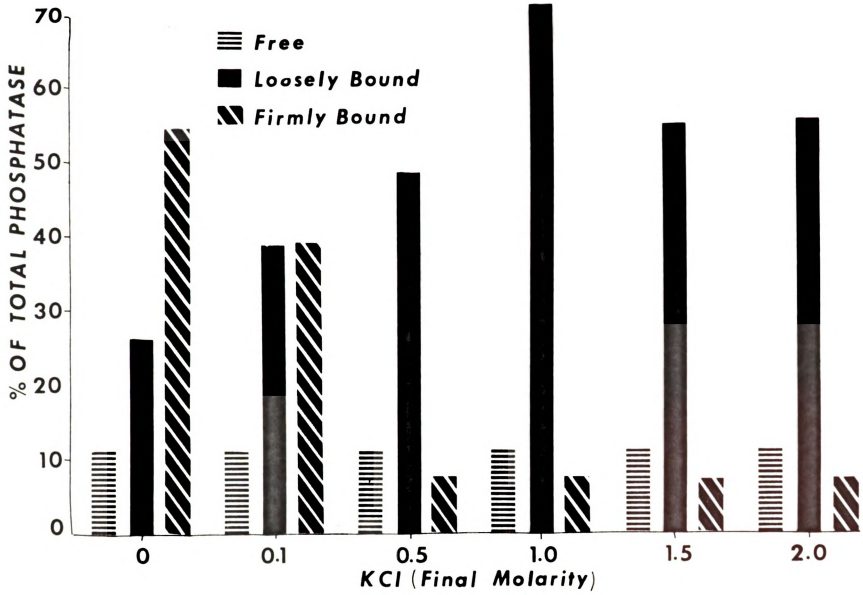
Free
Loosely Bound
Firmly Bound
% OF TOTAL PHOSPHATASE
70
60
50
40
30
20
10
0
0
0.1
0.5
1.0
1.5
2.0
KCl (Final Molarity)

Fig. 11

Fig. 12. Relative amounts of free, loosely bound, and firmly bound acid phosphatase in shake cultures (casein acid-hydrolysate medium supplemented with glycerophosphate). Enzyme elution was effected with different concentrations of KCl at pH 7.5.

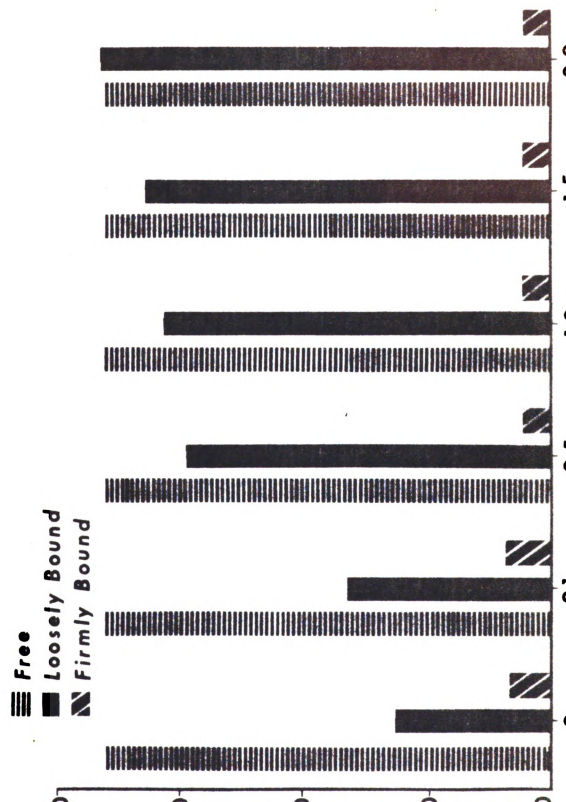
Free
Loosely Bound
Firmly Bound
% OF TOTAL PHOSPHATASE
40
30
20
10
0
0
0.1
0.5
1.0
1.5
2.0
KCl (Final Molarity)

Fig. 12.

Fig. 13. Solubility curve of acid phosphatase (fraction D-II-P, see Fig. 10) prior to gel filtration. Decreasing solubility of the enzyme was determined by observing at 625 mu the precipitate formed, and residual enzymatic activity was determined by assaying the supernatant fluid.

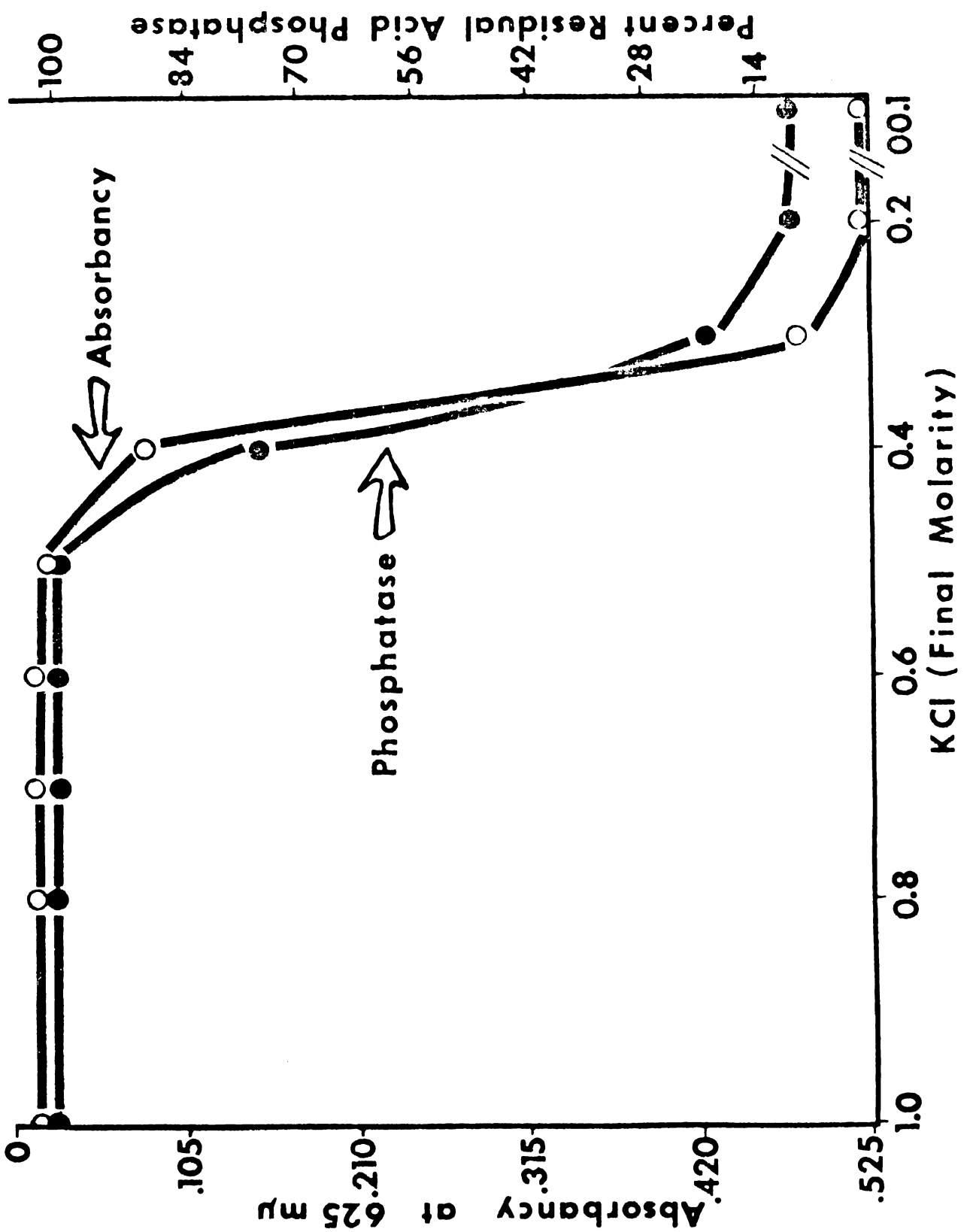
Absorbancy at 625 mμ
0
.105
.210
.315
.420
.525
1.0
0.8
0.6
0.4
0.2
0.1
KCl (Final Molarity)
Percent Residual Acid Phosphatase
100
84
70
56
42
28
14
Absorbancy
Phosphatase

Fig. 13.

which represented a 172-fold purification of enzyme. The 280/260 ratio was 0.87, and there was unexpected increase in percent recovery over the previous purification step.

Gel filtration. Figure 14 illustrates a typical elution pattern of acid phosphatase and protein when the redissolved precipitate (D-II-P, Fig. 10) obtained during dialysis was passed through a column of Sephadex G-100. One peak of enzymatic activity and two protein peaks were observed. The partition coefficient (K_{av}) was 0.20 for the major protein peak and 0.61 for the minor protein peak. Figure 15 represents the elution pattern following gel filtration of the fraction S-I-P (Fig. 10). Four-ml fractions were collected, and the K_{av} for the common protein and enzyme peak was 0.19.

The elution constant (V_e/V_o), where V_e is the elution volume and V_o is the void volume, for acid phosphatase was 1.46. Employing the data of Determan and Michel (1966) for globular proteins, a first approximation of the molecular weight (MW) of acid phosphatase passed through Sephadex G-100 was 54,000.

Efficiency of the purification procedure. Efficiency of the procedure is shown in Table 4. As seen here, purified product obtained after the second cycle of gel filtration accounted for 17.1% of the enzyme bound to the cells. A specific activity of 2,350 represented approximately a 300-fold purification. The 280/260 ratio of this fraction was 1.72. The second dialysis step eliminated lipase activity (Table 5), and repeated gel filtration produced a higher 280/260 ratio and specific activity.

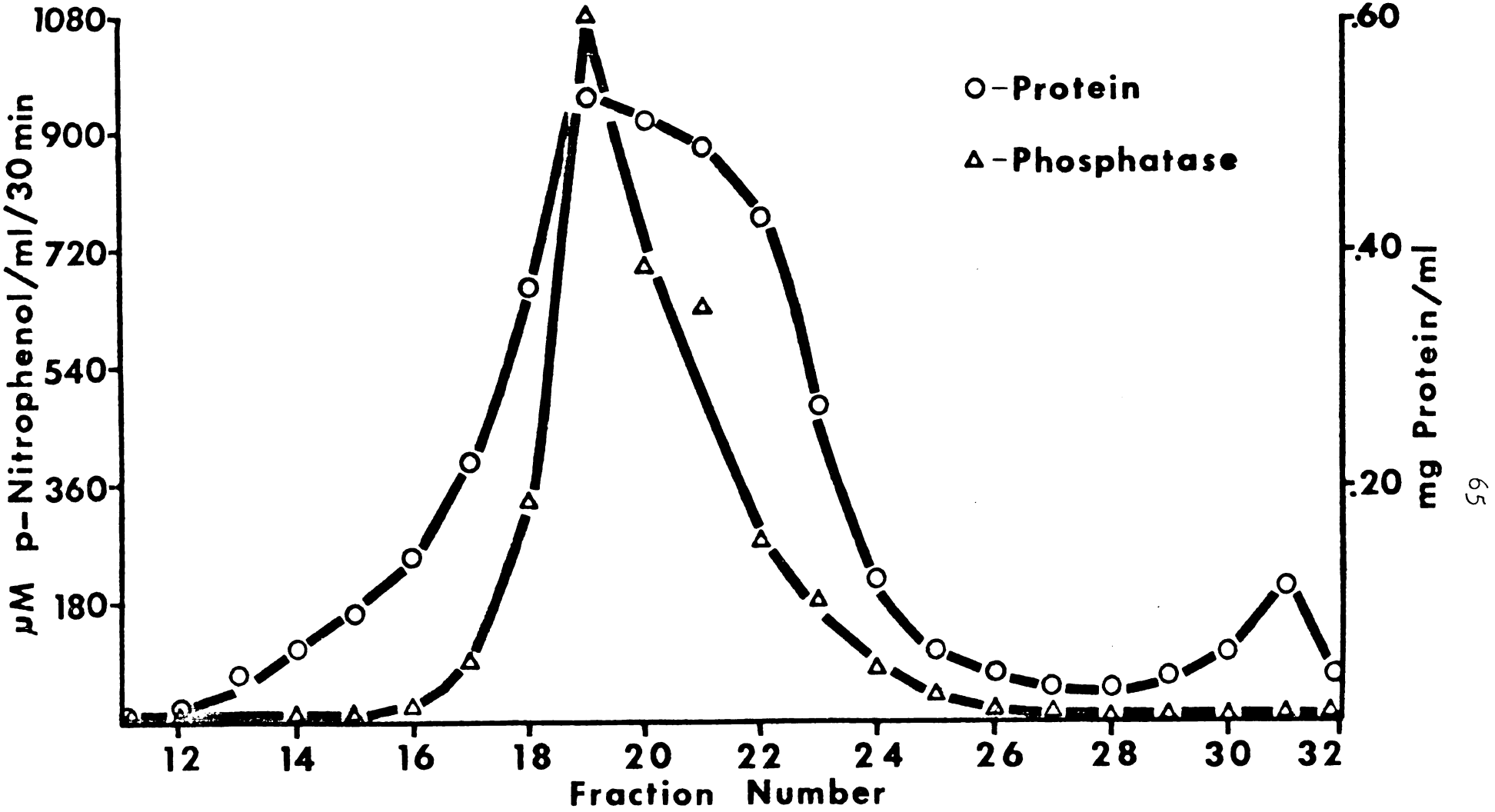

Fig. 14. Gel filtration (Sephadex G-100) of the fraction D-II-P using a 2.5 x 38 cm column at 5 C. Enzyme was eluted with 1.0 M KCl in 0.05 M Tris-chloride buffer, pH 8.5.

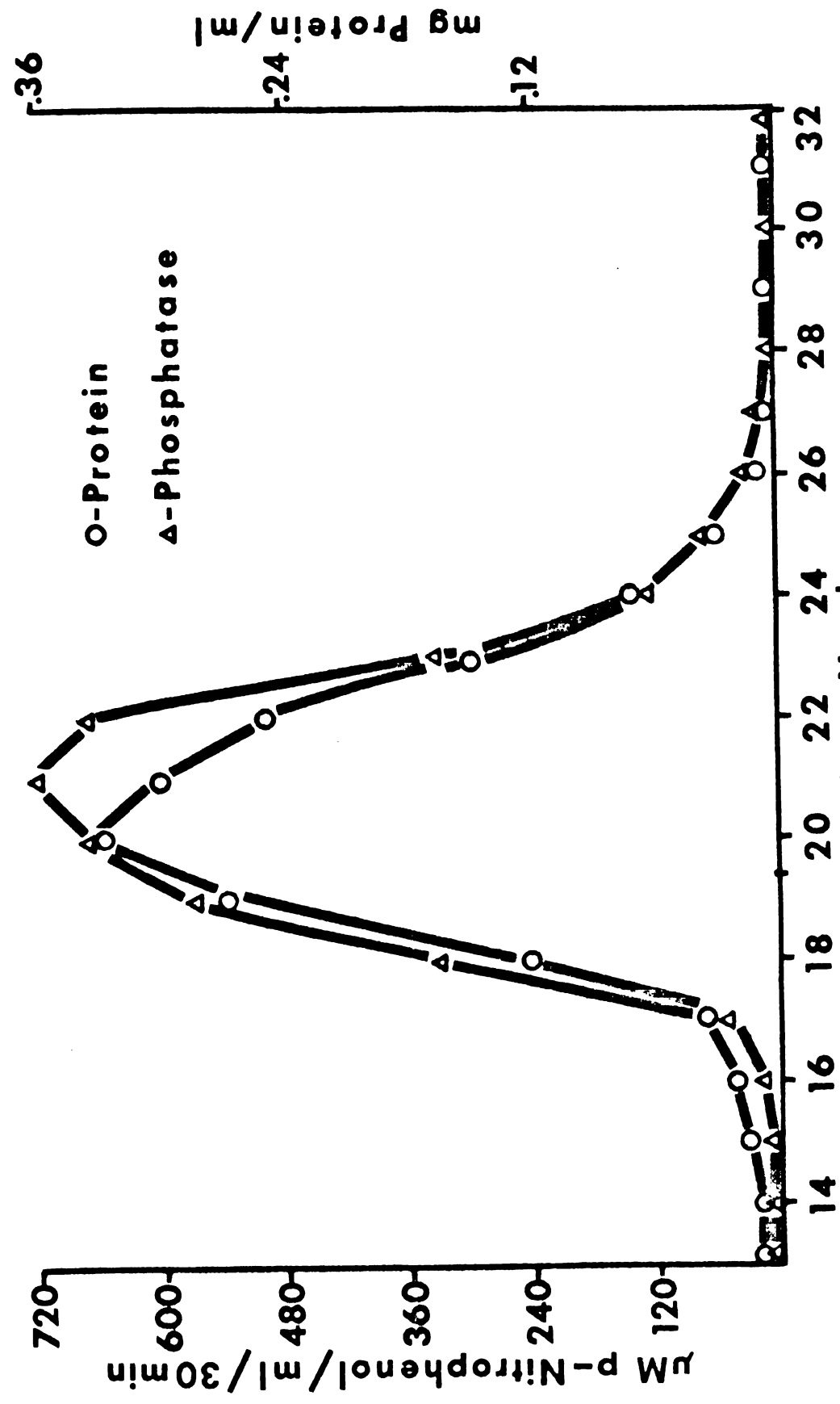

Fig. 15. Gel filtration (Sephadex G-100) of the fraction S-I-P using a 2.5 x 38 cm column at 5 C. Enzyme was eluted with 1.0 M KCl in 0.05 M Tris-chloride buffer, pH 8.5.

Table 4. Enzyme purification summary sheet for staphylococcal acid phosphatase (see Fig. 10).

Purification fraction	Specific activity[a]	Percent recovery	280/260	Purification factor
Washed cells	7.7	———	———	———
Eluted	54.1	33.3	0.71	7
D-I-P	1300	62.5	0.87	172
D-II-P	1350	64.6	0.90	175
G-100 (First cycle)	1580	21.7	1.43	205
G-100 (Second cycle)	2350	17.1	1.72	306

[a]Expressed as uM p-nitrophenol/ml/30 min/mg protein

Table 5. Presence or absence of some staphylococcal products during purification of acid phosphatase.

Staphylococcal product	Purification Fraction					
	Cells	Eluted	D-I-P	D-II-P	G-100 (1st Cycle)	G-100 (2nd Cycle)
Acid phosphatase	+	+	+	+	+	+
Coagulase	+	+	-	-	-	-
Lipase	+	+	±	-	-	-
DNase	+	+	+	+	-	-
Hemolysin	-	-	-	-	-	-
Fibrinolysin	-	-	-	-	-	-
Carbohydrate	+	-	-	-	-	-
Nucleic acid	+	+	+	+	+	-

Table 5 illustrates the presence or absence of some staphylococcal products during different stages of purification. Acid phosphatase, coagulase, lipase, deoxyribonuclease, and nucleic acid were simultaneously eluted from the cells with 1.0 M KCl. Carbohydrate was not removed from the cells which were initially devoid of hemolysin and fibrinolysin activities. During dialysis against dilute buffer, phosphatase, deoxyribonuclease, and nucleic acid were precipitated, but coagulase and lipase activities remained soluble. The first cycle of gel filtration eliminated nuclease, and the second cycle, increasing the 280/260 ratio to 1.72, essentially removed contaminating nucleic acid.

Characterization of Acid Phosphatase

Homogeneity of purified enzyme. Electrophoretic analysis on starch indicated that the preparation was homogeneous with respect to charge (Fig. 16). The basic nature of the phosphatase was apparent from its movement (2 cm from the origin) toward the cathode at pH 8.0.

Figure 17 illustrates the sedimentation pattern of acid phosphatase 8 min (top) and 40 min (bottom) after the rotor reached maximal speed. In the top figure, the major peak represented acid phosphatase and the minor peaks represented high molecular weight components (probably contaminating ribosomal material). Interestingly, the 280/260 ratio of the sample put into the analytical centrifuge was 1.21 as contrasted to 1.72, the characteristic value of highly purified

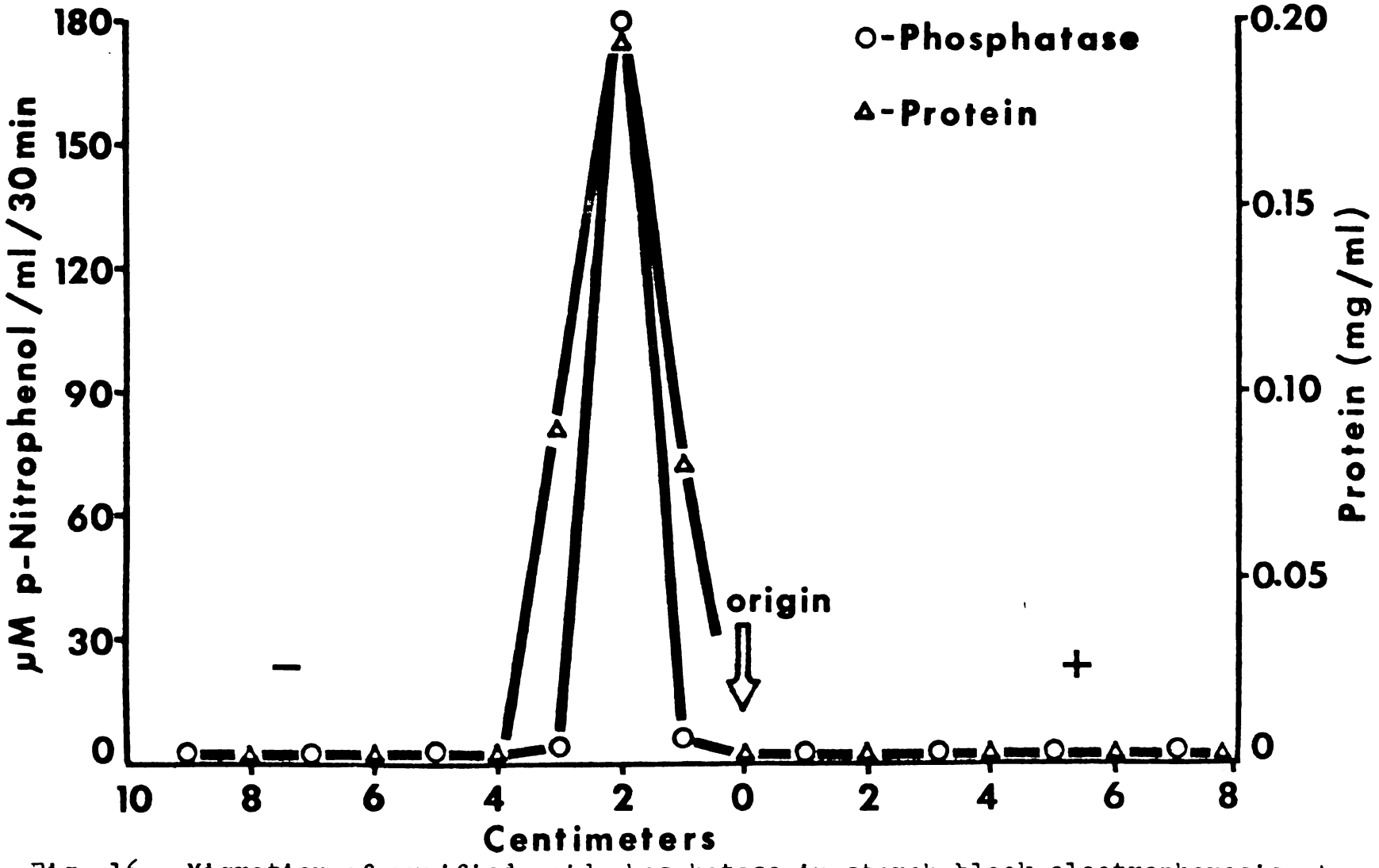

Fig. 16. Migration of purified acid phosphatase in starch block electrophoresis at pH 8.0, 4 C. Protein and enzymatic activities were determined in the material eluted from fractions consisting of one-cm wide segments which were cut from the starch block perpendicular to the direction of migration.

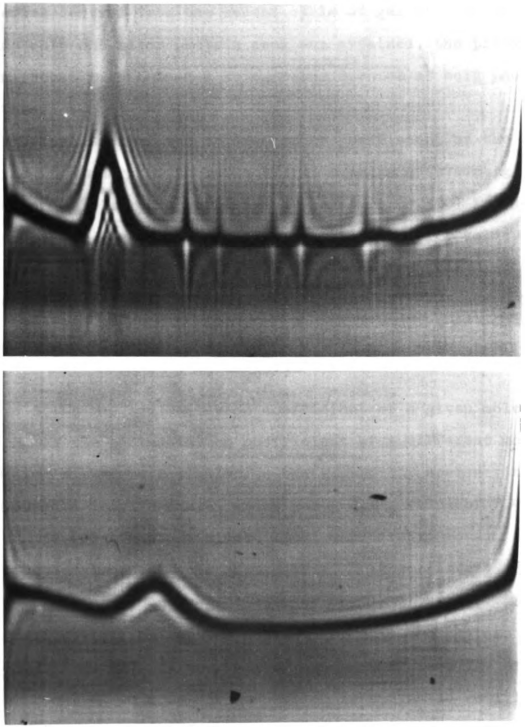

Fig. 17. Sedimentation pattern of partially purified acid phosphatase. The enzyme was in 0.6 M KCl-0.1 M Tris, pH 8.5. The upper photograph was taken after 8 min and the lower photograph was taken after 40 min, both at 56,100 rpm. The bar angle was 65°. The protein concentration was 3.5 mg/ml.

material derived from the second cycle of gel filtration. Since only one major protein peak was attained, the preparation probably contained a homogeneous species of acid phosphatase.

Calculation of the sedimentation coefficient of the enzyme is summarized in Table 6. An average S (svedberg) value of 2.36 at the corrected temperature (4.3 C) in the given buffer system was attained by using the $S_{t,b}$ values of pictures 3, 4, 7 and 8. The $S_{20,w}$ in this case was 3.68. An approximation of the molecular weight (MW) was made using the formula:

$$\frac{S_a}{S_b} = \frac{MW_a^{2/3}}{MW_b^{2/3}} ,$$

where S_a is the sedimentation coefficient of a given molecule and S_b is the sedimentation coefficient of a different molecule. Assuming a structure similar to the enzyme glyceraldehyde 3-phosphate dehydrogenase, which has a $S_{20,w}$ value of 7.4 and MW of 150,000 (Constantinides, 1967, unpublished data), the MW of staphylococcal acid phosphatase is approximately 53,000.

Effect of pH on the activity of acid phosphatase. The range of optimal pH for the activity of acid phosphatase was 5.2-5.3 (Fig. 18). A decrease of one pH unit from the optimal value resulted in about 50% reduction in enzymatic activity, and an increase of one unit caused a 30% reduction. The enzyme exhibited little or no activity in the alkaline range.

Effect of ionic strength on the activity of acid phosphatase. The optimal ionic strength for acid phosphatase activity was in the range 0.2-0.5 M (Fig. 19). With 1.0 M KCl

Table 6. Calculation of the sedimentation coefficient of staphylococcal acid phosphatase.

Picture	$\triangle R_p$	$\triangle r_p$	r_p	$r_{(t)}/r_{(t_o)}$	$\log r_{(t)}/r_{(t_o)}$	$t-t_o$	$\frac{\log r_t/r_{(t_o)}}{t-t_o}$	$S_{t,b}$
1	22.784	1.057	6.263	1.0000	0	0	----	----
2	22.718	1.054	6.266	1.0005	0.00022	2	1.100×10^{-4}	1.22
3	22.528	1.045	6.275	1.0019	0.00083	4	2.0750×10^{-4}	2.30
4	22.240	1.032	6.288	1.0040	0.00173	8	2.1625×10^{-4}	2.40
7	22.094	0.932	6.388	1.0200	0.00860	40	2.1500×10^{-4}	2.39
8	19.580	0.909	6.411	1.0236	0.01013	48	2.1104×10^{-4}	2.34

Avg. $S_{t,b} = 2.36$ $S_{20,w} = 3.68$

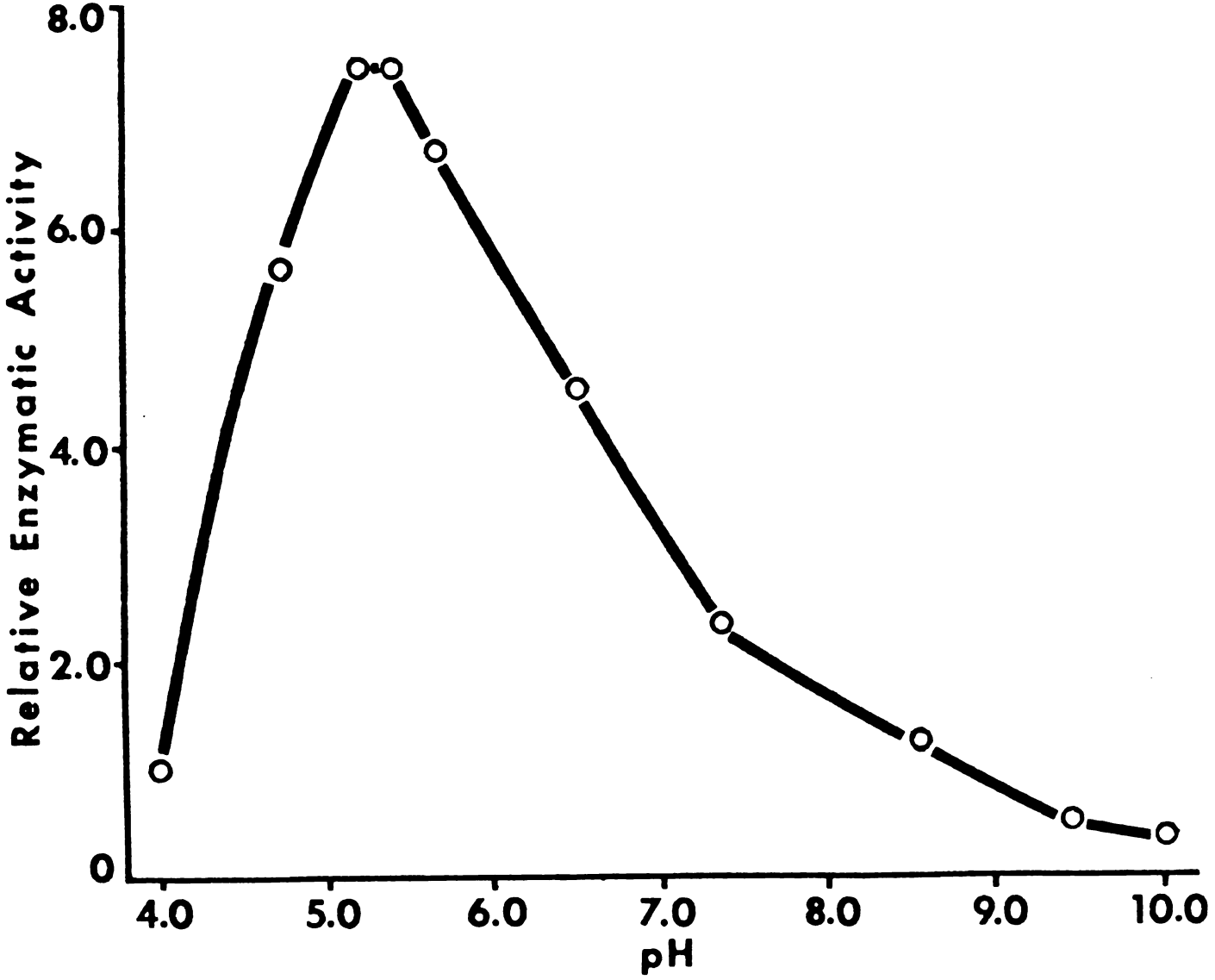

Fig. 18. Effect of pH on the activity of acid phosphatase using p-nitrophenyl phosphate as substrate.

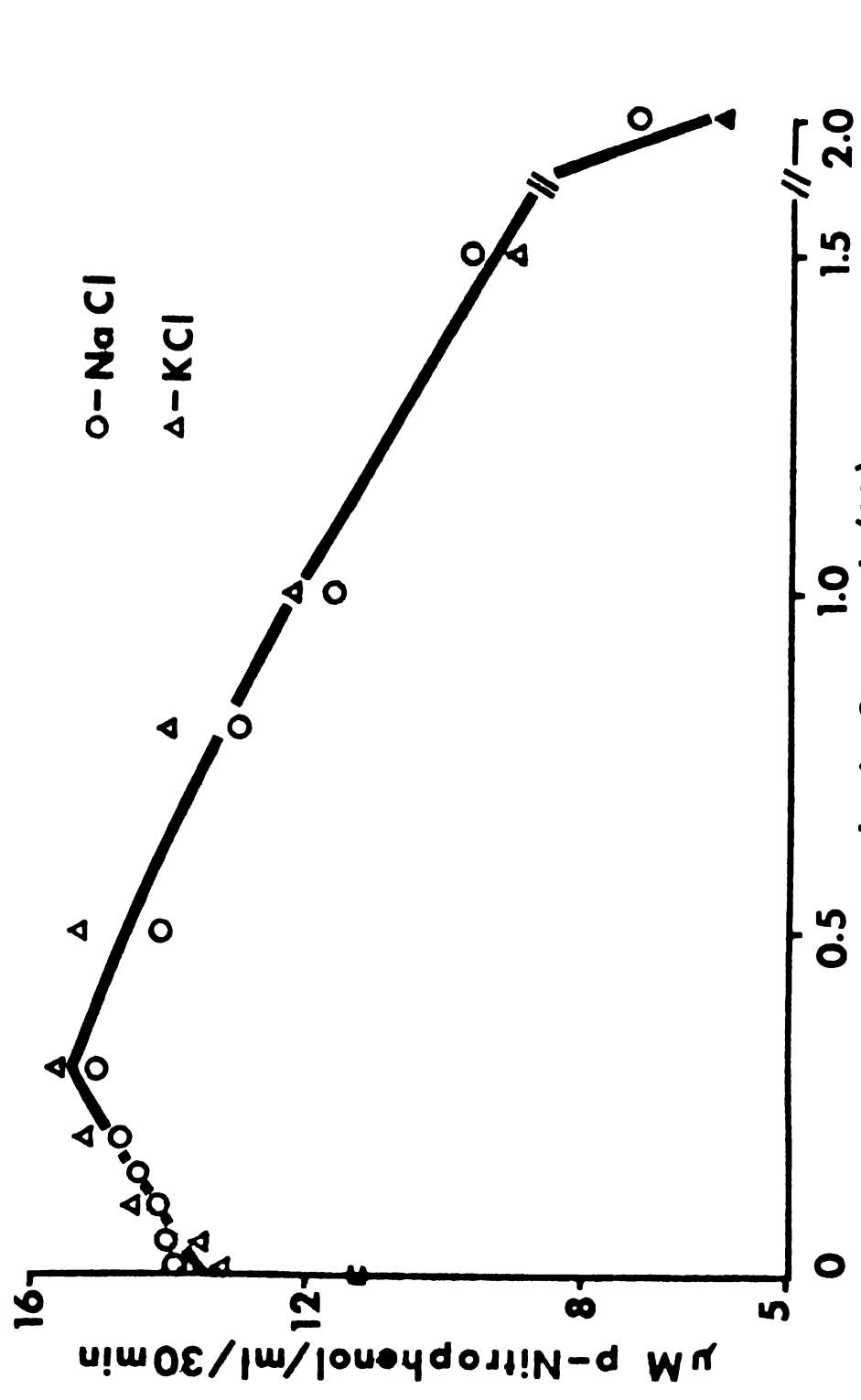

Fig. 19. Effect of ionic strength on the activity of acid phosphatase. Buffer (pH 5.2) concentration in all cases was 1 x 10^{-3} M acetate.

or NaCl, 80% of the optimal activity was still present, but with 2.0 M less than half the optimal enzymatic activity was apparent.

Effect of temperature on the activity of acid phosphatase. Maximal enzymatic activity (Fig. 20) appeared above 50 C. However, using an Arrhenius plot (Fig. 21) for the same data, maximal activity occurred at approximately 48 C. Between 15 C and 37 C there was a linear increase in initial activity, but below and above this range, the same relationship was not applicable. The slope of the solid portion (between 15 C and 37 C) of the plot was used to determine the Arrhenius function A (energy of activation) by the equation

$$m = \frac{A}{2.303\ R},$$

where m is the negative slope, and R is the gas constant. The value for A was 19.5 Kcal/mole.

Initial velocity of purified enzyme. Figure 22 is an Eadie-Hofstee plot of initial velocity of enzymatic activity. The slope of the line represents $-K_m$ for enzymatic activity against p-nitrophenyl phosphate. The K_m for the substrate was 4.5×10^{-4} M, and the V_{max} (intercept on Y axis) was 4.4×10^{-2} uM P_i liberated/min. Using a double reciprocal plot (Fig. 23) of the initial velocity and substrate concentration, values for K_m and V_{max} were the same.

Effect of different compounds on acid phosphatase. Of the compounds tested, mercaptoethanol was the only agent that stimulated enzymatic activity (Table 7). Inorganic phosphate,

μM p-Nitrophenol / ml / 30 .....

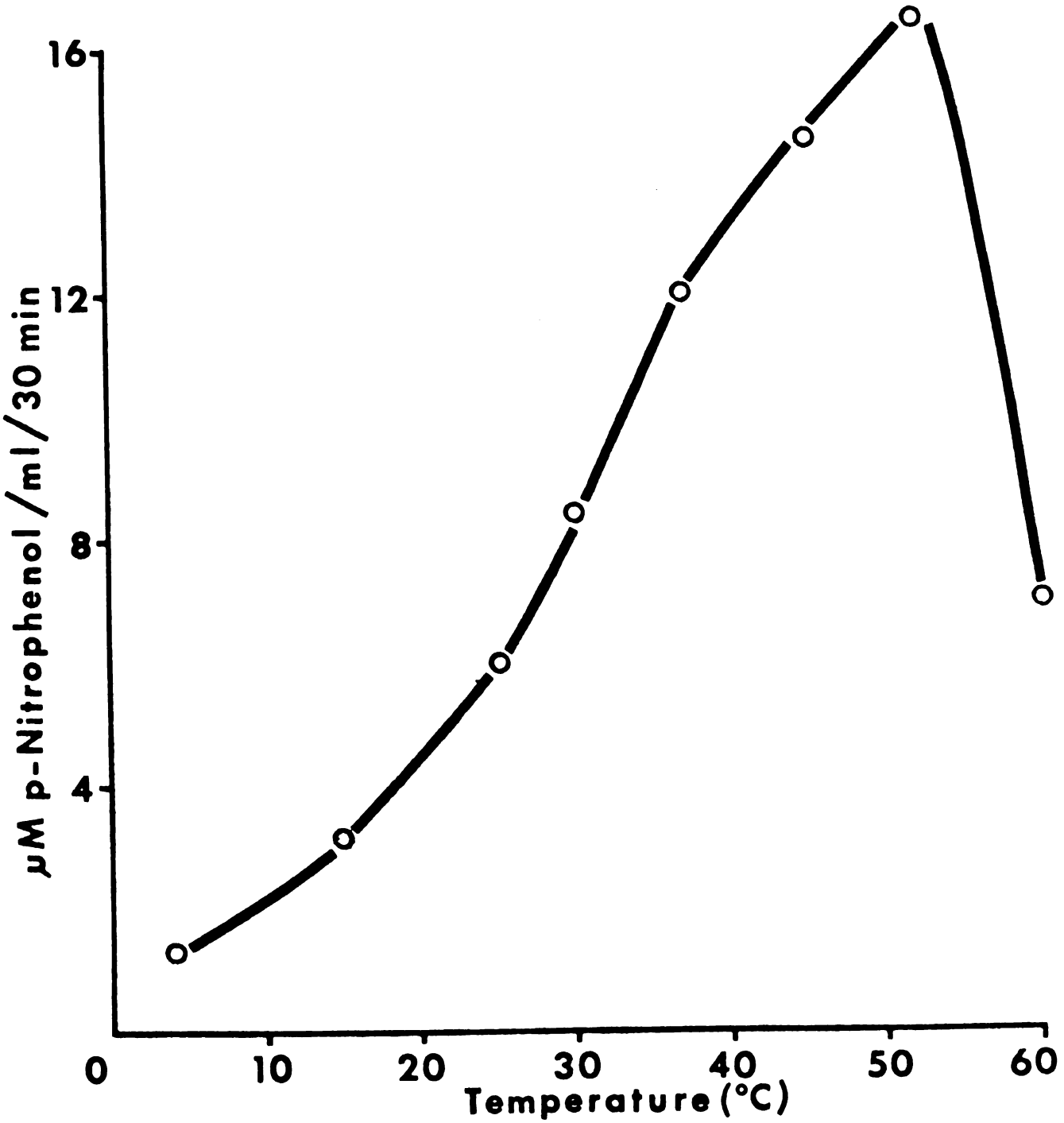

Fig. 20. Effect of temperature upon hydrolysis of p-nitrophenyl phosphate by acid phosphatase. The solutions were buffered with 0.1 M acetate, pH 5.2.

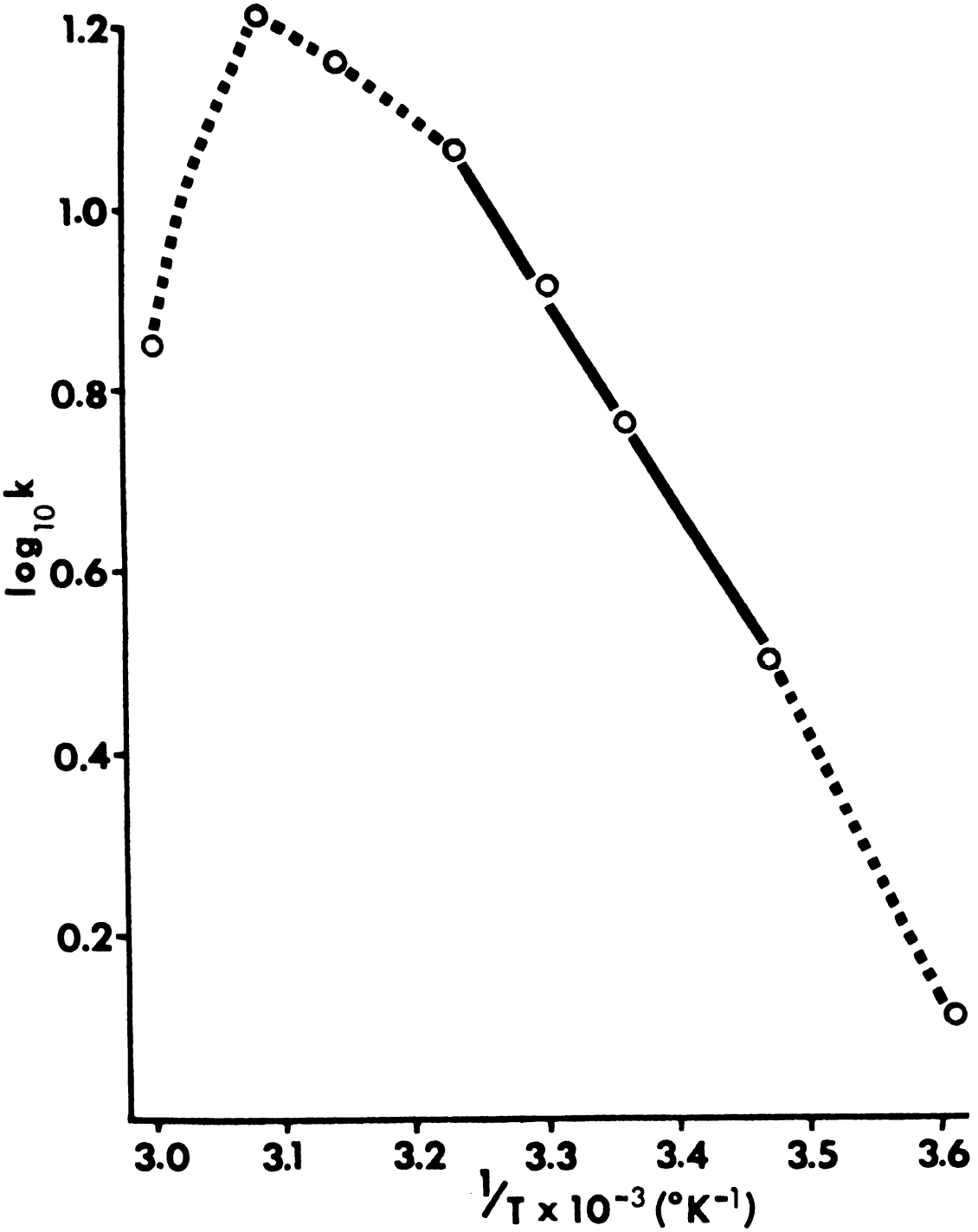

Fig. 21. Arrhenius plot of the effect of temperature upon hydrolysis of p-nitrophenyl phosphate by acid phosphatase. The slope of the solid portion (between 15 and 37 C) of the plot was used to determine the Arrhenius function A (energy of activation).

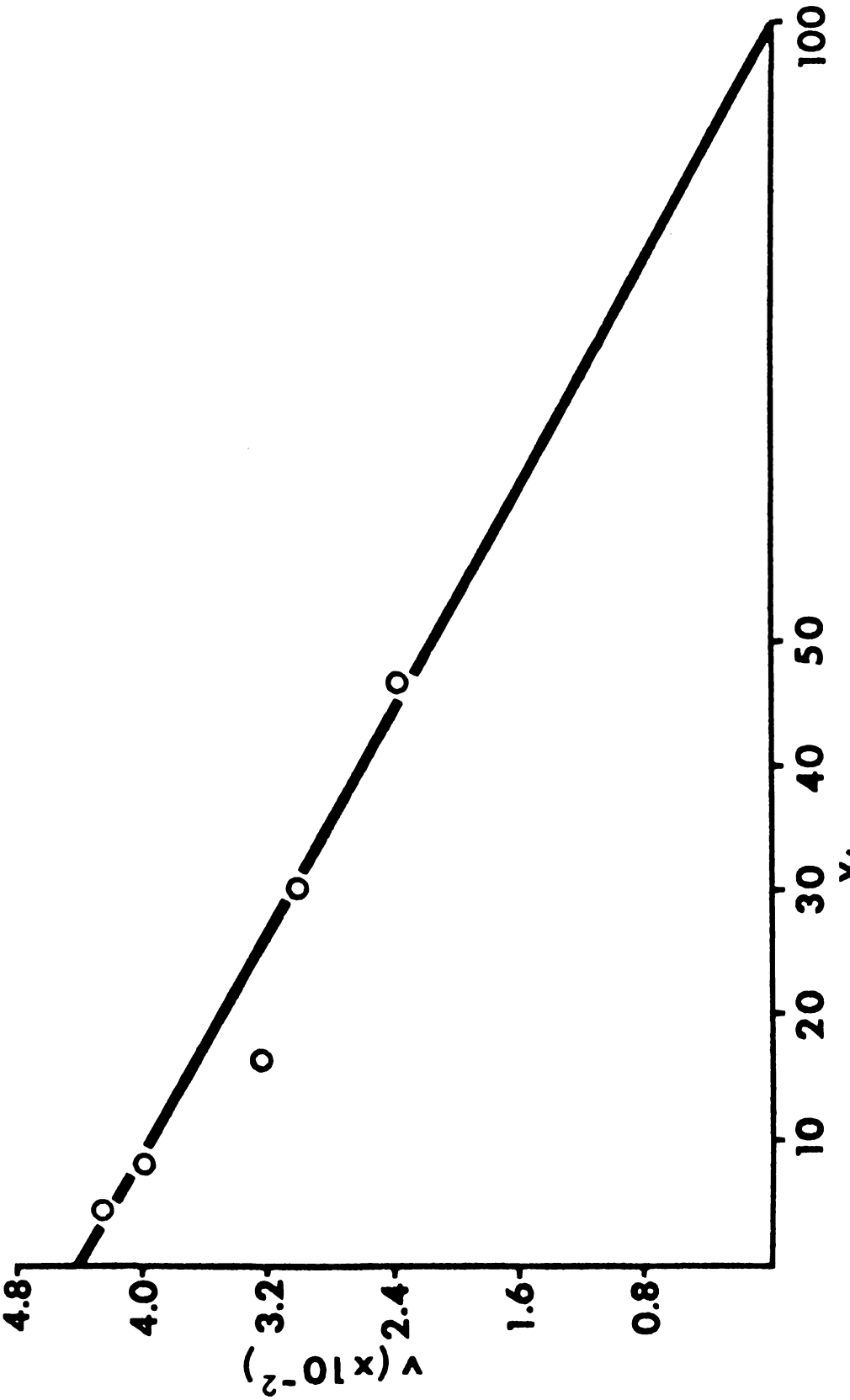

Fig. 22. Eadie-Hofstee plot of the relationship between initial velocity of acid phosphatase and rate per unit substrate (p-nitrophenyl phosphate). The reactions were run at pH 5.2 and 37 C.

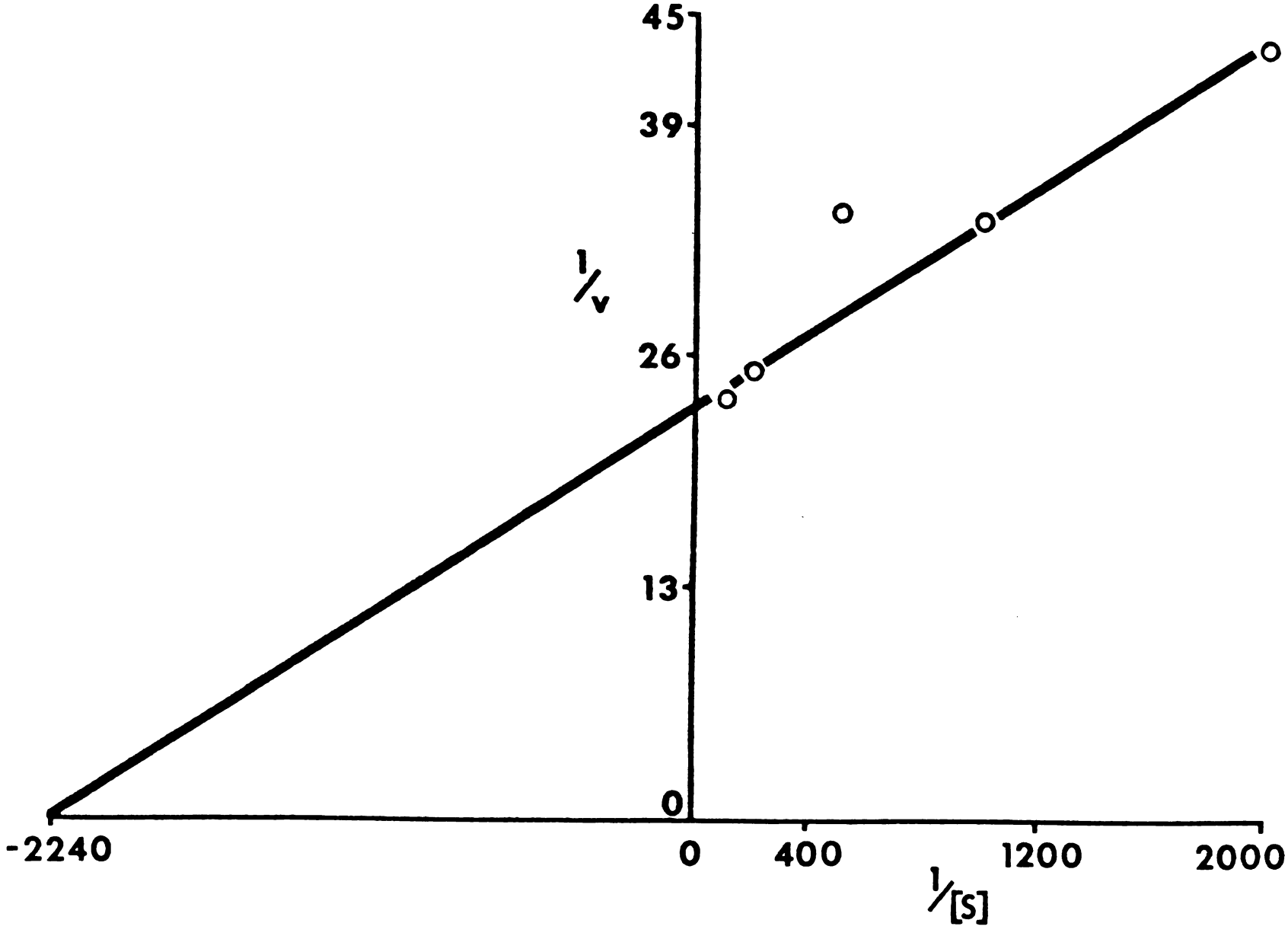

Fig. 23. Double reciprocal plot of the relationship between initial velocity of acid phosphatase and p-nitrophenyl phosphate concentration. The reactions were run at pH 5.2 and 37 C.

Table 7. Effect of different compounds on staphylococcal acid phosphatase. The concentrations of these compounds given in the table refer to final concentrations. Buffer concentration in all cases was 0.1 M acetate, pH 5.2.

Compound	Relative activity
None	100
0.003 M KH_2PO_4	104
0.020 M KH_2PO_4	97
0.034 M KH_2PO_4	79
0.032 M Mercaptoethanol	107
0.064 M Mercaptoethanol	132
0.128 M Mercaptoethanol	129
0.005 M Cysteine	86
0.005 M EDTA	62
0.005 M Sodium fluoride	79
0.005 M Sodium molybdate	87
0.005 M Tartaric acid	100
0.005 M Iodoacetate	55
8 M Urea	83

cysteine, sodium fluoride, sodium molybdate, iodoacetate, and urea inhibited the enzyme, but tartaric acid had no apparent effect.

Effect of divalent cations on acid phosphatase. The cations Co^{++}, Mn^{++}, Mg^{++}, Ca^{++}, Zn^{++}, and Ba^{++} had little or no effect on acid phosphatase activity (Table 8). Hg^{++} and Pb^{++}, on the other hand, reduced enzymatic activity significantly. Cu^{++} accelerated acid phosphatase activity two fold at its given concentration.

Heat stability of the enzyme. In the absence of Cu^{++}, stability of purified acid phosphatase under the conditions employed decreased rapidly between 40 C and 70 C, the temperature at which the enzyme was completely inactivated (Fig. 24). With Cu^{++}, the minimum temperature for complete inactivation was 80 C; thus the cation did afford some protection of acid phosphatase.

Stability of the enzyme under different conditions. Purified acid phosphatase was completely inactivated in 1.0 M acetic acid and 1.0 N NaOH (Table 9). When suspended in water for 6 days, 55% of the initial activity was lost. The enzyme appeared most stable in the alkaline range (8.5 and 9.5).

Activity of acid phosphatase with various substrates. Of the substrates tested, acid phosphatase was most active against p-nitrophenyl phosphate (Table 10). Substantial activity against glyceraldehyde 3-phosphate, and moderate activity against α-glycerophosphate, fructose 6-phosphate, and phenolphthalein diphosphate were also observed. Acid

Table 8. Effect of different divalent cations on the activity of acid phosphatase. Final concentration of the metal ions (used as chloride form) was 1 x 10^{-3} M.

Metal	Relative Activity
None	100
Co^{++}	95.5
Mn^{++}	80.0
Mg^{++}	95.5
Ca^{++}	76.4
Zn^{++}	93.9
Cu^{++}	206.4
Hg^{++}	18.5
Pb^{++}	17.9
Ba^{++}	95.5

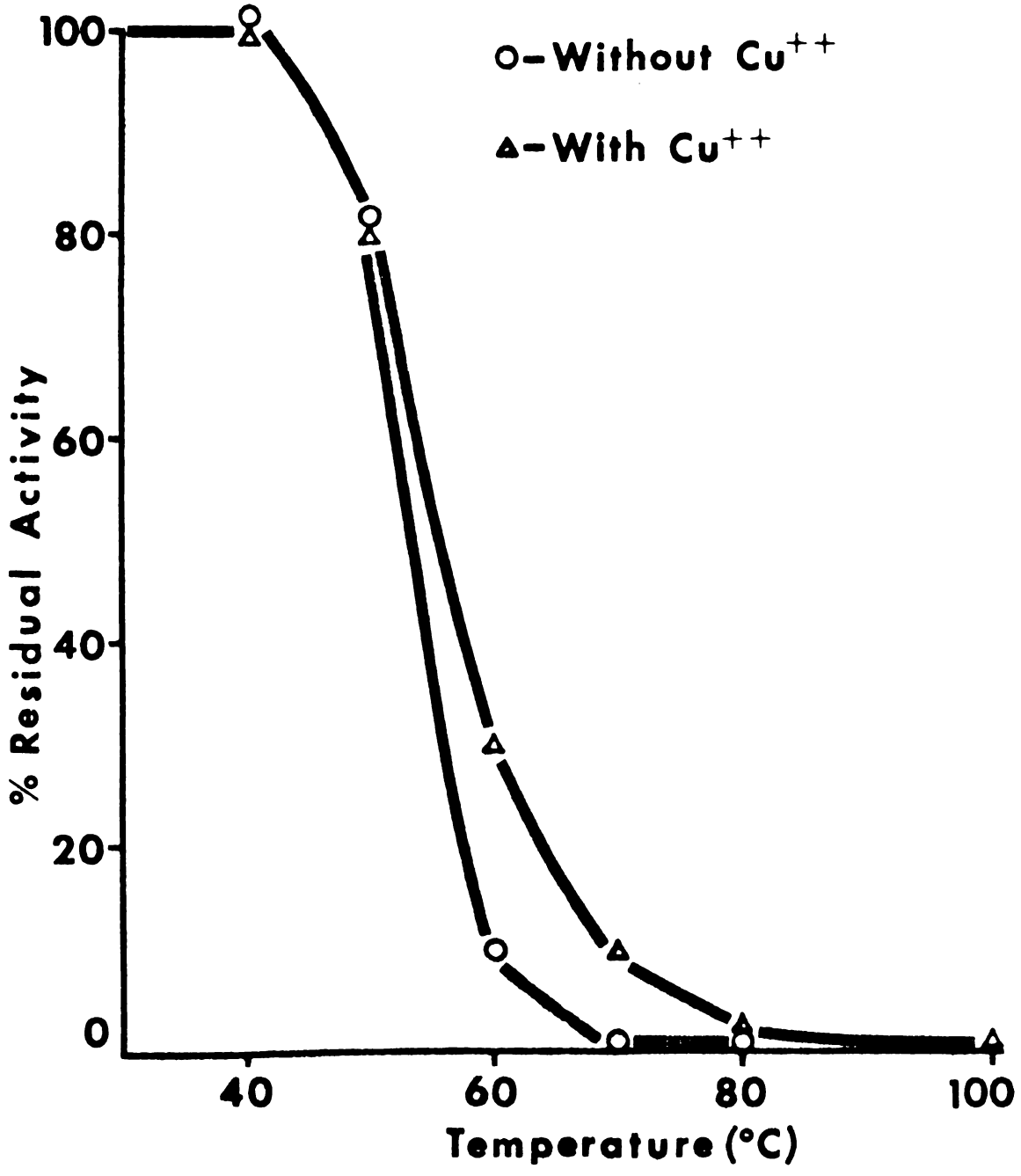

Fig. 24. Heat stability of staphylococcal acid phosphatase with and without Cu^{++} (1×10^{-3} M). The enzyme was heated at each temperature for 5 min in 0.01 M Tris-chloride (pH 8.5) and cooled quickly in ice water. Assays were carried out at 37 C and pH 5.2.

Table 9. Stability of staphylococcal acid phosphatase. Samples were stored at room temperature (25 C) for 6 days. The activities are relative to zero time.

Conditions	Relative Activity		
	1 day	3 days	6 days
Water (no buffer)	100	100	45
0.1 M Acetate, pH 5.2	24	27	10
0.1 M Tris-chloride, pH 7.5	21	9	2
0.1 M Tris-chloride, pH 8.5	95	71	54
0.1 M Glycine-NaOH, pH 9.5	100	85	10
1.0 M KCl, pH 5.2	47	25	14
1.0 M KCl, pH 7.5	23	27	22
1.0 M KCl, pH 8.5	103	88	42
1.0 M KCl, pH 9.5	84	80	52
1.0 M Acetic Acid	0	0	0
1.0 N NaOH	0	0	0

Table 10. Relative activities of staphylococcal acid phosphatase against different phosphate esters (1×10^{-2} M). Samples were incubated at 37 C for 30 min. A value of 100 was assigned to the activity of acid phosphatase against p-nitrophenyl phosphate.

Compound	Relative activity
p-Nitrophenyl phosphate	100
Glucose 6-phosphate	3.5
α-glycerophosphate	34.5
β-glycerophosphate	4.1
Dihydroxyacetone phosphate	0
Fructose 6-phosphate	38.0
Ribose 5-phosphate	0
Phosphoglyceric acid	0
Glyceraldehyde 3-phosphate	82.8
Fructose 1, 6-diphosphate	13.8
Guanosine diphosphate	17.3
Cytidine 3' (2') phosphoric acid	10.4
ATP	0
ADP	10.4
AMP	3.5
DNA	0
Sodium pyrophosphate	0
Phenolphthalein diphosphate	24.1
Casein	4.1
α-casein	0
Lecithin	3.5

phosphatase exerted little or no hydrolytic activity against the remaining substrates.

DISCUSSION

Acid phosphatase has been implicated in the virulent nature of Staphylococcus aureus and has been screened in many strains of the organism; yet, to this writer's knowledge, there is no report on purification and localization studies of the enzyme in S. aureus, and only limited characterizations have been made on one enzyme preparation consisting of whole cells. There is a need, therefore, to purify, characterize, and localize the acid phosphatase of S. aureus.

Experimental evidence indicates no restriction of high phosphatase activity to any particular group of phage-propagating strains of coagulase-positive staphylococci (Fig. 1). Relatively little alkaline phosphatase activity is observed in the strains of S. aureus used in our studies. In this regard, it must be noted that the growth media (Brain Heart Infusion, Trypticase Soy Broth, and even the casein acid-hydrolysate medium) contain a considerable amount of inorganic phosphorus, and formation of alkaline phosphatase by S. aureus (Shah and Blobel, 1967) is subject to repression by inorganic phosphorus in the medium. Production of acid and alkaline phosphatases by Escherichia coli (Torriani, 1960) is similar to that of S. aureus; acid phosphatase is always present, but alkaline phosphatase is formed only when the concentration of inorganic phosphorus is limiting in the

medium. However, neither acid nor alkaline phosphatase from E. coli is found in the culture medium.

Both Elek (1959) and Cannon and Hawn (1963) reported that staphylococcal phosphatase appears to be an intracellular enzyme and cannot be detected in the culture medium. Our studies contradict such findings; in fact, every strain that we tested elaborated extracellular enzyme (Fig. 2).

It is of interest that the relative amounts of acid phosphatase in the three fractions (free, loosely bound, and firmly bound) vary to such an extent in different strains of S. aureus. That such variation may occur even in the same strain is of greater interest. Initially, about 65-70% of the total phosphatase activity of phage-propagating strain (PS) 3A was loosely bound material. However, during later purification studies only 30% of the total activity was loosely bound, and therefore this strain was abandoned in favor of PS 55. The latter strain forming a greater percent of loosely bound acid phosphatase was retained as a routine source of enzyme for purification and characterization studies.

When S. aureus is grown in a complex medium (Trypticase Soy Broth), the rate of whole culture acid phosphatase production is a function of cell number. Barnes and Morris (1957), as well as Cannon and Hawn (1963) made the same observation in certain strains of S. aureus. When cells are grown in the casein acid-hydrolysate medium, whole culture enzyme activity again increases with an increase in cell number; but in the

same medium supplemented with glycerophosphate, there is an unexpected increase in enzymatic activity during the stationary phase of the growth cycle (Fig. 4). This latter increase cannot be due to increased cell number since cessation of cell division coincides with glucose depletion from the medium. Hofsten (1961) described a repressible effect of carbohydrates (glucose, glycerol, and glycerophosphate) on acid phosphatase production in E. coli. However, similar repression is not observed in our system. Increased phosphatase activity in the stationary phase is probably the result of enzyme induction, since a similar increase in activity is not observed in the absence of glycerophosphate (Fig. 5).

Because acid phosphatase is easily eluted from the surface of S. aureus, we became interested in the cellular location of this enzyme. Our method for localizing acid phosphatase in S. aureus depended upon the formation of spheroplasts. Unlike most gram-positive bacteria, cells of S. aureus are lysozyme-resistant (Mandelstam and Strominger, 1961 and Virglio et al., 1966). The protoplasts of S. aureus formed by an autolytic system described by Mitchell and Moyle (1957) were permeable to glycerol, but not to NaCl and sucrose. Hash and his coworkers (1964) stabilized protoplasts that were formed by a fungal N-acetylhexosaminidase with 0.5 M sucrose. And recently, Schuhardt et al. (1967) formed protoplasts of S. aureus with 30% NaCl and lysostaphin. In our studies, 90% of the cells were lysostaphin-sensitive (Fig. 6). Polyethylene glycol (30%) as well as 30% NaCl were both

effective stabilizing agents, but spheroplasts could not be formed even in 2 M sucrose. Since any stabilizing agent must be unable to penetrate the cytoplasmic membrane, it is likely that sucrose, but not polyethylene glycol (PEG) and NaCl, did penetrate S. aureus PS 55.

Ultraviolet-absorption spectra of the supernatant fluids of intact cells, intact spheroplasts, lysed cells, and lysed spheroplasts indicate preservation of the osmotic barriers of spheroplasts (in 30% PEG). There is a great deal more 260 mu-absorbing material liberated when these spheroplasts are lysed in water. Apparently, increased absorption at 260 mu results from freed nucleic acid. Osmotic fragility is again demonstrated by measuring cell turbidity when spheroplasts are suspended in water. Reduction in turbidity (at 610 mu) then approaches a value comparable to that of cells broken down by lysostaphin.

When certain cellular fractions (cell wall, cytoplasmic membrane, and intracellular contents) of S. aureus were prepared after spheroplast formation, the fractions were analyzed for acid phosphatase activity. Half the activity was liberated with the lysostaphin-solubilized material (cell wall). Most of the remaining half of phosphatase activity was associated with the cytoplasmic membrane. Thus, the enzyme is not intracellular, but is located at the level of the cytoplasmic membrane. Since half the enzyme is liberated with the cell wall fraction, that half may well be combined with the cell wall, or at least be located outside the

the cytoplasmic membrane. The conclusions drawn from our studies are in agreement with those of Mitchell and Moyle (1956) who concluded that most of the acid phosphatase of S. aureus is found in the cytoplasmic membrane. A similar location for the same enzyme has been found for E. coli (Hofsten, 1961 and Dvorak et al., 1967). Neu and Heppel (1965) suggested the existence of a family of degradative enzymes on the cell surface of E. coli. Even alkaline phosphatase of E. coli is located at the cell surface (Kushnarev and Smirnova, 1966). Since the cytoplasmic membrane is considered impermeable to some phosphate esters, it is reasonable that phosphatase be located at the cellular surface (Malamy and Horecker, 1961). The same investigators noted that such a location for alkaline phosphatase of E. coli would account for the ability of the cell to preserve its intracellular pool of phosphate ester intermediates. This is a type of compartmentalization which has sometimes been suggested to account for coexistence of enzymes and substrates in bacterial cells. As noted by Cedar and Schwartz (1967), all of the bacterial enzymes so far reported to be situated in the periplasmic region are degradative, and would probably inhibit cellular function if they were not separated from the cytoplasm.

Elution of acid phosphatase from S. aureus is a function of pH and ionic strength. Salt concentrations of 1.0 to 2.0 M at alkaline pH values are optimal for elution of the enzyme. Our system of elution necessitates a higher salt concentration than 0.5 M KCl which is reported for acid phosphatase elution

from Saccharomyces mellis (Weimberg and Orton, 1965). The enzyme of yeast cells could not be eluted at higher salt concentrations unless a thiol compound were added to the eluting menstruum. The same authors (Weimberg and Orton, 1966) found great variation for conditions of acid phosphatase elution among related species of yeast cells.

Elution of acid phosphatase with a salt solution indicates the enzyme is associated at least in part with the cells through electrostatic interactions. Weimberg and Orton (1965) noted that a requirement of ionic compounds for elution of acid phosphatase from yeast cells meant the enzyme was held to the cell wall by electrostatic forces. The fraction which we eluted from S. aureus with salt solution is probably loosely bound to the cell surface and gives rise to the free fraction found in the culture medium. Rogers (1956) suggests that extracellular enzymes of S. aureus are extruded into the growth medium as a "capsular-like" material that later dissolves.

The ionic binding properties of the surface of S. aureus were studied by Cutinelli and Galdiero (1967). They showed that divalent cations combined with the cell wall of S. aureus more readily than monovalent ions. In fact, the cell wall of S. aureus behaved like a weak ion-exchange resin. It appears then, the surface of S. aureus bears a negative charge as does most bacteria. Since phosphatase is eluted with potassium and sodium ions, the enzyme is expected to have basic properties at pH 7.5. Our studies indicate that this is

indeed the case; because the purified enzyme migrates toward the cathode at pH 8.0 in a starch block (Fig. 16). Therefore, since the enzyme is associated with the cells by electrostatic interaction, it probably undergoes ionic exchange with the cations of the eluting menstruum.

Also employing ionic elution, Takeda and Tsugita (1967) liberated alkaline phosphatase from Bacillus subtilis with Mg^{++}. The one-step elution caused an increase in specific activity of the enzyme which subsequently required high salt concentration for its dissolution. The eluted enzyme which we obtained from S. aureus required at least 0.5 M KCl for its dissolution (Fig. 13). Kidwai and Murti (1965) also noted that certain oxidative enzymes of E. coli which were associated with the cytoplasmic membrane could not be solubilized by conventional means. Thus, it appears that some enzymes that are part of the cytoplasmic membrane have unusual solubility properties once they are liberated from their native site.

Solubilization of acid phosphatase in a protein-free ionic solution provides us with a major step in enzyme purification. Neu and Heppel (1965) recognized that a good first step in purification of an enzyme is its selective removal from the cells. They were able to elute certain enzymes from E. coli by their "osmotic shock" procedure.

Hofsten and Porath (1962) demonstrated limited effectiveness of precipitation procedures for purification of acid phosphatase of E. coli, but they were able to purify the

enzyme 200-fold by gel filtration and zone electrophoresis. Dvorak et al. (1967) observed two acid phosphatases in E. coli, one (hexose phosphatase) which was purified 870-fold by column chromatography, and the second (nonspecific phosphatase) which resisted purification. We were able to purify staphylococcal acid phosphatase 300-fold by employing the mild procedures of elution, dialysis, and gel filtration (Fig. 10, Table 4).

A 280/260 ratio of 1.72 indicated the purified material is essentially free from contaminating nucleic acid, and 17% of the original enzymatic activity associated with washed cells was recovered. It is noteworthy that the fraction D-I-P has a higher percent recovery (62.5%) of enzyme than the previous fraction (33.3%). Thus, some small molecular weight inhibitor(s) may be removed during dialysis. Another possibility is the disaggregation of acid phosphatase from other macromolecules that remain soluble during dialysis against dilute buffer while phosphatase is precipitated. Such aggregates could function by masking the active sites of acid phosphatase.

Purified staphylococcal acid phosphatase appeared as a homogeneous protein by gel filtration (second cycle on Sephadex G-100), starch-block electrophoresis, and analytical ultracentrifugation. Attempts to characterize purified enzyme by disc-gel electrophoresis at pH 8.3 and 7.5 were unsuccessful because the sample failed to migrate. Most of the purified material which displayed a high 280/260 ratio ($>$ 1.70) was

soluble in dilute buffer (0.05 M Tris-chloride, pH 8.0), but material with a lower 280/260 ratio (< 1.70) required a solvent having an ionic strength of at least 0.5 M for dissolution. Thus, it appears that contaminating 260 mu-absorbing material affects the solubility of acid phosphatase by requiring solutions of high ionic strength. We did obtain some evidence concerning the nature of the contaminating 260 mu-absorbing material. When a sample of the purified enzyme (dissolved in 0.6 M KCl-0.1 M Tris, pH 8.5) which had a 280/260 ratio of 1.21 was analyzed by the sedimentation velocity method in the ultracentrifuge, contaminating material was of high molecular weight, and acid phosphatase appeared as one sharp symmetrical schlieren pattern (top of Fig. 17). The contaminating fraction was probably ribosomal material. The presence of ribosomes is reasonable because in order to obtain sufficient protein (3.5 mg/ml) for adequate resolution in the ultracentrifuge, we combined chromatographic fractions (second cycle) which had high enzymatic activity and a low 280/260 ratio (1.10) with those that had high enzymatic activity and a high 280/260 ratio (1.72). However, it is unlikely that the ribosomal material is present in purified fractions having a 280/260 ratio of 1.72.

Approximations of the molecular weight (MW) of purified acid phosphatase by two different methods were comparable: 54,000 by gel filtration and 53,000 by analytical ultracentrifugation. The acid phosphatase purified by Hofsten and Porath (1962) from E. coli has a MW of 13,000 (estimated

by amino acid analysis). Their enzyme is quite different because it is unstable in dilute solutions, stable in 1 M acetic acid, and denatured in the presence of neutral salts. Staphylococcal acid phosphatase, on the other hand, is relatively stable in water and 1 M KCl, but inactivated in the presence of 1 M acetic acid (Table 9).

Dvorak et al. (1967) isolated two acid phosphatases from E. coli: hexose phosphatase and nonspecific phosphatase. The latter enzyme more closely resembles the staphylococcal acid phosphatase. Like the enzyme from E. coli, acid phosphatase from S. aureus has a pH optimum near pH 5 (Fig. 18), is inhibited by EDTA (Table 7), and readily hydrolyzes p-nitrophenyl phosphate (Table 10). The two enzymes differ in that activity of the staphylococcal enzyme is stimulated 2-fold by Cu^{++} (Table 8), whereas metals have no effect on the enzyme of E. coli. Cu^{++} not only stimulated enzymatic activity, but also gave greater stability to the enzyme between 50 and 80 C (Fig. 24). The native staphylococcal enzyme may contain Cu^{++}, just as alkaline phosphatase of E. coli contains Zn^{++} (Plocke and Vallee, 1962).

Ionic strength has little effect on staphylococcal acid phosphatase activity up to 1.0 M KCl or NaCl (Fig. 19). However, progressive loss of enzymatic activity does occur at higher salt concentrations. There is a rather narrow pH range (5.2 to 5.3) for optimal activity (Fig. 18). Though optimal activity occurs at acidic pH values, the enzyme is more stable in a slightly alkaline menstruum (Table 9). Both

extremes of the pH scale completely inactivate staphylococcal acid phosphatase.

As noted by Dixon and Webb (1964), the effects of temperature on enzyme reactions are very complex. Deleterious effects due to instability of the enzyme itself can be studied by first exposing the enzyme to various temperatures for a definite period of time and then measuring its activity at a temperature in which it is stable. Discontinuity of the slope of an Arrhenius plot (Fig. 21) at 48 C suggests irreversible inactivation of the enzyme since it loses its stability rapidly at temperatures above 50 C (Fig. 24). The Arrhenius function A (energy of activation) for staphylococcal acid phosphatase is 19,500 cal/mole. This value is reasonable, because the values of A for ordinary chemical reactions (including catalytic ones) range from a few thousand to 40,000 cal/mole, with the majority in the neighborhood of 15,000 to 25,000 cal/mole (White, Handler, and Smith, 1964).

Values for either the Michelis constant (K_m) or maximal velocity (V_{max}) were the same on both Eadie-Hofstee and double reciprocal plots of initial velocities of acid phosphatase against p-nitrophenyl phosphate. The K_m was 4.5×10^{-4} M, and V_{max} was 4.4×10^{-2} uM P_i liberated/min (Fig. 22 and 23). Barnes and Morris (1957) reported the K_m for p-nitrophenyl phosphate was 2.0×10^{-4} M. However, as previously noted, the enzyme preparation used in their studies was a suspension of whole cells. Statistical methods for obtaining K_m and V_{max} were not applied to our data since the graphical methods proved

adequate. In this regard, Dixon and Webb (1964) state that for nearly all purposes, graphical methods suffice for determining K_m and V_{max}. These authors prefer the double reciprocal plot to the Eadie-Hofstee plot.

Staphylococcal acid phosphatase probably requires a free sulfhydryl (-SH) group for maximal activity. Iodoacetate which usually reacts with thiol groups to give alkylated derivatives (Dixon and Webb, 1964) proved to be the most effective inhibitor (Table 7), and mercaptoethanol which usually preserves -SH groups had a stimulatory effect. Since EDTA (a metal chelator) also inhibited enzymatic activity, the enzyme may be more active in the presence of certain metals. As noted earlier (Table 8), acid phosphatase is twice as active in the presence of Cu^{++} than in its absence. The Law of Mass Action could explain the inhibition of inorganic phosphate in high concentration. Other compounds tested had little or no inhibitory effect on staphylococcal acid phosphatase.

Relative activity of the enzyme with the different substrates tested (Table 10) gives little insight into the natural substrate and role of the enzyme in vivo. Acid phosphatase was most reactive against p-nitrophenyl phosphate and glyceraldehyde 3-phosphate. This writer is aware of no biological system where the latter compound is a normal substrate for phosphatase.

The role of acid phosphatase in microorganisms is still a matter of conjecture. The enzyme in S. aureus may play some

important biological and ecological role, since some strains produce significant amounts of the enzyme. The enzyme may render some compounds more readily available to the cells, for many phosphorylated esters cannot cross the cytoplasmic membrane. The presence of enzyme in the cytoplasmic membrane and in the culture medium suggests that it may degrade organic phosphates present in the growth medium. A similar function has been proposed for acid phosphatase of E. coli. Yet, the cytoplasmic membrane of the same organism is permeable to glucose 6-phosphate, and alkaline phosphatase is not related to the entry of the phosphorylated ester into the cell (Fraenkel, Kelly and Horecker, 1964). Staphylococcal acid phosphatase may even be a part of the "translocase" mechanism involved in phosphate transfer across the cell membrane as proposed by Mitchell (1957). Heppel (1967) recently noted that proteins of E. coli found at the "surface of the cell are capable of binding with substances in the medium, and these proteins may be components of active transport systems responsible for the concentrative uptake of these nutrients." We believe, as does Kedzia and his coworkers (1966), that staphylococcal acid phosphatase plays an important role in aiding the penetration of certain phosphorylated compounds into the bacterial cell by hydrolyzing the esteric bond, thereby making the products more readily available for uptake by the cells.

SUMMARY

Strains of Staphylococcus aureus from the International-Blair and the Seto-Wilson Series of phage-propagating strains (PS) were examined for acid phosphatase activity. All selected strains showed enzyme production after 24 hr of growth, and when all samples were adjusted to the same optical density (0.5), PS 3A surpassed all others in enzyme activity. Acid phosphatase occurred in varying amounts in three different fractions: free (6 to 60%), loosely bound (25 to 82%), and firmly bound (0 to 40%).

Rate of whole culture enzyme production paralleled cell growth in Trypticase Soy Broth. In the casein acid-hydrolysate medium supplemented with glycerophosphate, initial production of enzyme during logarithmic growth was followed by increased production during the stationary phase of the growth cycle. This biphasic pattern was not observed in the absence of glycerophosphate.

Spheroplasts of PS 55 were made in the presence of lysostaphin and 30% polyethylene glycol. Following spheroplast formation and controlled lysis of the spheroplasts, half the total phosphatase was located in the cell wall fraction, and most of the remaining enzyme (48%) was associated with the cytoplasmic membrane.

Loosely bound acid phosphatase was purified 300-fold by elution, dialysis, and two passages through Sephadex G-100.

The specific activity of purified enzyme was 2350 and approximately 17% of the initial activity (loosely and firmly bound) was recovered. Purified acid phosphatase appeared homogeneous by gel filtration, starch-block electrophoresis, and analytical ultracentrifugation.

Maximal enzymatic activity occurred at pH 5.2, between 45 and 50 C. The enzyme was most stable in the alkaline pH range and at temperatures below 50 C. Iodoacetate and EDTA were potent inhibitors, but mercaptoethanol and Cu^{++} stimulated enzymatic activity. Purified enzyme was basic in nature since it migrated toward the cathode at pH 8.0 in a starch block. The enzyme was most active against the substrates p-nitrophenyl phosphate and glyceraldehyde 3-phosphate. K_m for p-nitrophenyl phosphate was 4.5×10^{-4} M, and the Arrhenius function A (energy of activation) for the hydrolytic reaction was 19.5 Kcal/mole. Approximations of the molecular weight made by gel filtration and analytical ultracentrifugation were 54,000 and 53,000 respectively.

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