AN ABSTRACT OF THE THESIS OF

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Title: PURIFICATION AND CHARACTERIZATION OF AN ALKALINE PHOSPHATASE FROM HALOBACTERIUM SALINARIUM

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The halophilic alkaline phosphatase of Halobacterium salinarium has been purified to a specific activity of 3,000-3,200 units per mg of protein. The purification scheme consisted of acetone and ammonium sulfate fractionations, and molecular sieving and adsorption chromatography on Sephadex G-50. The enzyme was stabilized during purification by the presence of 0.01 M manganous chloride. The purified preparation exhibited a constant specific activity across the final protein peak on Sephadex G-50 and appeared well over 95% pure upon disc-gel electrophoresis.

Amino acid analysis of the purified enzyme revealed a composition very similar to other halophilic proteins which have been studied. The phosphatase was similarly high in the acidic amino acids, glutamic and aspartic acids.

A detailed kinetic study revealed the enzyme's obligatory requirement of manganous ion for activity. Its affinity for this metal ion was found to be strongly pH and salt concentration dependent. The salt concentration dependency was interpreted as reflecting the charge shielding of electrostatic interactions on the enzyme surface which when unshielded distort the conformational features of the manganese binding site. When saturated with manganese, the enzyme exhibited an optimal activity at pH 8.5.

Stability studies on the apoenzyme demonstrated that the stability of this species was strongly dependent on the nature and concentration of the stabilizing salt. The apoenzyme was stabile only in sodium and ammonium sulfates and in sodium and potassium chlorides. The salt concentrations required for apoenzyme stability were all well in excess of 3 N. The function of high salt concentration in the stabilization of the alkaline phosphatase was interpreted as being the result of salting-out effects of salt solutions. These effects were believed to diminish solvent-protein interactions thereby strengthening the intramolecular interactions required for the conformational stability of the enzyme.

Purification and Characterization of an Alkaline Phosphatase from *Halobacterium salinarium*

by

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PURIFICATION AND CHARACTERIZATION OF AN ALKALINE PHOSPHATASE FROM HALOBACTERIUM SALINARIUM

I. INTRODUCTION

The extremely halophilic enzymes are unique proteins in that they often require the presence of as much as 4.5 M sodium chloride for structural stability. The major objectives of this work were to purify such an enzyme, to study the basic features of salt stabilization, and to interpret such stabilization on the bases of the nature of the salt and the physical and chemical nature of the enzyme. An alkaline phosphatase of Halobacterium salinarium was selected for this study.

Aspects of Halophilism

The aspects of "halophilism" have been reviewed by Larsen (18, 19). Obligately halophilic organisms require the presence of sodium chloride to exhibit optimum growth. Based on the level of the salt required, they may be designated as slightly, moderately, or extremely halophilic. The extreme halophiles have been of most interest, for they exhibit optimum growth only in culture medium containing over 20% sodium chloride. This group is restricted to three bacterial genera: Halobacterium of the family Pseudomonadaceae and Micrococcus and Sarcina of the family

Micrococcaceae (4). The species of the genus Halobacterium are perhaps the most well characterized of the extreme halophiles. They grow optimally in the presence of 25% sodium chloride and lyse when exposed to hypotonic solutions. The intracellular salt level of the Halobacterium species has been shown to approach that of the growth medium (7, 19). The metabolic apparatus must then be constructed to withstand the presence of high salt concentrations and function under these conditions.

To date, the cell envelopes, ribosomal particles, a number of enzymes, and an amino acid incorporating system (2) of Halobacterium species have been studied. The results from these studies indicate that exceedingly high salt levels, often approaching 4.5 M sodium chloride are required to maintain the integrity or activity of the system. It then appears that halophilic systems actually require high salt levels to maintain their structural integrity. The role of salt in such stabilization has not been clearly demonstrated. The results of amino acid analyses of halophilic cell envelope (17) and ribosomal protein (1) show a high content of glutamic and aspartic acids, which suggest that the function of salt is to shield destabilizing electrostatic interactions, much like salt stabilization of DNA. Several observations (12, 19), however, suggest that additional salt effects are also exceedingly important in the halophilic system.

Studies on Extremely Halophilic Enzymes

The high intracellular salt concentration of the Halobacterium species presents a number of interesting aspects concerning the synthesis, function, and stability of halophilic enzymes in the presence of such salt levels. The folding of the polypeptide chain in the presence of 25% sodium chloride is an interesting aspect of protein synthesis. The reactivation studies with malate dehydrogenase of H. salinarium are of particular interest in this regard (12). Reactivation of the enzyme was found to be a strong function of the nature and concentration of the salt. Only potassium and sodium chlorides above 2.5 M reactivated the enzyme at measurable rates. These results suggest that the high salt concentration actually induces proper folding of the enzyme.

The functional salt requirements of a number of halophilic enzymes have been reviewed (18, 19). The enzymes studied were found generally to exhibit optimal activity somewhere between one and four molar sodium chloride. A number of salts other than sodium chloride were also found capable of meeting the functional salt requirements of these enzymes. When compared with similar enzymes from lesser halophilic and non-halophilic organisms, the halophilic enzymes were also found to exhibit a greater functional salt-tolerance to high salt concentrations.

Studies concerning salt requirements for halophilic enzyme stability remain incomplete. However, there appears at the moment to be little correlation in regard to the nature and concentration of the salt required for optimal activity and those required for optimal stability. The malate dehydrogenase of H. salinarium is an interesting example. It exhibits an optimal activity in about 1 M sodium chloride and an optimal stability in 4.5 M sodium chloride (12). The enzyme has been reported to be stabile only in sodium and potassium chloride.

Purification of extremely halophilic enzymes has been an exceedingly difficult task because of the high salt levels required for the stability of these enzymes. Holmes and Halvorson (13) have lately reported the purification of the malate dehydrogenase from H. salinarium. The enzyme was purified as an inactive, low-salt form which could be reactivated to 60% of the original activity by dialysis against 4.5 M sodium chloride. However, the overall recovery of malate dehydrogenase activity was very low, and the procedure did not appear technically attractive for this work.

Salt Effects on the Structural Stability of Protein

Salt effects on the conformational stability of macromolecules are of particular interest in the extremely halophilic system. These effects have been reviewed by von Hippel (30). To date, salt effects

on several fibrous and globular proteins, and on a number of model polymers and small molecular weight compounds have been studied. These studies suggest that salt effects on the conformational stability of proteins are the result of electrostatic and lyotropic or Hofmeister effects. Electrostatic effects are simply charge shielding effects exhibited by all ions in solution. Such effects are a function of the charge character of the ions and are of major importance below 1 M ion concentration. As previously stated, charge shielding has been suggested as the major role of salt in the halophilic system.

In contrast to electrostatic effects, lyotropic effects appear independent of charge character and arise from other ionic properties. Schrier and Schrier have suggested (27) that lyotropic effects are actually the result of salt-peptide interactions and "salting-out" effects. Salt-peptide interactions appear to be purely electrostatic in nature arising from the interaction of ions with the peptide dipole. These interactions are a function of the charge character of the ions and tend to pull peptide linkages from the interior of the protein into contact with the solvent where such interactions may be maximized. The effect would therefore be primarily disruptive in nature.

In contrast to salt-peptide interactions, salting-out effects are independent of charge character of the ion and account for such apparent charge independence of lyotropic effects. Salting-out effects are indirect in nature and arise from salt induced changes in

solvent properties. The result is a decrease in solvent-protein interactions accompanied by strengthening of the intramolecular hydrophobic interactions within the protein. Salting-out is then primarily stabilizing in nature. The difference in the magnitudes of the salt-peptide interactions and the salting-out effects is then believed to determine the direction and magnitude of lyotropic effects.

The electrostatic and lyotropic effects are obviously of particular interest in the interpretation of "halophilism", for halophilic enzymes might be expected to use either or both the charge shielding and salting-out features of salt for stabilization. An alkaline phosphatase of Halobacterium salinarium was selected to determine the importance of these effects in the salt stabilization of halophilic enzymes. The phosphatase was purified using organic solvent fractionation, salt fractionation, molecular sieving, and adsorption chromatography techniques. The amino acid composition of the enzyme was determined. The kinetics and stability of the enzyme were studied in considerable detail.

II. MATERIALS AND METHODS

Materials

Halobacterium salinarium was a gift of Dr. H. O. Halvorson, Department of Microbiology, University of Illinois, Urbana, Illinois. Sodium chloride for the cultivation of H. salinarium was a bulk grade purchased from Diamond Salt Company, St. Clair, Michigan. Yeast extract and casamino acids were purchased from Difco Laboratories, Detroit, Michigan. Phosphatase substrate (p-nitrophenyl phosphate) was Sigma's "104" phosphatase substrate purchased from Sigma Chemical Company, St. Louis, Missouri. Phenol reagent (Folin and Ciocalteu reagent) was purchased from Van Waters and Rogers Inc., San Francisco, California. All other salts used for cultivation and enzyme work were reagent grade purchased from Baker-Adamson, Allied Chemical Company, Morristown, New Jersey.

Tris(hydroxymethyl)aminomethane (Tris), and imidazole were purchased from Sigma Chemical Company. Diethanolamine, disodium ethylenediaminetetraacetate (EDTA), and trichloroacetic acid (TCA) were from J. T. Baker Chemical Company, Phillipsburg, New Jersey. Glycylglycine was a product of Calbiochem, Los Angeles, California.

Sephadex G-50 fine was purchased from Pharmacia, Uppsala, Sweden. Columns were purchased from Kontes Glass Company,

Vineland, New Jersey.

UM-1 and UM-2 ultrafilters were purchased from Amicon Corporation, Lexington, Massachusetts. Millipore filters one inch in diameter with a 0.1μ pore size were purchased from the Millipore Corporation, Bedford, Massachusetts.

Reagents used in disc-gel electrophoresis were reagent grades made up as recommended by Canalco (5). N,N'-methylenebisacrylamide, acrylamide, and riboflavin were EastmanOrganic Chemicals purchased from Distillation Products Industries, Rochester, New York. Glycine was from Matheson, Coleman and Bell, Norwood, Ohio. All other materials for disc gel were purchased from Canalco, Canal Industrial Corporation, Bethesda, Maryland.

Size 18/32 and size 1-7/8 S.S. dialysis tubing were products of Union Carbide, Food Products Division, Chicago, Illinois.

Methods

Operational Assay

Halophilic alkaline phosphatase activity was measured by a method of Schlesinger (26) adapted to the halophilic enzyme. The assay solution contained 2 M NaCl, 0.1 M Tris at pH 8.5, and 0.2 mg per ml p-nitrophenyl phosphate. During purification the enzyme was diluted to 0.1-1 units per ml with a solution containing 0.2 M

NaCl, 0.1 M Tris at pH 8, and 0.01 M $MnCl_2$ at 0°C. Because of the instability of $MnCl_2$ at this pH, it was added just prior to the addition of enzyme by appropriate dilution of a 1 M solution. One tenth of one ml of diluted enzyme was added to three ml of the assay solution and the mixture incubated at 25°C for ten minutes. Eight-tenths of one ml of 13% K_2HPO_4 was then added and the optical density determined at 400 mμ using a Spectronic 20 (Bausch & Lomb, Model 340) with Hycel cuvettes (Hycel Corporation, Houston, Texas). One unit of activity was defined as one optical density change per minute. Since 0.1 ml of diluted enzyme was used for the assay, the optical density reading after ten minutes gave directly the number of units present per ml. Enzyme activity assayed in this fashion was proportional to the amount of enzyme between one tenth and one unit per ml.

Protein Determination

Protein was determined by the method of Lowry (20) using bovine serum albumin as a standard. Protein in samples containing high ammonium sulfate levels was first precipitated by adding 50% trichloroacetic acid (TCA) to a final concentration of 10%. The precipitated protein was centrifuged in a clinical centrifuge, and the pellet washed once with one ml of 10% TCA. Sufficient glass distilled water was then added to bring the protein concentration within

20-150μ g per ml. A small amount of saturated NaOH was added to samples requiring more than one ml of water to dissolve the protein. Lowry alkaline-copper reagent was added directly to samples taken up in one ml of water. The specific activity of all preparations are given in units per mg of protein.

pH Adjustments

The pH of all solutions used in enzyme assay and purification were adjusted at room temperature using a Corning Model 12 pH meter equipped with Corning glass-calomel electrodes. Solutions used in kinetic and stability studies were adjusted at room temperature using a Corning semi-micro combination electrode.

Disc-gel Electrophoresis

Disc-gels were prepared as recommended by Canalco (6). The standard 7% acrylamide gel which stacks at pH 8.9 and runs at 9.5 was selected for all work. Electrophoresis was done at 4°C using one milliampere per tube.

III. EXPERIMENTAL AND RESULTS

Studies on Crude Enzyme Preparations

Cell Growth and Enzyme Production

Growth of H. salinarium at 37°C in the complex media of Halvorson and Kushner and in the defined medium of Gibbons[1] was followed over a 40 hour period. Five ml aliquots of each culture were taken at convenient time intervals, and the optical densities measured at 525 mμ using a Spectronic 20 with Hycel cuvettes. The cells were then collected by centrifuging at 8,000 x g and the cellular levels of alkaline phosphatase determined. Each cell pellet was taken up in one ml of 4 M NaCl containing 0.1 M Tris at pH 8. The cells were lysed by the slow addition of four ml of Tris buffer pH also 8. Cell collection and lysis were done at 0°C. One-tenth of one ml of this lysate was added to three ml of assay solution and the alkaline phosphatase activity assayed as described under operational assay. $MnCl_2$ was omitted. Cell growth and alkaline phosphatase production in these media are shown in Figures 1 and 2. Enzyme level is given in units of enzyme per optical density at 525 mμ and represents a cellular specific activity.

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[1] For media see Appendix, pages 78-79.

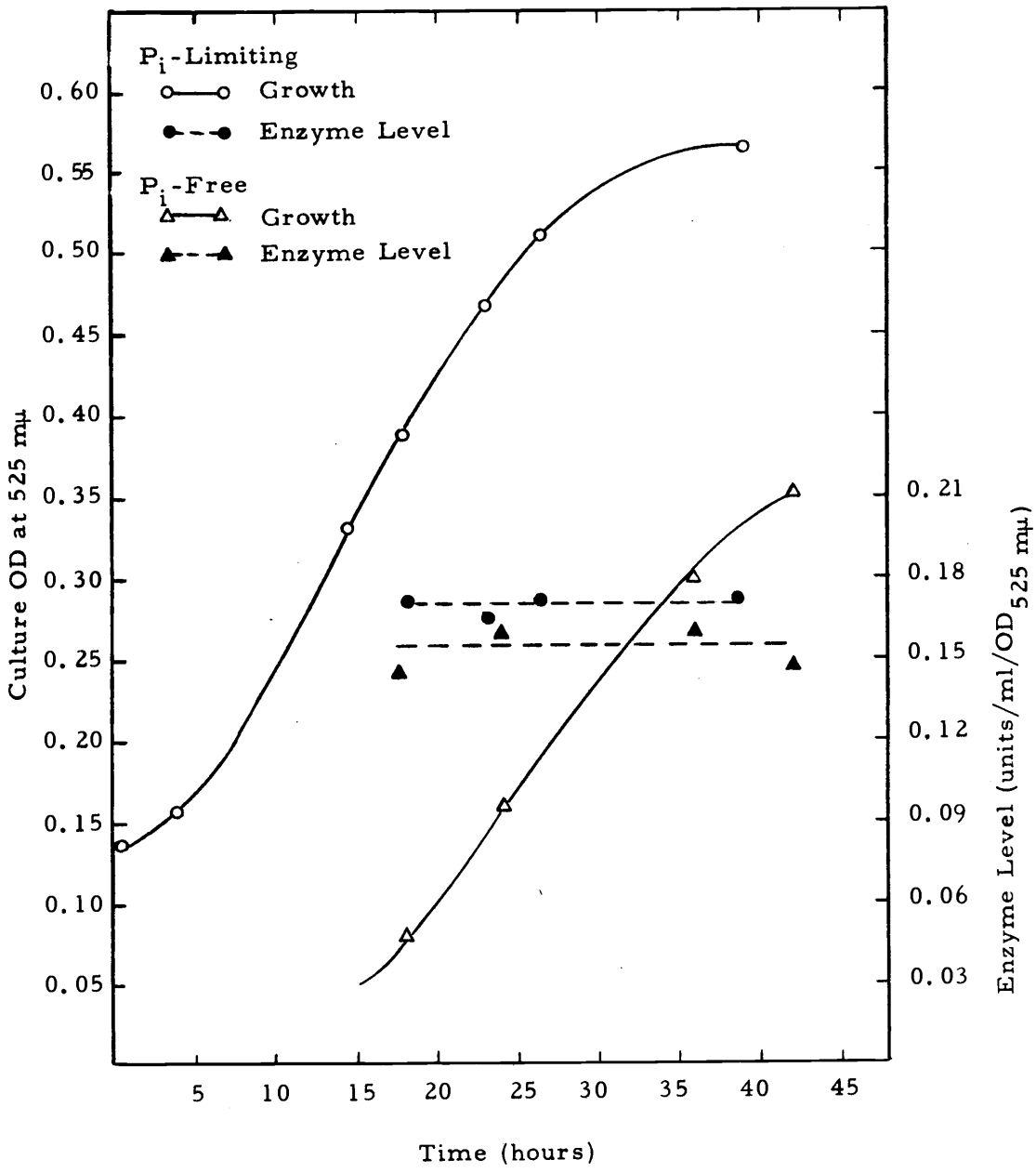

Figure 1. Cellular Alkaline Phosphatase Level During Phosphate Limiting Growth of H. salinarium in Defined Media.

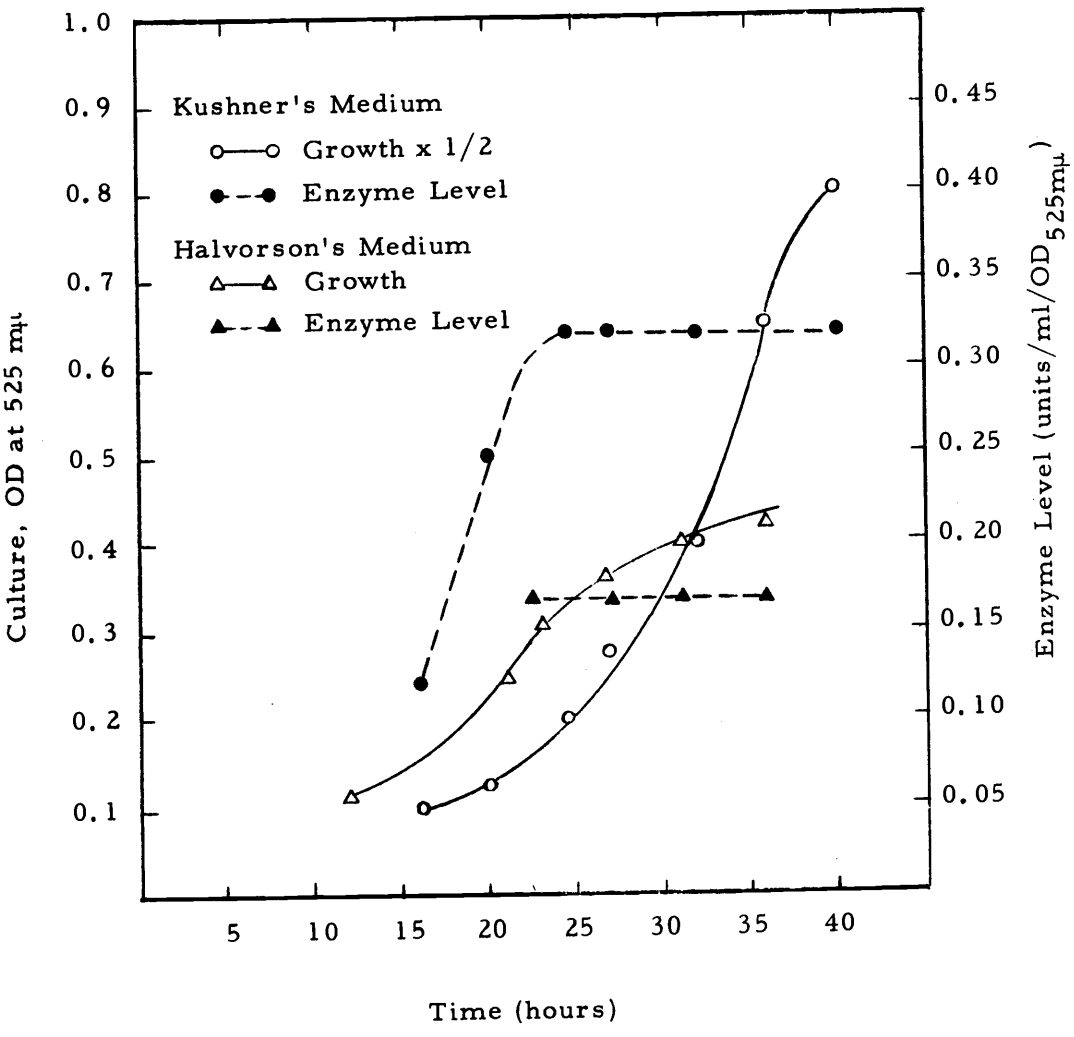

Figure 2. Cellular Alkaline Phosphatase Level During Growth of H. salinarium in Complex Media.

Enzyme Activation and Inhibition

The following salts were tested for activation and inhibition of halophilic alkaline phosphatase activity in crude preparations: $MnCl_2$, $ZnCl_2$, $MgCl_2$, and $CaCl_2$. The crude extract was prepared by lysing the cells as described. The lysate was then centrifuged at 30,000 x g to clarify the solution. One ml of this preparation was transferred to one ml of a solution containing 4.5 M NaCl, 0.1 M Tris at pH 8, 0.01 M of the metal being tested, and sufficient solid NaCl to bring the final concentration to 4.5 M. The crude preparations were incubated at 25°C for one minute. One-tenth ml aliquots were then transferred to three ml of assay solution, and the enzyme activity was measured as described. The final metal ion concentration in the assay solution was 0.16 mM. Results are shown in Table 1.

Table 1. Activation and Inhibition of the Halophilic Alkaline Phosphatase.

(M^{++})	Metal	Units of Activity
0.16 mM	$MnCl_2$	0.27
"	$ZnCl_2$	0
"	$MgCl_2$	0.19
"	$CaCl_2$	0.19
0	None	0.17

Enzyme Stabilization by Manganese

Manganous ion was tested for its ability to stabilize halophilic alkaline phosphatase in 4.5 M NaCl at 25°C and in 0.2 M NaCl at 0°C. Both solutions contained 0.1 M Tris at pH 8. A crude extract was prepared as before and centrifuged to clarify the solution. One ml of the extract was transferred to one ml of 4.5 M NaCl containing sufficient solid NaCl to bring the final level to 4.5 M, or was diluted with Tris buffer to 0.2 M NaCl. $MnCl_2$ was added in 0.1 ml of a solution at the appropriate manganese concentration to give the levels shown in Figures 3 and 4. The activity of the preparations was determined as a function of time using the operational assay.

Lysis of H. salinarium

Larsen (19) reported that cell lysis was complete at 5% (.85 M) NaCl. However, in the presence of 0.01 M $MnCl_2$, added to stabilize the enzyme, complete lysis did not appear to occur at this level. To obtain more detailed information, the release of alkaline phosphatase activity during cell lysis was studied in the presence of sodium and potassium chlorides. Cells grown in 400 ml of Kushner's medium were collected by centrifuging at 8,000 x g, washed with 25% NaCl containing 1% $MgSO_4$, and resuspended in 25 ml of 4 M NaCl for NaCl lysis or 18 ml of 3 M KCl for KCl lysis. Each

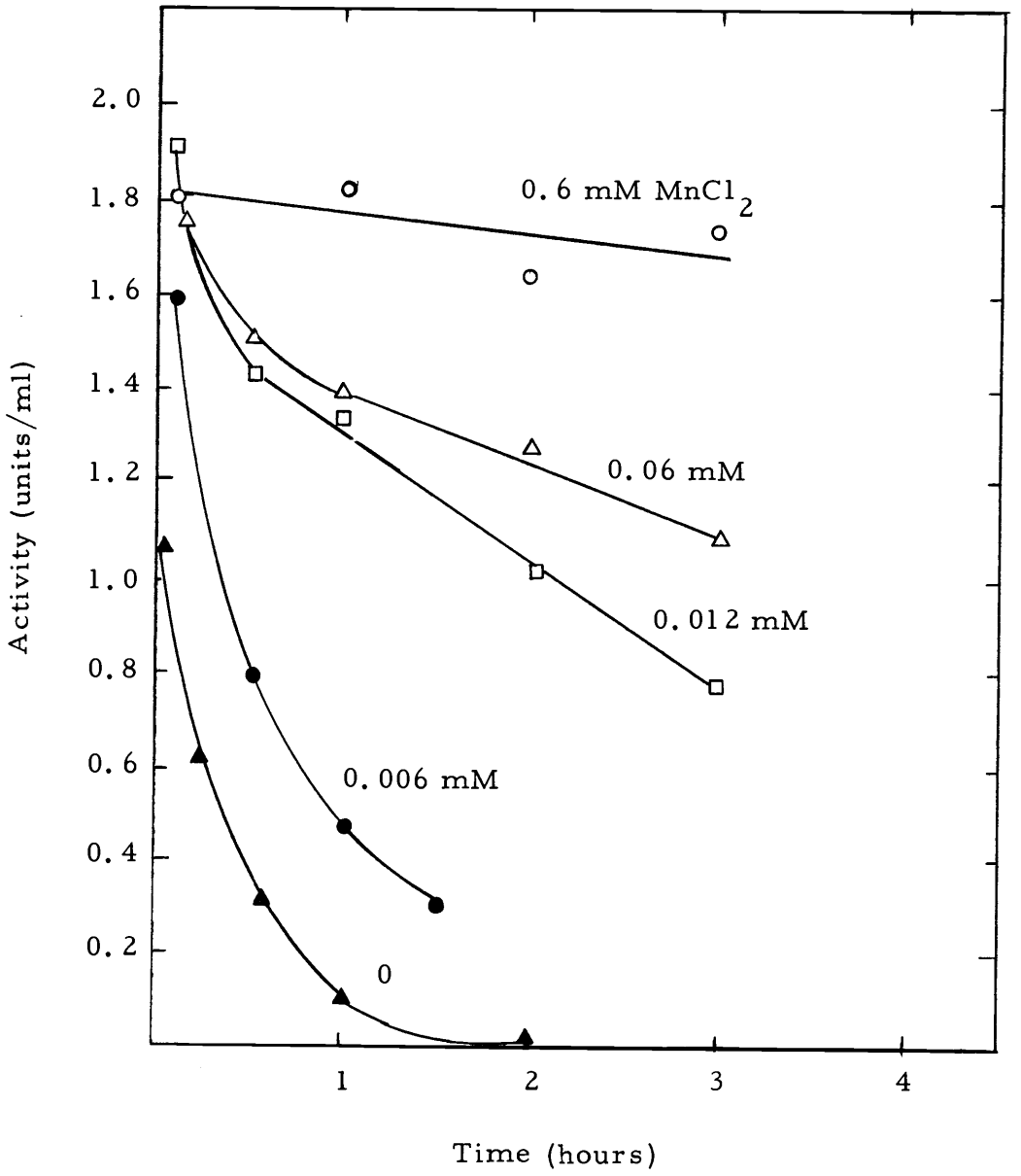

Figure 3. Effect of Mn^{++} on the Stability of Halophilic Alkaline Phosphatase in 4.5 M NaCl.

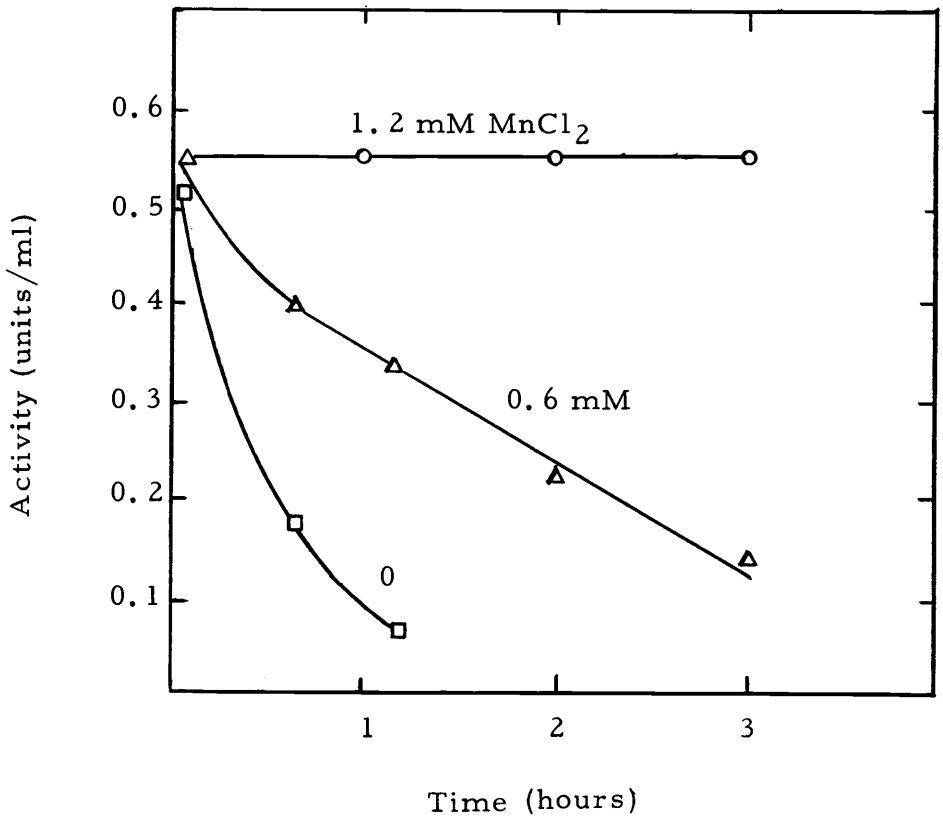

Figure 4. Effect of Mn^{++} on the Stability of Halophilic Alkaline Phosphatase in 0.2 M NaCl.

solution also contained 0.1 M Tris at pH 8 and 0.01 M $MnCl_2$. Higher potassium chloride levels were not used, for 3 M KCl was near saturation at $0^{o}C$. A five ml aliquot was withdrawn and the cell suspension lowered by 0.5 M in salt concentration by the slow addition of 0.1 M Tris buffer at pH 8 containing 0.01 M $MnCl_2$. A second five ml aliquot was taken and this procedure repeated until the cell suspension reached 0.5 M in salt concentration. Adequate buffer was then added to reduce the salt level to 0.2 M and the final aliquot taken. Aliquots were centrifuged to remove unlysed cells. One-tenth of one ml from each lysate was then transferred to three ml of assay solution and the activity measured as described. Results were corrected for dilution during lysis and are recorded in Figure 5 as relative percent of the total activity released. Enzyme release was 86% complete in 0.2 M NaCl and 95% complete in 1 M KCl.

Enzyme Purification

Cultivation of H. salinarium

Cells were grown in ten liter batches using Kushner's medium supplemented with 20 mg per liter $MnCl_2$. Thirteen 2800 ml Fernbach flasks containing 800 ml of autoclaved medium were each inoculated with 50 ml of an inoculum culture grown for 30 hours and

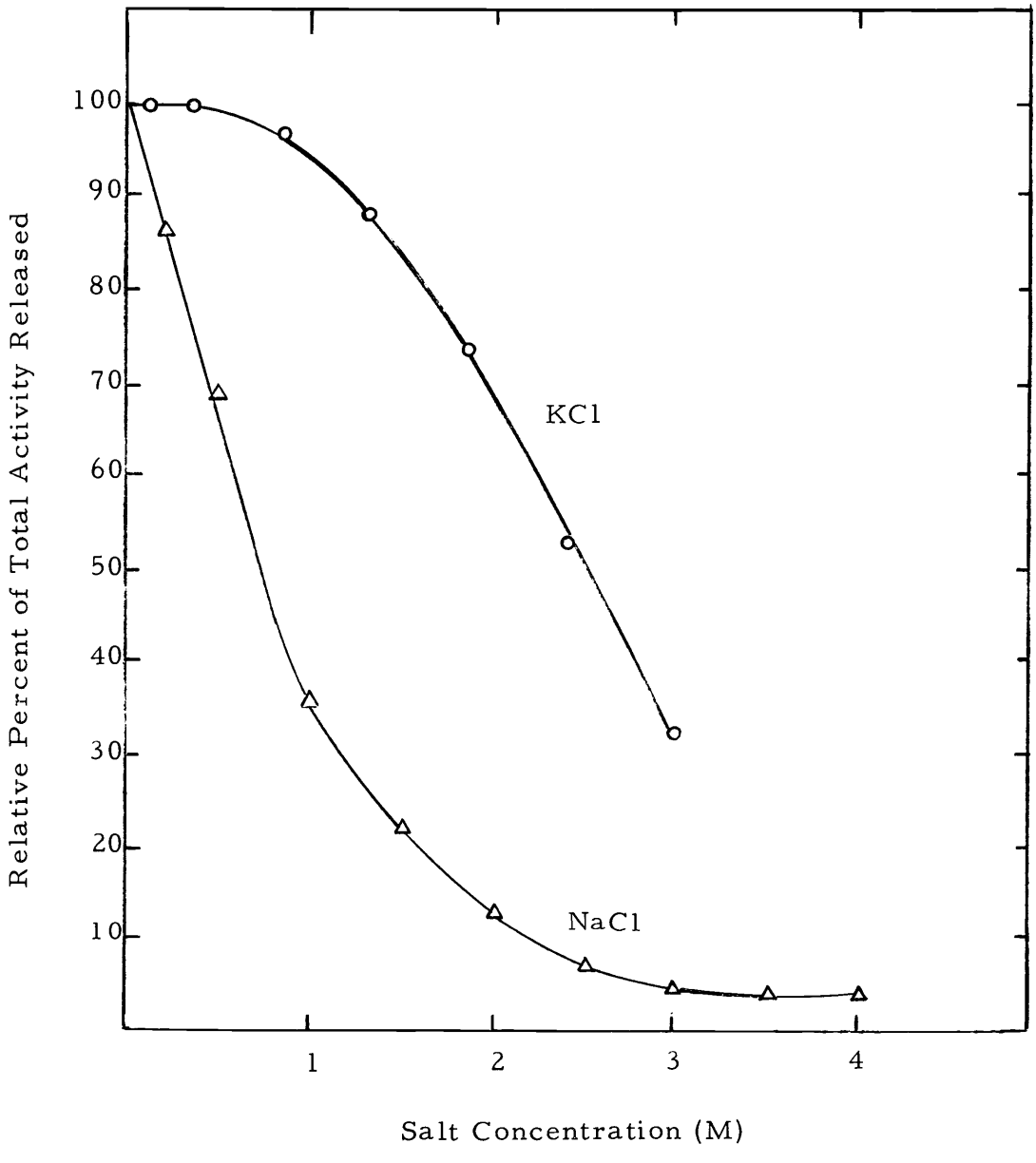

Figure 5. Lysis of H. salinarium.

stored at 4°C until used. The inoculated Fernbach flasks were shaken on a shaker table at 37°C until the optical density at 525 mμ reached 1.7-1.8. This generally required about 30-36 hours. Optical densities were measured by withdrawing two ml of culture medium and diluting it with two ml of a solution containing 25% NaCl and 1% $MgSO_4$. Cultures were then removed from the shaker and stored at 4°C. Cells were collected within three days from the beginning of storage in six 250 ml bottles using a Sorvall GSA rotor cooled to 4°C. Cells were centrifuged at 8,000 x g for 15 minutes, and the supernatant solutions discarded. Additional culture medium was poured over the previous cell pellet and centrifuged as before with pellets outward. This procedure was repeated until ten liters of cells were collected.

Cell Lysis and Preparation of the Crude Extract

Each pellet above was taken up in 30 ml of 3 M KCl solution at 0°C containing 0.1 M Tris at pH 8 and 0.01 M $MnCl_2$. Manganous chloride was added by appropriate addition of a 1 M solution just prior to use. The bottles were washed with an additional 70 ml of the KCl solution described, and the pellets and washings combined. The cells were then evenly suspended by intermittent, vigorous stirring over a 30 minute period at 0°C. When suspension was complete, 500 ml of 0.1 M Tris buffer at pH 8 and 0°C containing 0.01 M

$MnCl_2$ were slowly added with vigorous stirring. The lysate was then stirred for an additional ten minutes, transferred to 50 ml centrifuge tubes, and centrifuged with an SS-34 Sorvall rotor at 30,000 x g for 30 minutes. The highly viscous supernatant solution was collected and dialyzed against 1,500 ml of a solution containing 0.2 M NaCl, 40 g per liter $(NH_4)_2SO_4$, 0.05 M Tris buffer at pH 8, and 0.01 M $MnCl_2$. Manganese was added as the dry salt just prior to dialysis. Dialysis was done at 0°C using size 1-7/8 S.S. dialysis tubing equipped with a stirring rod inside turned by an overhead stirrer, and a magnetic stirring bar on the outside. A two liter cylinder packed in ice was used as the dialysis vessel. After a period of four hours, the dialysis solution was replaced by an additional 1,500 ml of the solution described. Dialysis was continued for an additional eight to ten hours, and the extract treated as outlined.

Acetone and Ammonium Sulfate Fractionations

Acetone, cooled to -10°C in a salt-ice bath, was added slowly in 100 ml volumes to the dialyzed extract until it reached 40% by volume. During the fractionation procedure, the extract was cooled in a salt-ice bath and stirred with a thermometer. The rate of acetone addition was adjusted to maintain the temperature between 0-4° for the first fractionation. The precipitate was removed by centrifugation at 8,000 x g using 250 ml bottles and a Sorvall GSA

rotor refrigerated at -10°C. The supernatant fluid was collected and acetone added until the concentration was 55% by volume. The temperature for the second fractionation was maintained at -5°C. The precipitate was collected by centrifuging at 4,000 x g. Routinely, the rotor was accelerated to the desired speed (4,000 x g) and then immediately decelerated with the centrifuge brake system. This prevented hard packing of the pellet and permitted greater ease in resuspension of the precipitate as well as greater recovery of enzyme activity. Each pellet was resuspended in 20 ml of 60% saturated $(NH_4)_2SO_4$ containing 0.01 M $MnCl_2$. Because of the extreme insolubility of the precipitate in this solution, the pellets were first resuspended with a pipet, and then transferred to a Potter-Elvehjem type homogenizer and homogenized. The insoluble material was removed by centrifuging at 30,000 x g for 15 minutes. The supernatant solutions were pooled and taken to 82% saturation by the addition of 125 ml of saturated $(NH_4)_2SO_4$ per 100 ml of supernatant volume. Precipitation was complete within 15 minutes. The precipitated protein was collected by centrifuging at 30,000 x g for 15 minutes. The pellets were then taken up in 50 ml of 50% saturated $(NH_4)_2SO_4$ containing 0.01 M $MnCl_2$, and the insoluble material was removed by centrifugation. Seventy-five ml of saturated $(NH_4)_2SO_4$ were added slowly, and the precipitate was collected as before. The pellets were taken up in seven ml of 25% saturated $(NH_4)_2SO_4$ and centrifuged

to remove the insoluble material. The enzyme solution was then equilibrated by dialysis (size 18/32 dialysis tubing) against 40% saturated $(NH_4)_2SO_4$.

Ammonium sulfate fractionations were carried out at 0-4°C. Saturated $(NH_4)_2SO_4$ used in this work was prepared by adding 750 g of $(NH_4)_2SO_4$ and 12 g of Tris buffer to one liter of glass distilled water. The pH was adjusted to 8.3, and the solution stored at 4°C for several days before use. Solutions of lower saturation were prepared by appropriate dilution of the saturated solution. $MnCl_2$ was again added as a 1 M solution just prior to use.

Resolution on Sephadex G-50

Three columns were prepared with Sephadex G-50 fine. Column I was 2 x 100 cm with a bed volume of 300 ml and was equilibrated with 40% saturated $(NH_4)_2SO_4$ containing 0.01 M $MnCl_2$, and 0.1 M Tris buffer at pH 8. Column II was 2.5 x 100 cm with a 450 ml bed and was also equilibrated with this solution. Column III was 2 x 100 cm again with a bed of 300 ml and was equilibrated with 50% saturated $(NH_4)_2SO_4$ containing 0.01 M $MnCl_2$ and 0.1 M Tris buffer at pH 8. Ammonium sulfate solutions were prepared according to Green *et al.* (10). They were stored at 0-4°C for several days and filtered prior to use. $MnCl_2$ was added as a 1 M solution again just prior to use. All column work was done at 0-4°C.

The equilibrated enzyme was placed on Column I and eluted at 20-25 ml per hour. Fractions of seven to eight ml were collected and assayed for activity and protein. The elution profile is shown in Figure 6. Fractions containing 50 units per ml and above were pooled as noted, and concentrated to 15 ml using an Amicon Model 400 concentration cell equipped with a UM-1 membrane. The concentrated sample was then placed on the second column and eluted at 30-35 ml per hour. Fractions of ten ml were collected and assayed for activity and protein. Fractions shown in Figure 7 were pooled and again concentrated to 15 ml. The enzyme solution was then equilibrated by dialysis against 50% saturated $(NH_4)_2SO_4$ for several hours and placed on the third bed. It was eluted at 20-25 ml per hour, and ten ml fractions were collected. Fractions were assayed for activity and protein. The elution profile from this column demonstrated a constant specific activity across the protein peak as shown in Figure 8. This preparation was used for all studies on the enzyme. A summary of the yields and specific activities obtained from the last two purifications is shown in Table 2.

Criterion of Purity

Disc-gel electrophoresis was used as the major criterion of purity. One-half ml samples were taken from the dialyzed crude extract, and the redissolved 40-55% acetone and second ammonium

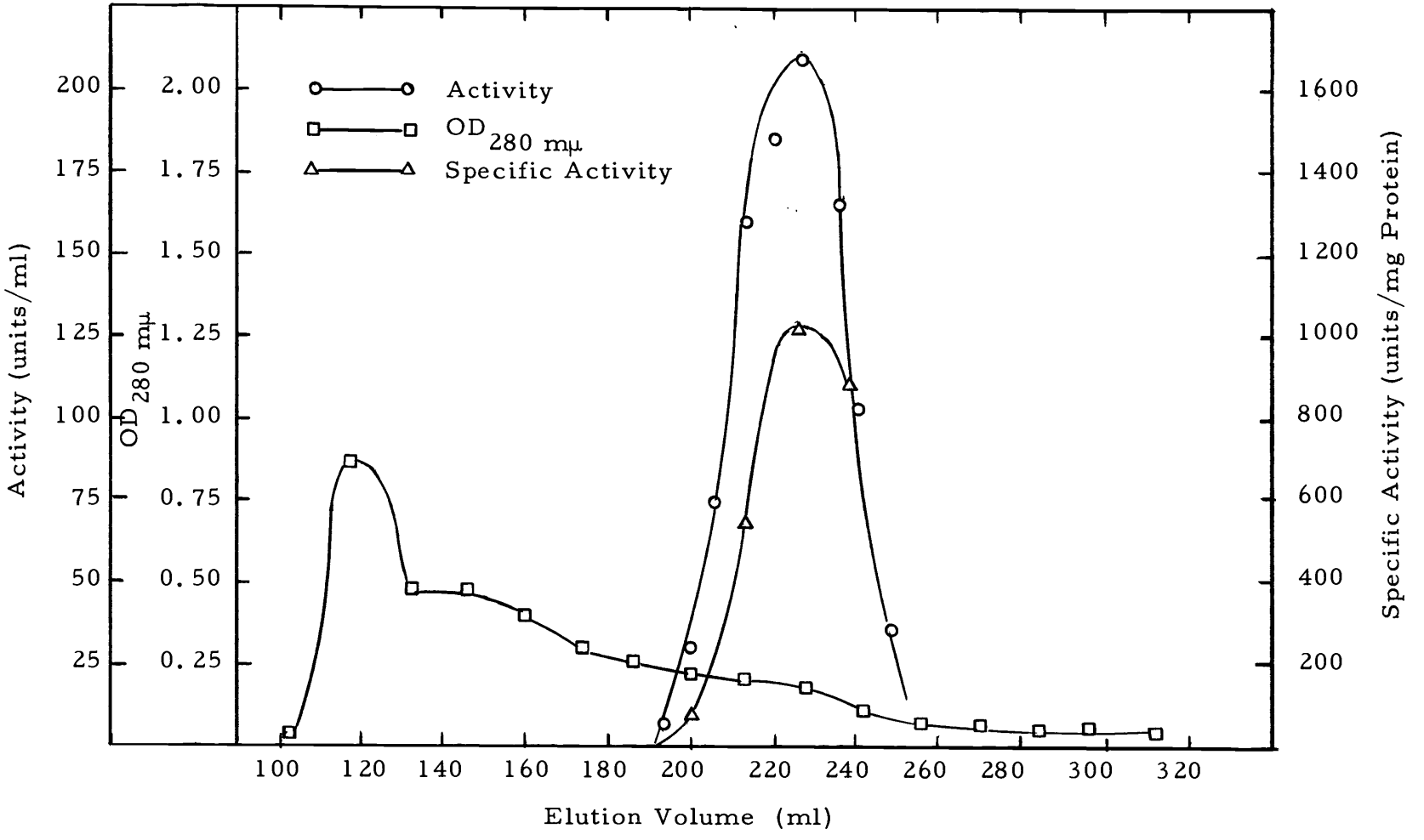

Figure 6. Chromatography of Halophilic Alkaline Phosphatase on Sephadex G-50 (Column I).

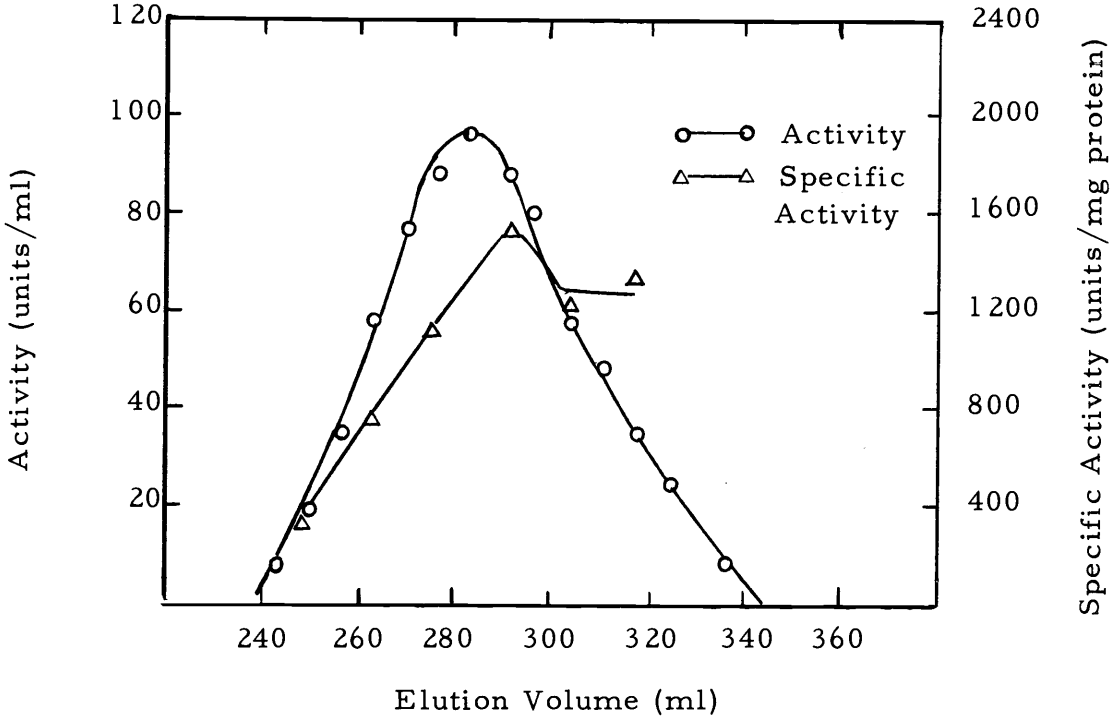

Figure 7. Chromatography of Halophilic Alkaline Phosphatase on Sephadex G-50 (Column II).

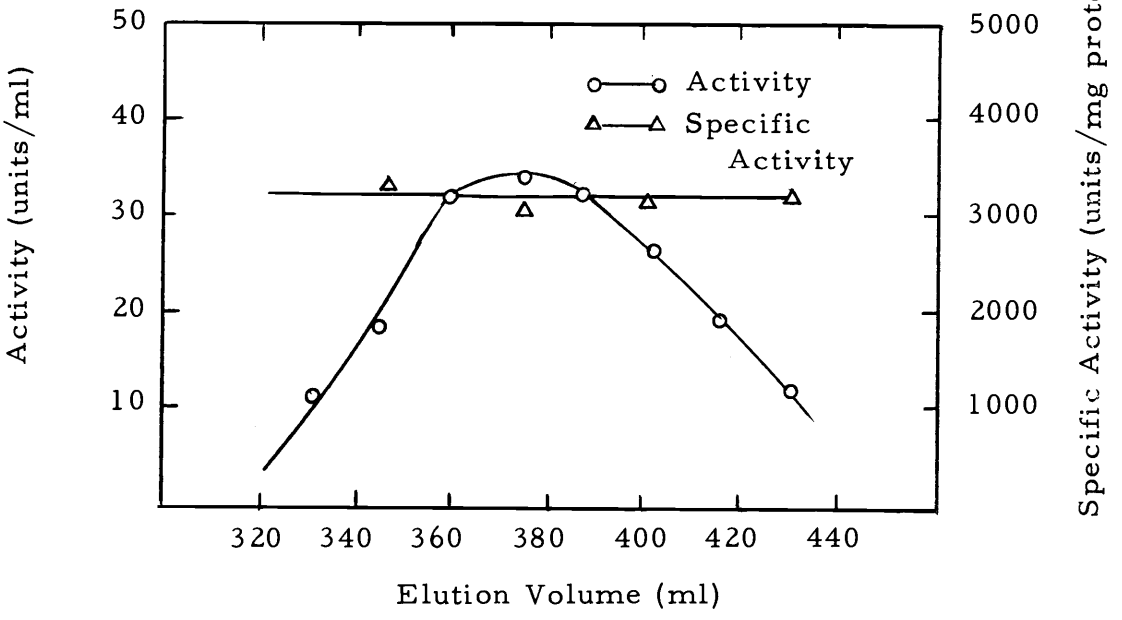

Figure 8. Chromatography of Halophilic Alkaline Phosphatase on Sephadex G-50 (Column III).

Table 2. Summary of Purification Data.

Fraction	I				II			
	Total Protein mg	Total Units of Enzyme	Specific Activity units/mg protein	Yield %	Total Protein mg	Total Units of Enzyme	Specific Activity units/mg protein	Yield %
Extract	6,120	17,750	2.9	-	6,110	15,275	2.5	-
Acetone	302	9,370	31	52	276	9,100	33	59
$(NH_4)_2SO_4$	62	7,000	113	39	80	7,500	94	49
Column I	7.6	6,600	866	37	6.6	6,600	1000	43
Column II	4	4,900	1200	27	3.1	4,900	1550	32
Column III	0.87	2,800	3200	15	1.03	3,100	3000	20

sulfate pellets. They were dialyzed for two hours against 0.2 M NaCl containing 0.05 Tris buffer at pH 8 and used as outlined. One ml aliquots were also taken from the concentrated preparations resolved on Columns II and III. These were dialyzed for 12 hours against 0.2 M NaCl containing 0.05 M Tris buffer at pH 8 and 32% sucrose. Sucrose dialysates were diluted to give about 2-300 units of enzyme activity per ml and used as described. Three parts of those samples dialyzed without sucrose were added to one part buffer, one part acrylamide, one part riboflavin, and two parts of 80% sucrose as outlined by Canalco (5). Five parts of the samples dialyzed against sucrose were added as above omitting the two parts of sucrose. This allowed enrichment of the enzyme for a more critical analysis on disc gel. Dialysis and electrophoresis were done at 4^{o}C. Results are shown in Figure 9.

A second disc gel of the final preparation was placed in an assay solution containing 4 M NaCl, 0.1 M Tris pH 8.5, 1 mM $MnCl_2$, and 0.2 mg per ml p-nitrophenyl phosphate. The assay was done at room temperature. A diffuse yellow band was observed within the region of the intense band shown in Figure 9.

Amino Acid Analyses

Samples for amino acid analysis were prepared by two methods. Three-thousand units of enzyme in 50% saturated

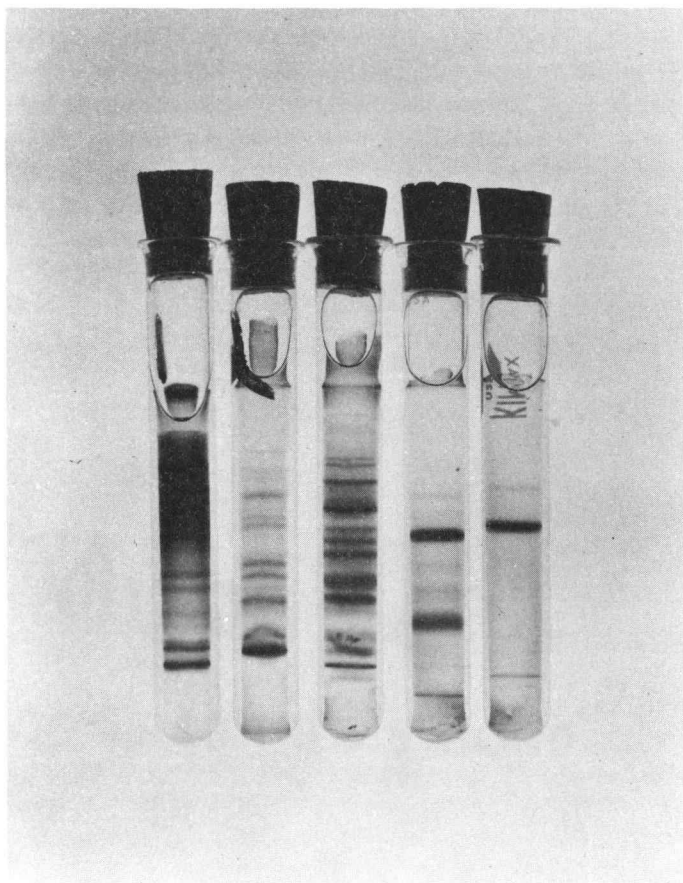

Figure 9. Disc-gel Electrophoresis of Halophilic Alkaline Phosphatase Preparations.
From left to right: crude extract, 40-55% acetone fraction, 60-80% ammonium sulfate fraction, enzyme fraction from Column II, and enzyme fraction from Column III.

$(NH_4)_2SO_4$ were concentrated as before to 15 ml. Then 1800 units of the concentrate were dialyzed exhaustively against glass distilled water until the protein precipitated. The precipitate was collected by centrifuging at 30,000 x g for 15 minutes and resuspended in 10 ml of 0.2 M NaCl. The suspension was dialyzed against one liter of 0.05 M NaCl with one change after 12 hours. The suspension was removed and dried in a drying ampoule (Kontes Glass Co., 5 ml capacity) by rotary evaporation under reduced pressure obtained with a water aspirator. This sample was used for the first amino acid analysis. For the second analysis, 1800 units of enzyme in 50% saturated $(NH_4)_2SO_4$ were dialyzed against 10 volumes of 0.05 M NaCl with one change after 12 hours. The dialyzed enzyme was concentrated to five ml with an Amicon Model 50 concentration cell equipped with a UM-2 membrane. The enzyme solution was removed, filtered through a Millipore filter (0.1μ pre size), and dialyzed against 500 ml of 0.05 M NaCl with four changes 12 hours apart. The dialyzed protein was removed and dried as before.

Amino acid analyses were done by the method of Moore and Stein (21). One ml of constant boiling HCl was added to each sample. The ampoules were evacuated and filled with nitrogen several times to displace all oxygen. The ampoules were then evacuated and sealed. The protein was hydrolyzed by heating for 22 hours in a refluxing toluene bath (110°C). The ampoules were opened and the

HCl was removed by rotary evaporation as before. The samples were dissolved in one ml of glass distilled water, re-evaporated, and then dissolved in 2.2 ml 0.2 M citrate buffer at pH 2.2. They were then filtered through glass wool to remove any precipitate. One ml aliquots of each hydrolyzate were chromatographed on a Beckman Spinco Model 120B Amino Acid Analyzer with an accelerated system (28). The results of these analyses are given in Table 3. The analyses of halophilic cell envelope and ribosomal protein, as well as several microbial alkaline phosphatases, are also given in Tables 3 and 4 for comparison. The mean-residue hydrophobicities, (Φ), for the alkaline phosphatases were calculated from the data of Bigelow (3) and are recorded in Table 4 for future reference.

Enzyme Kinetics

Enzyme activity toward p-nitrophenyl phosphate was studied in considerable detail in low (0.05 M), intermediate (0.5 M), and high (4 M) sodium chloride concentrations. p-Nitrophenol production was followed at 400 mμ using a Beckman DU equipped with a Gilford automatic cell changer and Honeywell recorder. The cell chamber was maintained at 25°C with a Heto thermostated bath. The molar extinction of p-nitrophenol was taken as 1.84×10^4 in 0.02 M NaOH at 400 mμ (23), and the extinction in solutions between pH 6.5 and 11 was determined relative to 0.02 M NaOH.

Table 3. Amino Acid Composition of Halophilic Proteins.

Amino Acid	Alkaline Phosphatase			Whole Ribosomal Protein (1)	Cell Envelope Protein (17)
	Mole Percent			Mole Percent	Mole Percent
	1	2	Mean		
Lysine	1.5	2.1	1.8	4.4	2.5
Histidine	4.4	3.7	4.0	2.2	1.3
Ammonium	(26)	(21)	-	-	(11.2)
Arginine	6.0	6.3	6.1	7.1	4.1
Aspartic Acid	14.0	13.3	13.6	13.5	17.2
Threonine	4.9	4.7	4.8	7.4	9.6
Serine	4.6	5.5	5.0	7.7	7.6
Glutamic Acid	12.4	13.6	13.0	14.7	13.4
Proline	4.8	5.6	5.2	4.5	4.1
Glycine	10.2	10.6	10.4	-	(11.2)
Alanine	11.7	10.4	11.0	11.7	10.7
Half-Cystine	0	0	0	0	0
Valine	11.2	9.3	10.2	10.0	9.5
Methionine	0	2.2	1.1	1.3	0
Isoleucine	3.8	4.1	4.0	4.4	5.2
Leucine	6.3	5.0	5.6	7.0	8.4
Tyrosine	2.2	2.2	2.2	1.9	2.8
Phenylalanine	1.8	1.4	1.6	2.2	3.6

( ) Not considered in determination of mole percent.

Table 4. Amino Acid Composition of Several Microbial Alkaline Phosphatases.

Amino Acid	H. salinarium	E. coli (25)	Neurospora crassa (14)	Aspergillus nidulans (8)
	Mole Percent	Mole Percent	Mole Percent	Mole Percent
Lysine	1.8	6.4	6.9	5.2
Histidine	4.0	2.1	2.3	4.8
Arginine	6.1	2.9	2.9	4.6
Aspartic Acid	13.6	10.0	11.8	12.1
Threonine	4.8	8.9	8.1	7.1
Serine	5.0	5.1	6.6	6.5
Glutamic Acid	13.0	10.0	7.8	9.1
Proline	5.2	4.6	5.8	4.2
Glycine	10.4	9.8	9.7	8.4
Alanine	11.0	14.0	8.0	7.4
Half Cystine	0	0.8	0.9	1.6
Valine	10.2	5.5	5.6	5.8
Methionine	1.1	1.5	1.2	1.0
Isoleucine	4.0	3.3	3.9	3.7
Leucine	5.6	8.4	7.8	8.5
Tyrosine	2.2	2.4	3.8	3.5
Phenylalanine	1.6	1.9	4.6	4.3
Tryptophane	--	0.8	1.5	1.1
(Φ)	(860)	(920)	(1070)	(1000)

(Φ) is the mean-residue hydrophobicity determined from data of Bigelow (3).

A preliminary pH optimum was determined for halophilic alkaline phosphatase using 0.2 mg per ml (.71 mM) p-nitrophenyl phosphate, 0.1 and 1 mM $MnCl_2$, and 0.05, 0.5, and 4 M NaCl. Three ml of each solution studied containing NaCl, buffer, and substrate were transferred to a cell followed by 0.1 ml of $MnCl_2$ and 0.1 ml of enzyme. All solutions were adjusted to give the final concentrations noted above in a total of 3.2 ml. Enzyme was diluted to two units per ml just prior to use with 0.2 M NaCl at $0^{o}C$ containing 0.05 M Tris buffer at pH 8. The results shown in Figure 10 indicated a pH optimum at 8.5 with a strong dependence on the manganese concentration in low salt.

The role of manganous ion in enzyme activity implicated in the above results was studied in greater detail at pH 8.5 in the presence of 0.005 M Tris buffer and 0.05 M NaCl. Appropriate volumes of NaCl-buffer and 30 mM p-nitrophenyl phosphate solutions were mixed so that in a volume of 2.86 ml the substrate was 3.00/2.86 that of the final desired concentration. The levels of substrate selected for this work were 2, 1, 0.7, 0.5, 0.25, and 0.2 mM. One-tenth of one ml of $MnCl_2$ was then added. This solution was prepared by diluting 0.1 M $MnCl_2$ to thirty times the desired cell concentration with NaCl-buffer solution. Manganese chloride levels were 3, 2, 1, 0.75, and 0.5 mM. The reaction was initiated by the addition of 0.04 ml of enzyme diluted to 5 units per ml with the

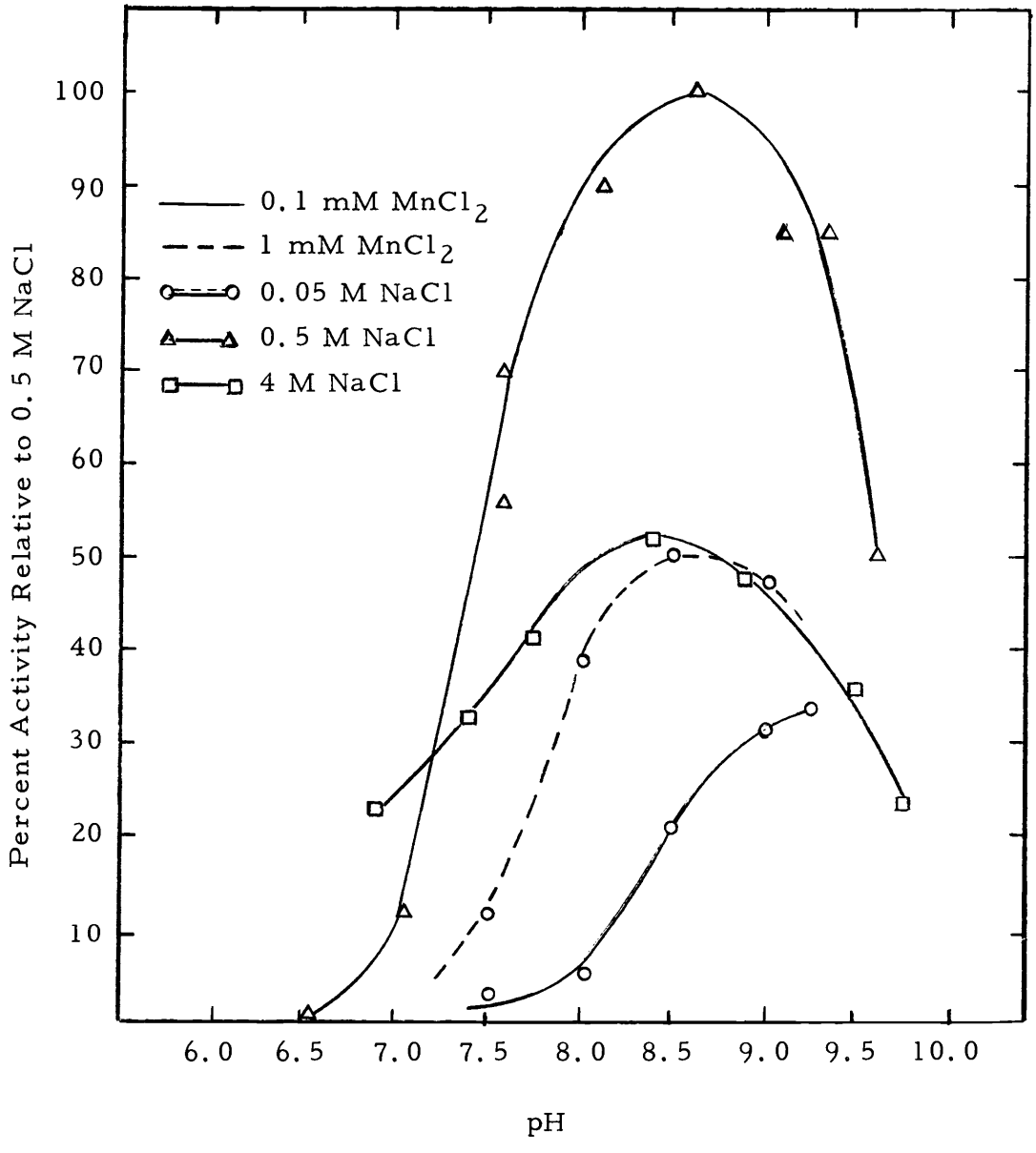

Figure 10. Effect of pH on the Activity of Halophilic Alkaline Phosphatase.

NaCl-buffer solution at 0°C. The total volume of the reaction mixture was then three ml. The initial rates of p-nitrophenol production were determined, and double reciprocal plots prepared from the data obtained. The results are shown in Figure 11. The slopes were determined by the linear least squares method using a computer program written by T. L. Miller[2]. The slopes were then plotted against the reciprocal of manganese concentration as shown in Figure 12. The data were consistent with an obligatory binding of manganous ion prior to the binding of substrate. K_a, V_{max}, and K_m could then be determined from the intercepts of the primary and secondary reciprocal plots and the slope of the secondary plot as indicated.

The kinetic parameters K_a, V_{max}, and K_m were determined in a similar fashion as a function of pH in the presence of 0.05, 0.5, and 4 M NaCl. Imidazole (6.5-7.0), Tris (7.5-9), and diethanolamine (9.5-10) buffers were used. Plots of these data are shown in Figures 13, 14 and 15. Above pH 9 the enzyme activity appeared to be independent of manganese concentration which suggested strong affinity of the enzyme for manganese. At pH and salt concentrations where K_a was not determined, sufficient $MnCl_2$ was added to assure saturation, and K_m and V_{max} were determined.

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[2]See Appendix, page 80.

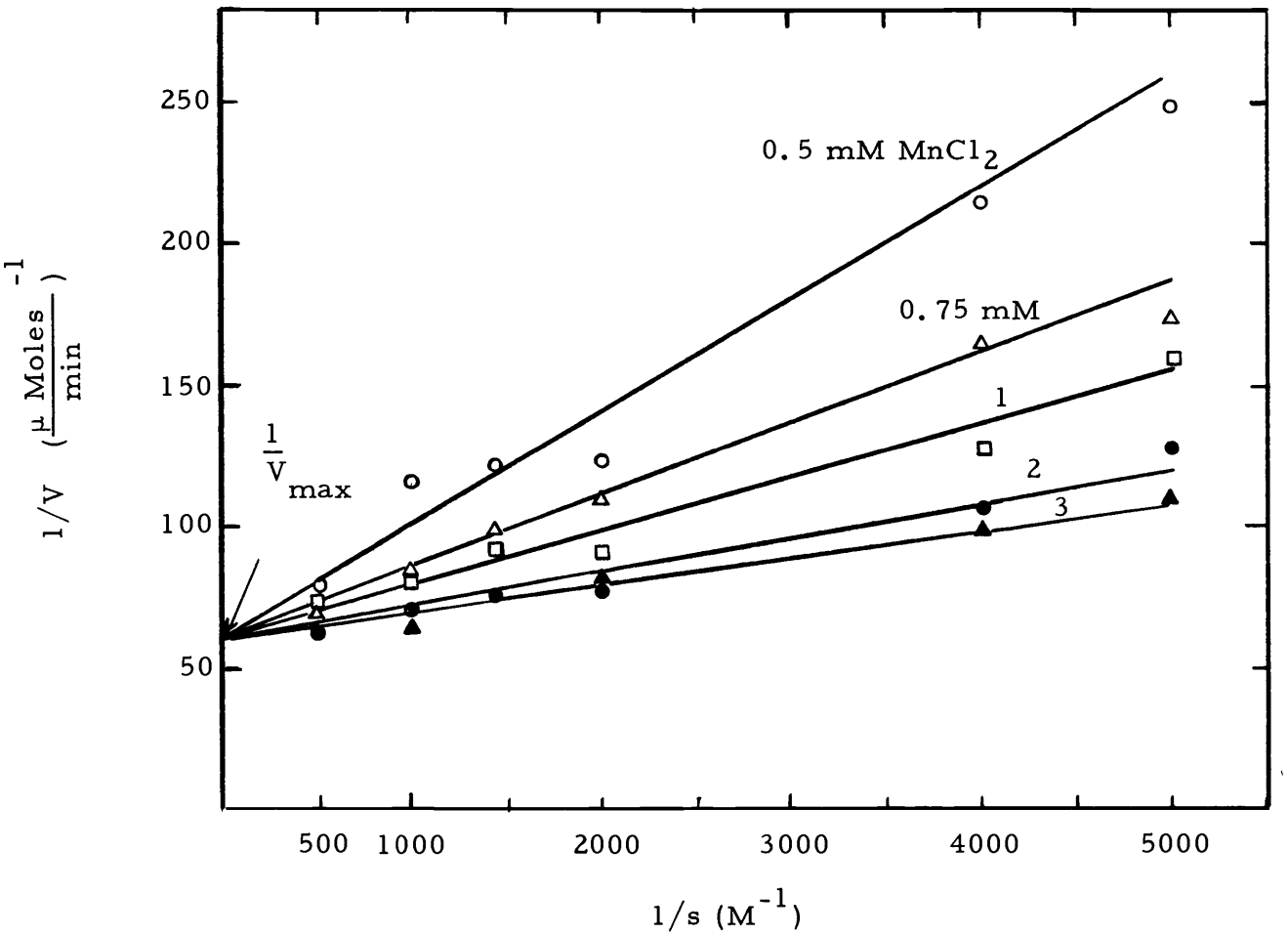

Figure 11. Primary Double Reciprocal Plots.

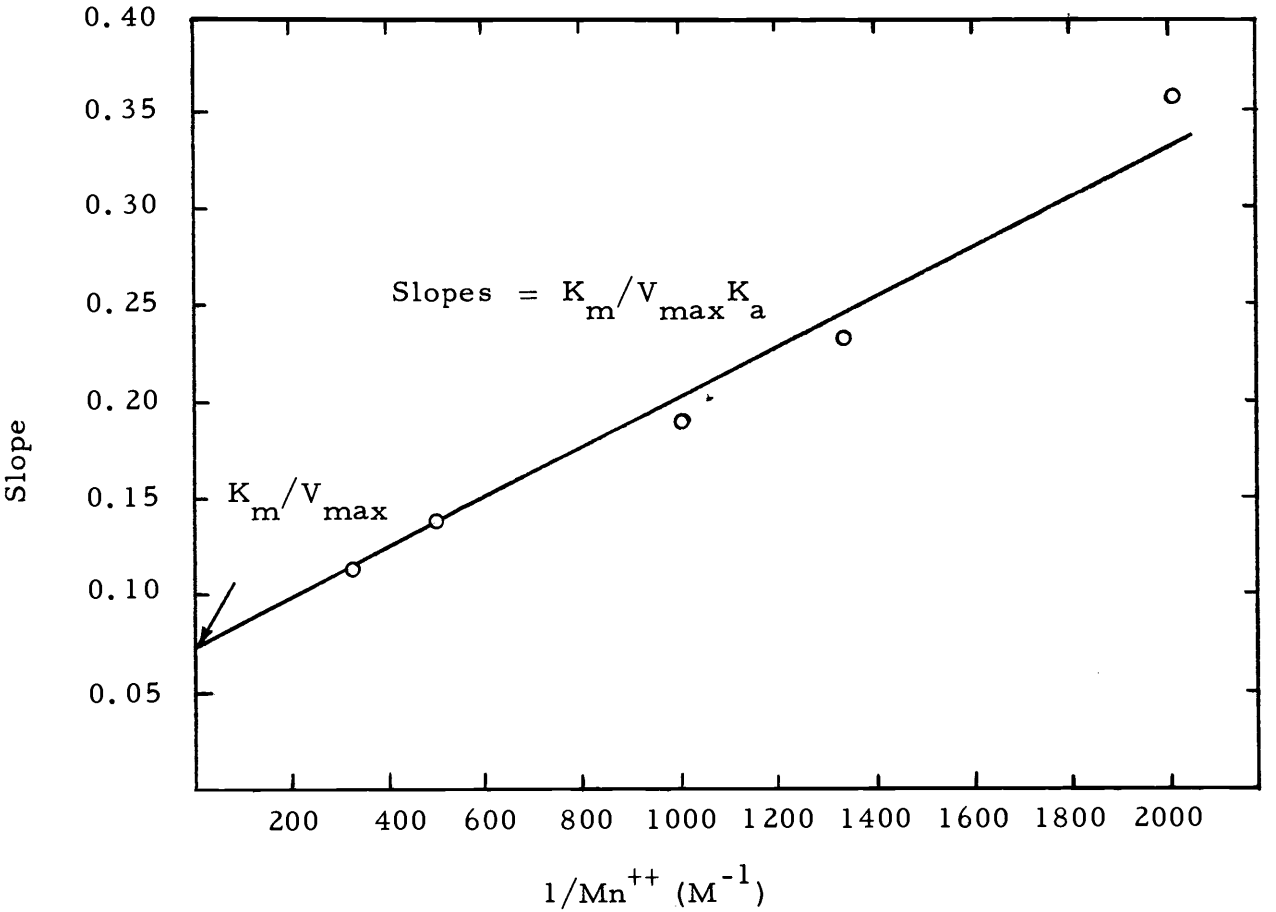

Figure 12. Secondary Reciprocal Plot.

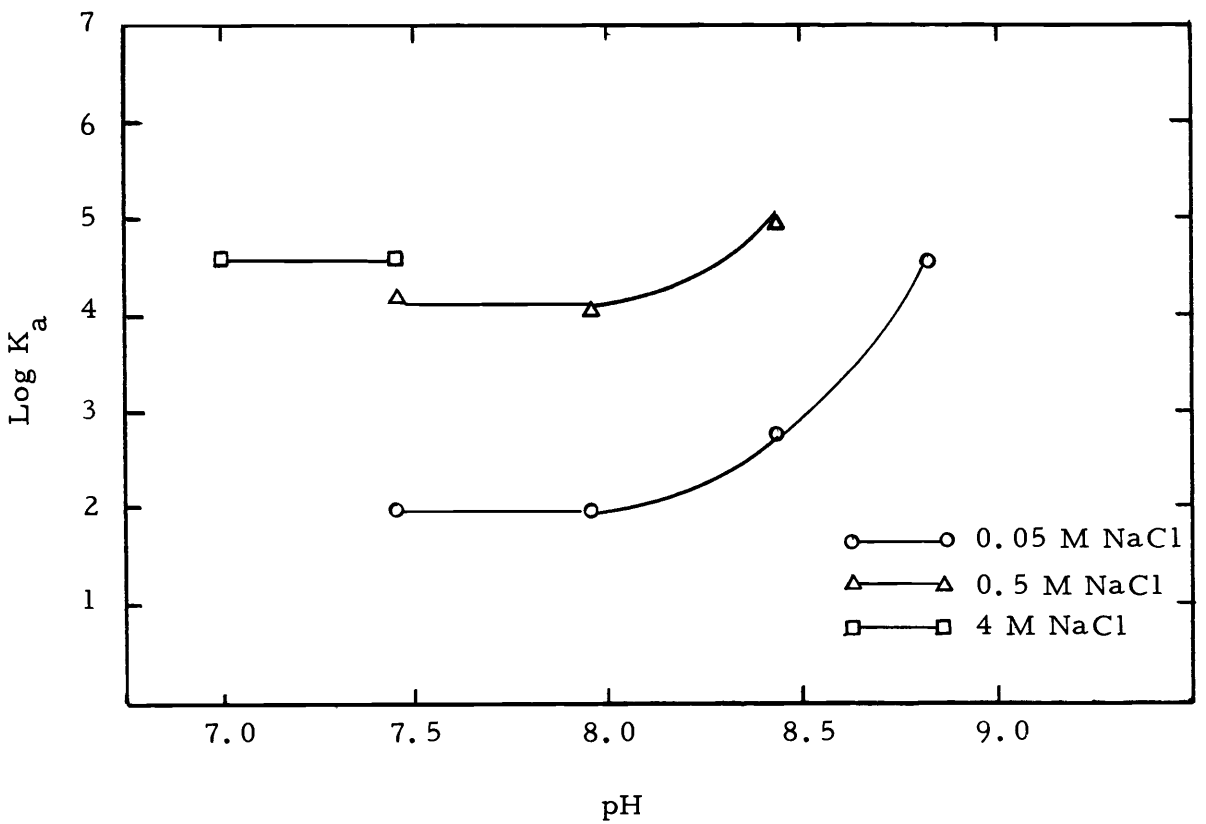

Figure 13. The Effect of pH on K_a.

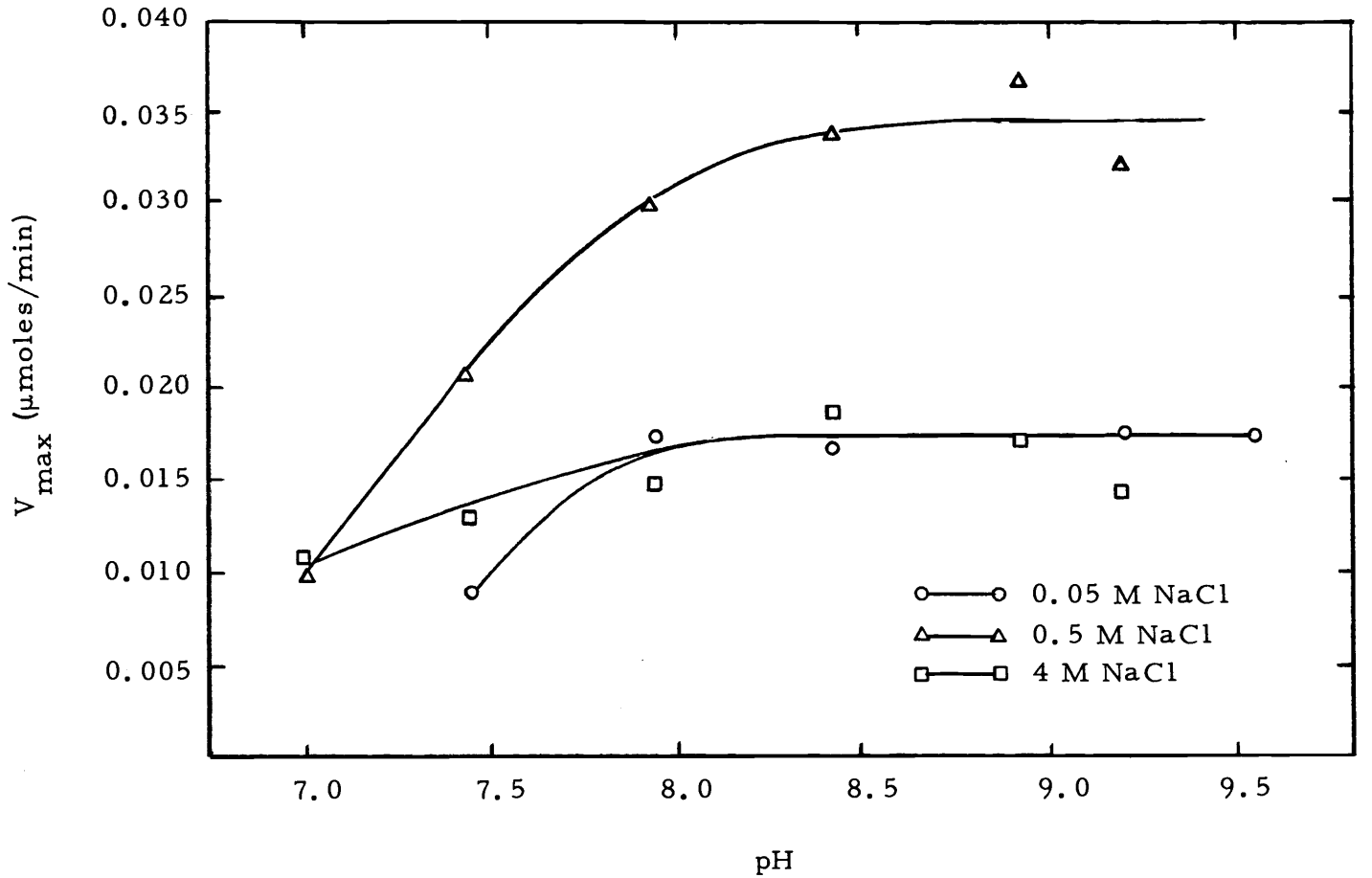

Figure 14. Effect of pH on V_{max}.

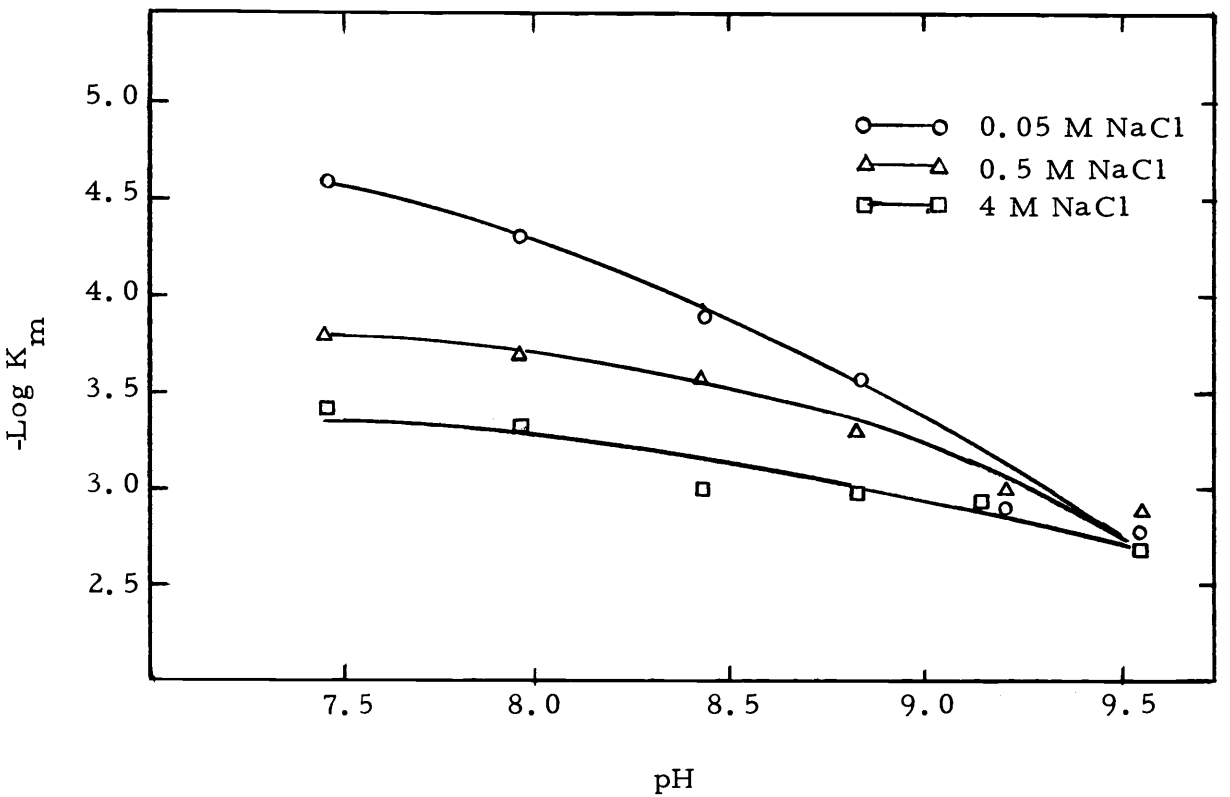

Figure 15. The Effect of pH on K_m.

K_a and V_{max} were also determined as a function of NaCl concentration at pH 8 and 9, respectively. The results are plotted in Figures 16 and 18. Data of Figure 16 were replotted as log K_a versus $1.02\sqrt{s}/(1 + 1.31\sqrt{s})$ as shown in Figure 17. The slope of this line equals the electrostatic factor, n. The value of n is believed to represent a complex measure of electrostatic interactions between charged groups on the enzyme surface which distort the manganese binding site and between manganous ion and its binding site.

One-hundredth molar buffer concentrations were used to avoid any chelation of manganous ion. As a result the addition of enzyme in 50% $(NH_4)_2SO_4$ even when diluted as described over 1000-fold was observed to considerably alter the pH of the reaction mixture above pH 9. The pH data given were corrected for this effect.

Enzyme Stability Studies

The stability of manganese containing holoenzyme and the production of apoenzyme were studied in some detail in 4 M NaCl. Eight-hundredths of one ml of enzyme (50 units per ml) in 50% saturated $(NH_4)_2SO_4$ was transferred to two ml of 4 M NaCl containing 5 mM EDTA and 0.1 M buffer adjusted to the pH noted below. The resulting ammonium sulfate concentration was then 0.08 M.

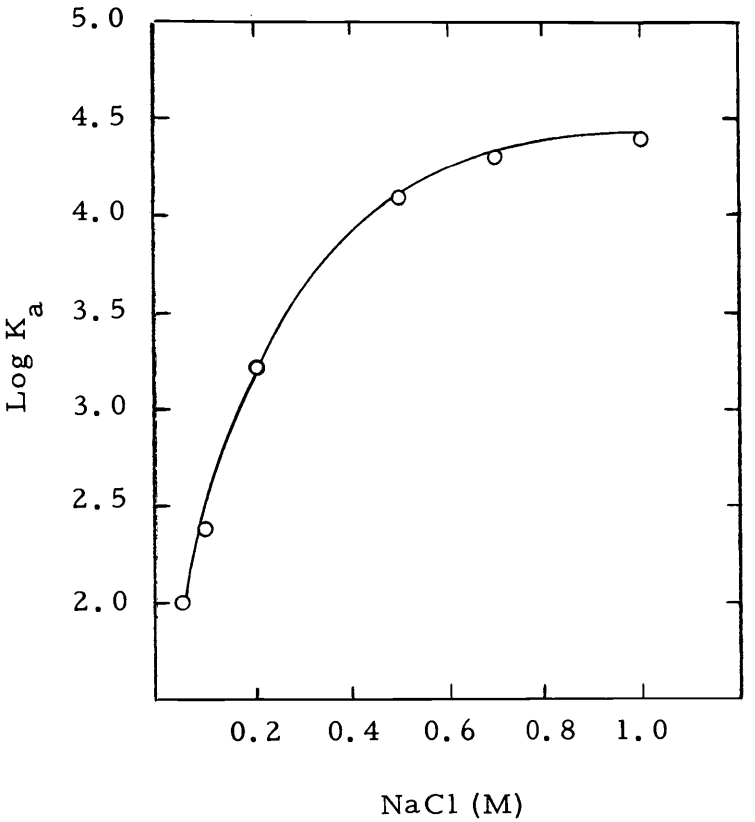

Figure 16. Effect of Sodium Chloride Concentration on K_a.

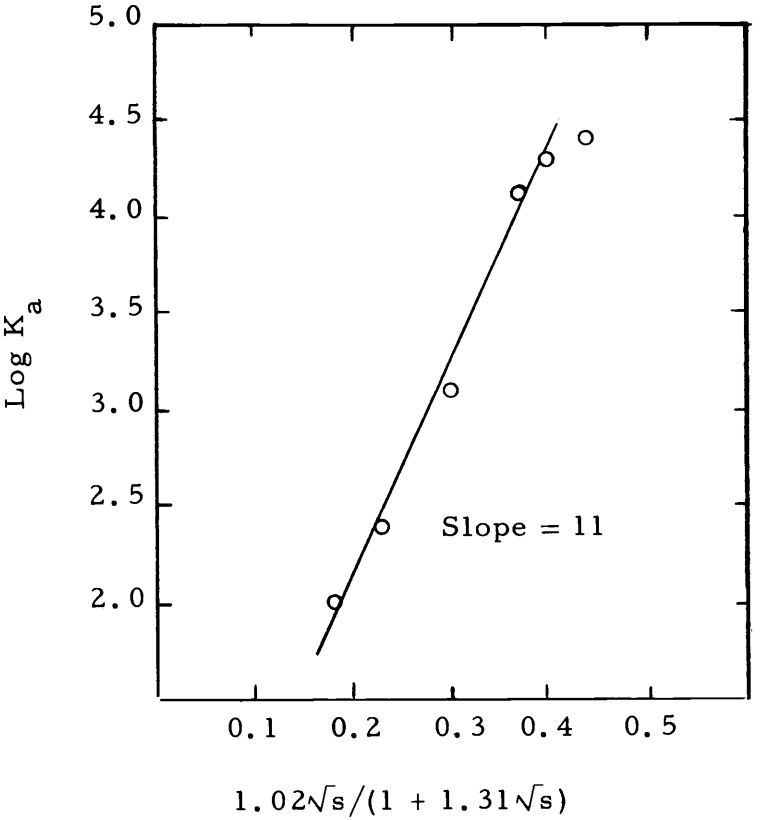

Figure 17. Secondary K_a Plot.

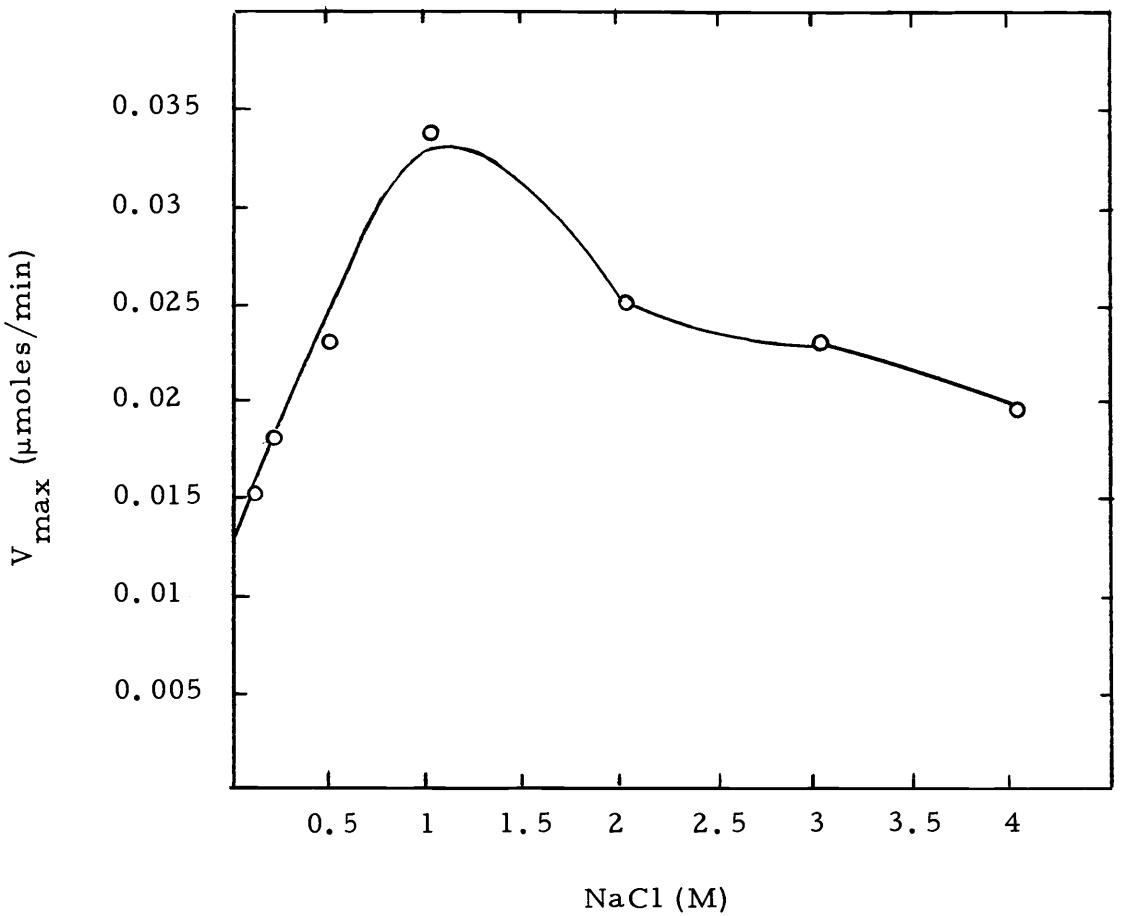

Figure 18. Effect of Sodium Chloride Concentration on V_{max}.

Imidazole, Tris, and diethanolamine buffers were again used. Because of the high buffering capacity of the enzyme solution, sufficient 1 M NaOH was added to the buffer solutions prior to enzyme to maintain a pH near that desired. The pH was checked afterward for the resulting value to assure proper pH adjustment. One-tenth of one ml of the mixed enzyme solution was transferred at convenient time intervals to 2.9 ml of assay solution containing 4 M NaCl, 0.1 M diethanolamine at pH 9.5, and 1 mM p-nitrophenyl phosphate. Under these conditions only the holoenzyme was found to exhibit activity; hence, the rate in ΔOD/min at 400 mμ was set proportional to the amount of holoenzyme remaining at the time the aliquot was taken. At the same time an additional 0.1 ml was transferred to a similar assay solution which also contained 0.3 mM $MnCl_2$. Manganese was added in a volume of 0.01 ml from a 0.1 M solution just prior to the addition of enzyme. The added manganese was sufficient to saturate EDTA and the apoenzyme generated by the loss of manganous ion from the holoenzyme. The rate in ΔOD/min was set proportional to the sum of holoenzyme and apoenzyme present at the time the aliquot was taken. The level of apoenzyme was then simply the difference between the second and first assays. The change in holoenzyme and apoenzyme levels in 4 M NaCl at pH 7, 8, and 9 are shown in Figure 19. Similar studies were conducted using 0.05 M NaCl in place of 4 M NaCl. Under these conditions,

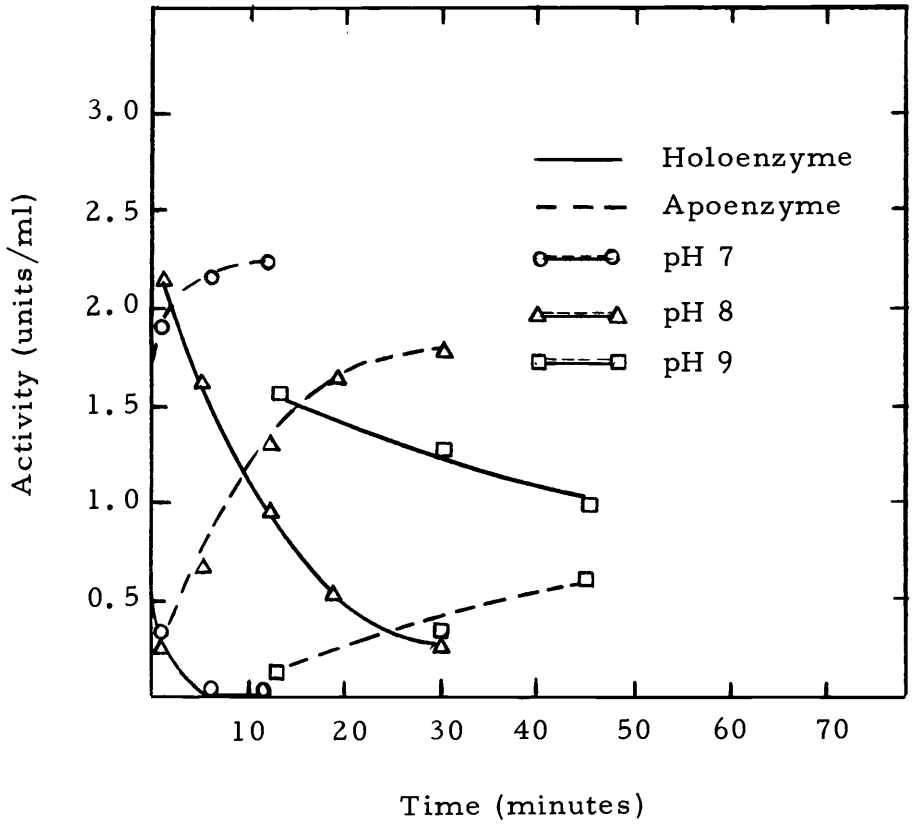

Figure 19. The Effect of pH on the Conversion of Holoenzyme to Apoenzyme.

apoenzyme was not observed to accumulate because of its extreme instability in low salt concentrations. The release of manganese from the surface of holoenzyme was then studied in greater detail as a function of pH in the presence of 4 M and 0.05 M NaCl. The log of the ratio of the remaining activity to the original activity was plotted as a function of time and gave the linear first-order plots shown in Figures 20 and 21. The half-life was determined from the slope of each plot. The data obtained are shown as a function of pH in Figure 22.

The stability of the apoenzyme in several salts was also studied. Apoenzyme was generated by adding 0.08 ml of enzyme as before to an appropriate volume of 4.5 M NaCl, 1.5 M Na_2SO_4, 3.5 M KCl, or 2 M $(NH_4)_2SO_4$ containing 5 mM EDTA, and 0.1 M imidazole buffer at pH 7. The enzyme was then incubated for two minutes at 25°C. Holoenzyme was fully converted to the apoenzyme under these conditions as indicated by a total loss in activity without addition of manganese. Sufficient buffer solution containing 5 mM EDTA was then added to reduce the salt to the desired level and to initiate denaturation of the apoenzyme. Volumes were adjusted to bring the sample to a final volume of two ml. One-tenth of one ml aliquots of enzyme were transferred at selected time intervals to 2.9 ml of the assay solution described containing 0.3 mM $MnCl_2$, and the rate in ΔOD/min was set proportional to the amount of

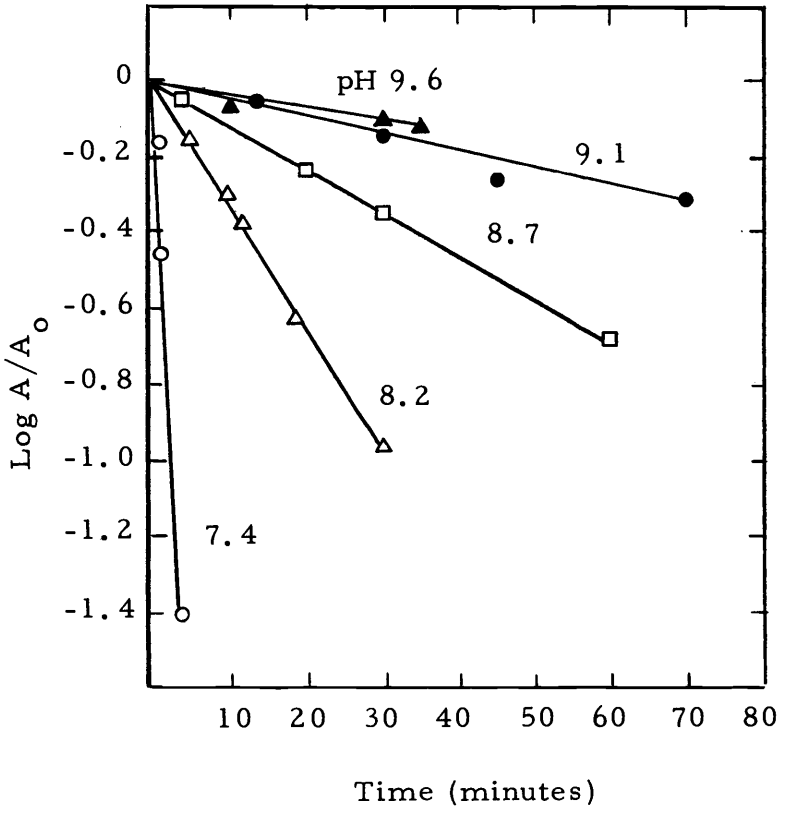

Figure 20. Effect of pH on the Release of Mn^{++} from the Holoenzyme in 4 M NaCl.

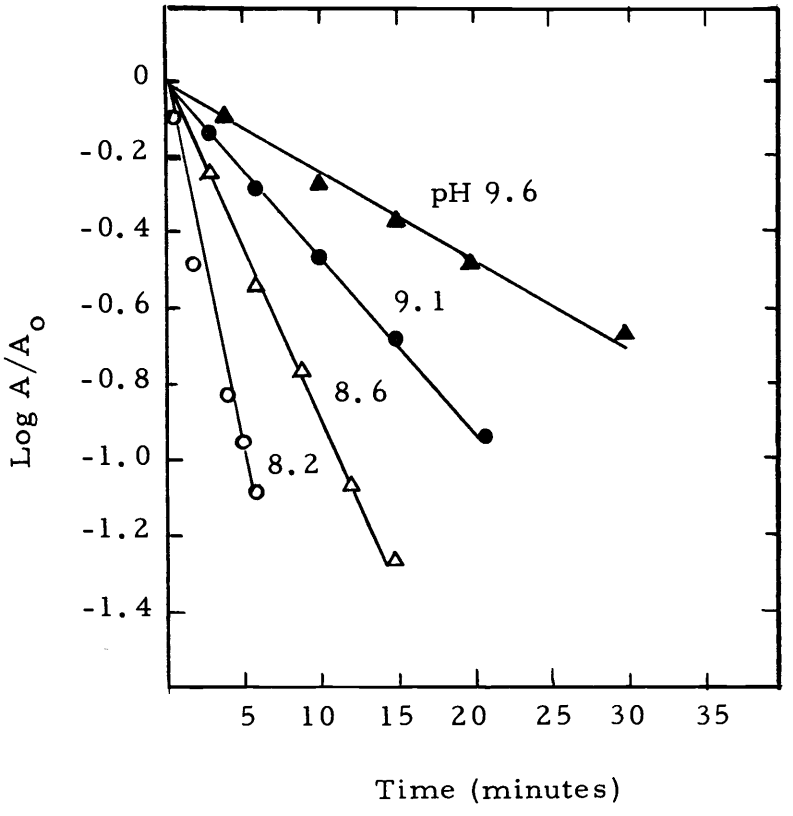

Figure 21. Effect of pH on the Release of Mn^{++} from the Holoenzyme in 0.05 M NaCl.

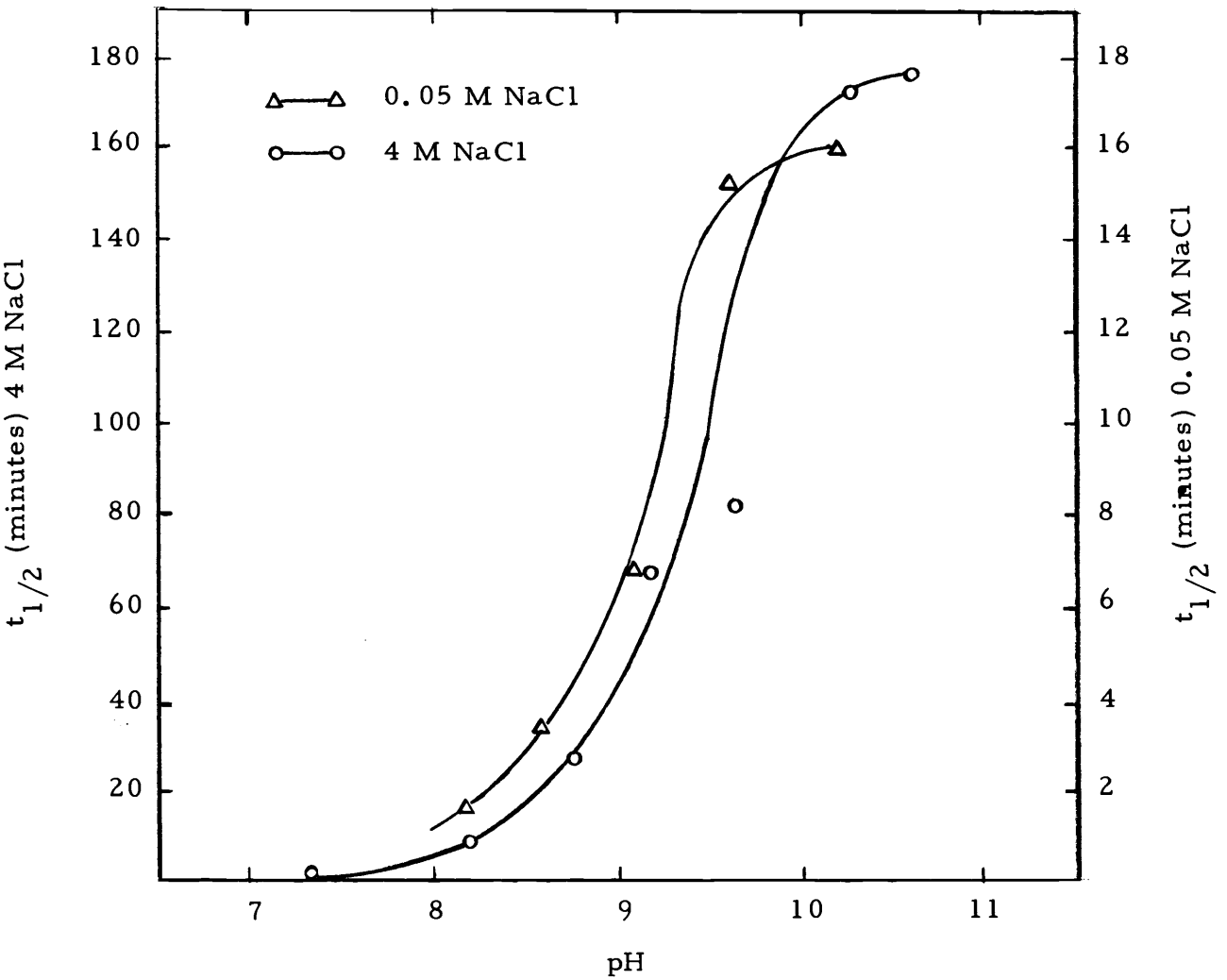

Figure 22. Effect of pH on the Half-life of the Holoenzyme.

reactivatible apoenzyme present. Time intervals were selected to give four determinations of apoenzyme level in the first order region of the reaction as shown in Figure 23.

Stability of the apoenzyme in NaBr and NaI was also studied. However, because of its instability in these salts, apoenzyme stability was only studied in the presence of 1 M NaBr and 0.5 M NaI in which additional sodium ion was added as NaCl. Apoenzyme was generated in 4.5 M NaCl and bromide or iodide anion added with the buffer to initiate denaturation. Initial first-order rates were taken as described and the half-life of the apoenzyme as a function of salt type and concentration was determined. The results are shown in Figure 24. Apoenzyme stability in KCl and $(NH_4)_2SO_4$ were comparable to NaCl and are not shown in Figure 24. The rates of denaturation were too rapid to measure in NH_4Cl, NaSCN, and LiCl.

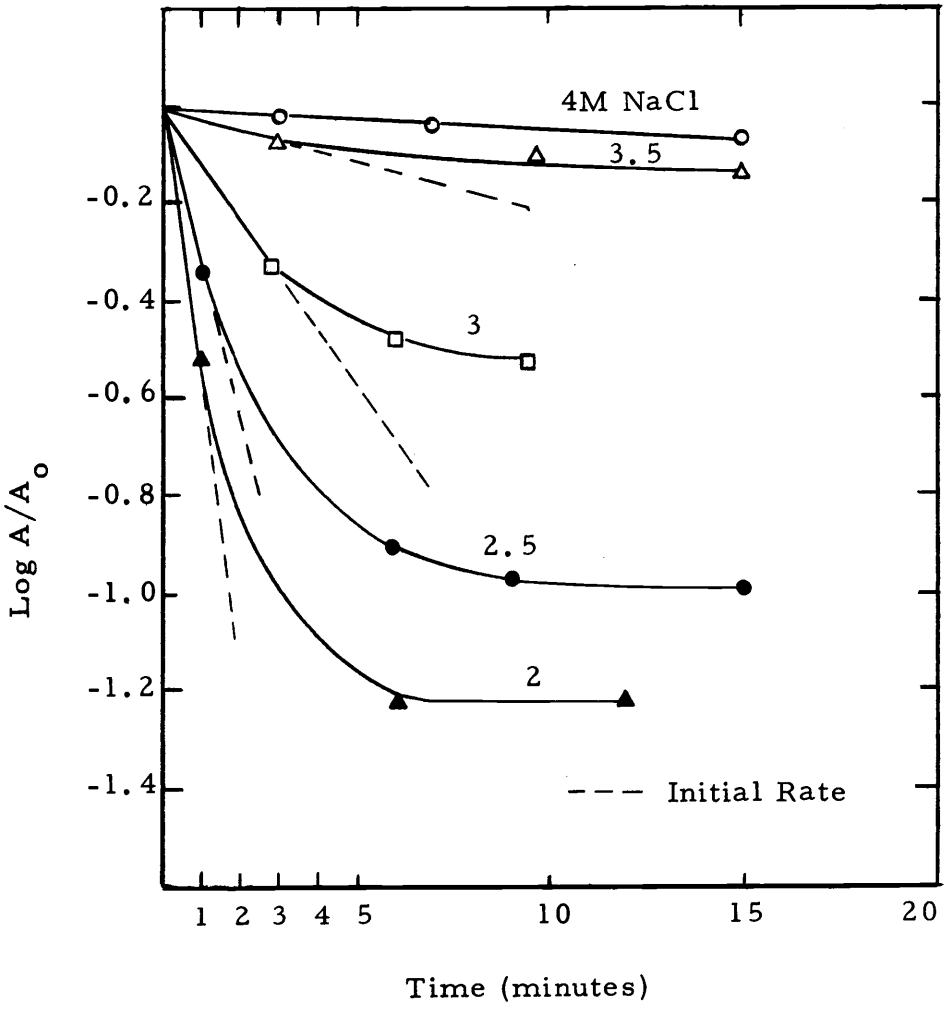

Figure 23. Apoenzyme Stability in Sodium Chloride.

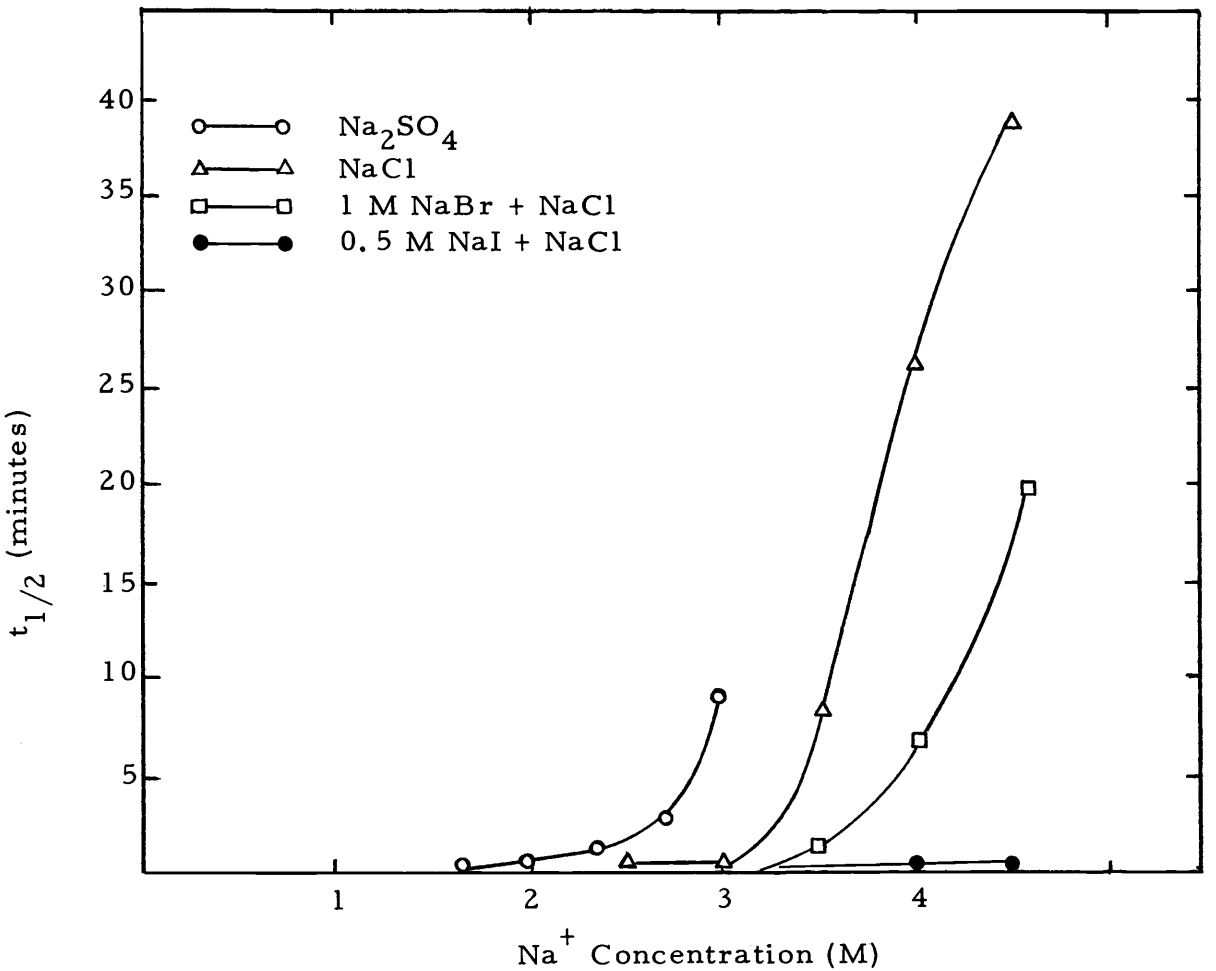

Figure 24. Effect of Several Anions on Apoenzyme Stability.

IV. DISCUSSION

Studies on Crude Enzyme Preparations

Preliminary studies on halophilic alkaline phosphatase in crude preparations were conducted in an effort to establish suitable conditions for its production and purification. Enzyme production was checked for derepressible synthesis under conditions similar to derepression of the alkaline phosphatase in E. coli (29). Gibbon's defined medium supplemented with 200 mM inorganic phosphate and devoid of all inorganic or organic phosphates was used. Under both conditions enzyme synthesis was found to remain constant. Enzyme production in Halvorson's medium, which is higher in all forms of phosphate, was identical to the production in Gibbon's medium under limiting phosphate. In Kushner's medium, enzyme production increased at the beginning of log phase and became constant near mid-log phase at an enzyme level twice that in Gibbon's or Halvorson's. All observations argue strongly against the repressible synthesis of alkaline phosphatase in H. salinarium. Because of the higher cell yield and enzyme level, Kushner's medium supplemented with 20 mg per liter $MnCl_2$ was used routinely for cultivation of H. salinarium.

Metals commonly required in enzyme catalysis involving organic phosphates as substrates were tested for their ability to

activate or inhibit halophilic alkaline phosphatase activity. Of those studied only manganous ion was observed to appreciably activate the enzyme. In addition, manganese was found to stabilize the enzyme in both high and low sodium chloride concentrations. These results were similar to observations made by Holmes and Halvorson (12) in which $NADH_2$ dramatically stabilized halophilic malate dehydrogenase in low salt.

Since the Halobacterium species generally lyse in low salt concentration (19) and the alkaline phosphatase of H. salinarium could be stabilized under such conditions in the presence of manganese, low ionic strength appeared to be a suitable means of rupturing the cells and extracting the enzyme. Cell lysis was studied in considerable detail using sodium and potassium chlorides. In agreement with Kushner (15), lysis was found to be more efficient in potassium chloride and was nearly complete at 1 M. In addition, the presence of 0.01 M manganese chloride appeared to stabilize the cell envelope structure sufficiently to prevent complete disintegration. Potassium chloride lysis was then used as outlined as a routine means of extracting the alkaline phosphatase from H. salinarium.

Enzyme Purification

Cells were cultivated in Kushner's medium supplemented with

20 mg per liter manganese chloride as outlined. The yield was 6-7 g of cells per liter of medium. The cells contained about 1500 units of enzyme. Several features appeared important in the cultivation of H. salinarium to give extracts suitable for purification by the scheme outlined. If growth exceeded an optical density of 1.8 at 525 mμ, the extract obtained was refractory to the acetone step. An oily product released during acetone fractionation appeared responsible for the observed inactivation of the enzyme. In addition, if cells were stored considerably longer than three days at 4°C in their culture medium, the extract again became refractory to acetone fractionation. During the three day storage period, enzyme level increased considerably making such storage advantageous.

Cell lysis by low ionic strength was scaled up for preparation of the crude extract. The extract obtained was exceedingly viscous. It was therefore dialyzed with stirring devices inside and outside the dialysis bag. A 12 hour dialysis period was used to bring the extract to the proper conditions for acetone fractionation. The dialysis period also offered a period for denaturation of ionically unstabile protein which was later removed by acetone and ammonium sulfate fractionations. Ammonium sulfate was added to the dialysis solution to stabilize manganous ion which was otherwise quite unstabile at pH 8. Ammonium sulfate also aided in stabilizing the alkaline phosphatase.

Acetone fractionation of the enzyme in low salt in the presence of manganese was highly reproducible and required few precautions. The only important technical precaution was the proper control of temperature as described. Attempts to perform such fractionations in high salt levels led to relatively inactive preps.

Routinely, the procedure did not require any longer than 45 minutes. Nucleic acid was the first component to precipitate and could often be removed by winding it on the thermometer. Resuspension of the 40-55% acetone fraction in 60% saturated ammonium sulfate removed considerable amounts of protein denatured during dialysis in low ionic strength and acetone fractionation. Other salts tested were considerably less effective and yielded preparation of lower specific activities. An additional feature was that the preparation was ready for higher ammonium sulfate fractionations.

Two ammonium sulfate fractionations were made to remove all of a red protein component which interfered with resolution on Sephadex. The final solution was pale yellow and gave only one visible, yellow band on the first column.

High resolution on Sephadex was exceedingly simple and reproducible. Resolution was obtained by a combination of molecular sieving (40% saturated ammonium sulfate) and adsorption chromatography (50% saturated ammonium sulfate). The degree of adsorption was also found to be a function of pH and manganese concentration.

A three to four fold dilution of enzyme concentration was observed with 40% and an eight to ten fold with 50% saturated ammonium sulfate. The concentration of enzyme which could be placed on Sephadex was limited by the ammonium sulfate level used. The limit for good recovery at 50% saturation was about 500 units per ml. At 40% it was about 1500-2000 units per ml.

Concentration of the pooled fractions from the Sephadex columns represented the only real technical difficulty in the procedure, for a considerable amount of enzyme penetrated the membrane and became inactivated during this process. In addition, after repeated exposure to ammonium sulfate the membranes appeared to deteriorate. Several other means of concentrating were therefore studied without much success. The enzyme could be adsorbed onto the matrix of Sephadex G-25 in the presence of 80% ammonium sulfate and then eluted at lower ammonium sulfate levels. The procedure was, however, rather irreproducible. With the proper selection of adsorption material, this technique may offer considerable promise. Dialysis against 20% sucrose may also offer a convenient means of concentrating.

Starting with the collection of cells, five days were required for purification. Best results were obtained if the preparations were worked up without long storage periods. The enzyme was, however, quite stabile when frozen at -20^{o}C in the dialyzed crude extract and

at 0-4°C in 40% saturated ammonium sulfate after chromatography on the first Sephadex column. Cells were collected and lysed, and dialysis begun by the evening of the first day. Acetone and ammonium sulfate fractionations were started in the morning of the second day, and required about six hours for completion. The enzyme was equilibrated and placed on Column I late in the afternoon of the second day. Nearly all activity was eluted within 12-14 hours. Fractions were assayed, pooled, and concentrated. The concentrate was then placed on Column II by the afternoon of the third day. All activity was eluted by the morning of the fourth day. Fractions were assayed, pooled, concentrated, and equilibrated. The concentrate was then placed on the final column by the afternoon of the fourth day. Purified enzyme was collected on the fifth day. Separate purification runs could be spaced two to three days apart.

The overall recovery was about one mg of purified enzyme per ten liters of cells. This was sufficient for most of the preliminary work reported here. Several points were noted during purification attempts where larger amounts of material could be handled. Since the enzyme was fairly stabile in ammonium sulfate after acetone fractionation, several consecutive acetone fractionations could be made on larger quantities of crude extract. The dissolved pellets could then be pooled and fractionated with ammonium sulfate. Only 14,000-18,000 units of enzyme in nine ml could be placed on

Column I in the presence of 40% saturated ammonium sulfate. Since resolution on this column was primarily a result of molecular sieving, lower saturations of ammonium sulfate may be used. This would allow higher quantities of enzyme to be placed on the column. The sample placed on Column III was only 6% of the bed volume. Because of the high resolution of this step, larger volumes might be used with comparable resolution.

Criterion of Purity

Disc-gels of samples taken at points in the purification scheme gave results which compared very well with specific activities of the preparations. An intense band representing over 95% of the total protein was observed with the preparation eluted from Column III. Under the assay conditions described, enzyme activity was observed in the region of this band.

Amino Acid Analysis

A detailed time course amino acid analysis was not attempted. The results of two analyses using low amounts of material demonstrated a high level of glutamic and aspartic acids which comprised about 27% of the total amino acid composition of the protein. Amide level has not been unequivocally established. Ammonium level in the first analysis was somewhat higher than in the second analysis.

This presumably represents occlusion of ammonium sulfate in the precipitate during preparation of the sample. Ammonium level in the second analysis suggested that at least 20% of the acidic amino acids exist in the protein as free carboxylates. An acidic character of the enzyme has, however, not been demonstrated.

Comparison of the alkaline phosphatase with halophilic cell envelope and ribosomal protein shows rather remarkable similarities. All halophilic proteins appear high in acidic amino acids and in valine, but low in cysteine content. Comparison of the halophilic alkaline phosphatase with other microbial alkaline phosphatases demonstrates the halophilic enzyme to be 35% higher in the acidic amino acids and 100% higher in valine. The higher valine content appears to be a common feature of all halophilic protein thus far analyzed. The mean-residue hydrophobicity of the halophilic enzyme was somewhat lower than other non-halophilic alkaline phosphatases.

Enzyme Kinetics

The kinetics of the halophilic alkaline phosphatase toward p-nitrophenyl phosphate were studied in some detail. When saturated with manganese, it exhibited a pH optimum at pH 8.5 in the presence of all salt concentrations. The pH optimum was found to be the result of a decrease in V_{max} below pH 8.5 and a decrease

in K_m above pH 8.5. The manganese binding constant, K_a, was studied in 0.05 M sodium chloride at pH 8.5 to determine the nature of the observed activation. The data obtained was consistent with an obligatory binding model described by Reiner (24) in which manganous ion is bound prior to the binding of substrate. The obligatory model is represented by the following mechanism,

$$E + Mn^{++} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EMn^{++} + S \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EMn^{++}S \xrightarrow{k_3} EMn^{++} + \text{Products}$$

where E represents free enzyme and S substrate. Steady state equations may then be formulated and the velocity, v, written as a function of substrate and manganese concentrations giving

$$v = V_{max} / (1 + (k_{-2} + k_3)/k_2(S) + (k_{-1}/k_1)(k_{-2} + k_3)/k_2(S)(Mn^{++}).$$

The basic feature which separates this model from all others is that as the substrate level approaches infinity the velocity approaches V_{max} independent of the manganese concentration providing of course that it exceeds the concentration of the enzyme. In other words, the high levels of substrate drive the reaction to EMn^{++} S locking Mn^{++} to the enzyme surface. The above equation may be written in reciprocal form giving

$$1/v = 1/V_{max} + (1/V_{max})(K_m)(1 + 1/K_a (Mn^{++}))(1/S)$$

where $K_m = (k_{-2} + k_3)/k_2$ and $K_2 = k_1/k_{-1}$. Double reciprocal plots should then go to a common intercept at all manganese concentrations. Plots shown in Figure 11 indeed demonstrate this feature.

The slope of 1/v versus 1/S equals $(K_m/V_{max})(1 + 1/K_a(Mn^{++}))$. A plot of the slopes obtained at several manganese concentrations against $1/(Mn^{++})$ should then give a straight line with an intercept equal to K_m/V_{max} and a slope equal to $K_m/(V_{max})(K_a)$. This was also observed as shown in Figure 12.

The kinetic parameters K_a, V_{max}, and K_m were then determined as a function of pH and sodium chloride concentration and are shown in Figures 13-18. The dependence of these parameters on pH and salt concentration demonstrated a complex, interwoven array of features. V_{max} fell below pH 8.5 at all salt levels which presumably reflected changes in the charge character of the substrate. The magnitude of the V_{max} plateau (8.5-9.5) was a strong function of salt concentration and was greatest at 1 M sodium chloride. The increase in V_{max} between 0.05 M and 1 M sodium chloride was believed to reflect shielding of electrostatic interactions which distort the active site. A similar salt effect on K_a was interpreted in the same way. This effect will be discussed later. K_m fell from pH 7 to 9.5. The decrease in K_m was interpreted as reflecting a complex array of changes in the charge character of the enzyme, substrate, and manganese binding site. K_m also decreased with increase in salt

between pH 7.0 and 9.5. This was believed to reflect salt effects on the electrostatic interactions between substrate and enzyme. The apparent independence of K_m on salt above pH 9.5 is not yet clear. K_a increased above pH 8 which suggested the presence of at least two distinct enzyme species that differ in level of protonation and affinity for manganous ion. The presence of these two forms was demonstrated more clearly by the rate of release of manganese from the holoenzyme as a function of pH. This also will be discussed in some detail later.

The binding of manganese as a function of salt at pH 8 represented one of the most interesting kinetic features of the halophilic enzyme, for over a one-hundred fold increase in magnitude was observed between 0.05 M and 1 M sodium chloride. One interpretation was that the addition of sodium chloride shielded repulsive electrostatic interactions between manganous ion and a positive charge or charges in the vicinity of its binding site on the enzyme surface. The addition of salt would then result in a primary electrostatic shielding effect and would be expected to follow the relationship

$$\log K_a = 1.02 Z_{Mn}{}^{++} Z_s \sqrt{s}/(1 + C\sqrt{s}) + Bs + \log (K_a)_o .$$

B and C are empirical constants, $Z_{Mn}{}^{++}$ is the charge of the manganous ion and equals 2, Z_s is the charge at the binding site

shielded by the added salt, s is the molar concentration of any univalent salt, and $\log (K_a)_o$ is the binding constant of manganese in the absence of salt. B and C are difficult to estimate particularly when the interactions of interest are between cations or anions of the salt and a charged group on the protein surface. Webb (31) has estimated C to be equal to 1.31 for shielding effects of potassium chloride on enzyme-inhibitor interactions. Assuming C for sodium chloride to be similar to potassium chloride, the above expression may then be rewritten giving

$$\log K_a = 1.02 Z_{Mn}{}^{++} Z_s \sqrt{s}/(1 + 1.31\sqrt{s}) + \log (K_a)_o$$

The contribution of the B term has been ignored. With the other gross assumptions, this would represent only a small portion at best. A plot of $\log K_a$ versus $1.02\sqrt{s}/(1 + 1.31\sqrt{s})$ should give a linear plot with a slope equal to the product of $Z_{Mn}{}^{++} Z_s$. Since $Z_{Mn}{}^{++}$ equals 2, Z_s may be determined. The slope obtained was 11. Z_s would then equal 5.5. Since the amino acid composition of the enzyme was found to be low in the basic amino acids, this seems an unreasonable concentration of positive charge in the vicinity of the binding site.

A second interpretation was that a number of electrostatic interactions on the enzyme surface tend to distort the conformational features of the manganese binding site. The addition of sodium

chloride would shield these interactions and allow the binding site to assume a proper conformation for maximum binding of manganese. The resulting increase in K_a with increasing salt concentration would then reflect a secondary electrostatic shielding effect. This effect would be expected, however, to be related to the primary shielding effect and to follow

$$\log K_a = 1.02\, n\sqrt{s}/(1 + 1.31\sqrt{s}) + \log (K_a)_o$$

where n is a proportionality or electrostatic factor relating the binding constant, K_a, to the primary shielding effect. A plot of $\log K_a$ versus $1.02\sqrt{s}/(1 + 1.31\sqrt{s})$ would again give a straight line with a slope equal to n.

A third and perhaps more realistic interpretation was that the addition of salt had two effects: 1) an effect on the interaction of groups on the enzyme surface which distort the binding site, and 2) an effect on the electrostatic interactions between manganous ion and its binding site. The addition of salt would shield the interactions between manganese and its binding site and superimpose a negative salt effect upon the observed K_a. The value of n would then be a function of the shielding of interactions on the enzyme surface which increases K_a and the shielding of manganous ion from its binding site which decreases K_a. Without knowledge of the charge character of the binding site, it is impossible to separate

these two contributions.

There is then sufficient evidence to support the existence of electrostatic interactions on the enzyme surface which are of importance in the stability of the halophilic enzyme. Since the binding of manganese is involved in the stability of the enzyme in low salt, the interactions which effect its binding ability also dramatically effect the stability of the enzyme. As will be discussed later, the salt requirements for the stability of the enzyme without bound manganese far exceed those required to shield the electrostatic component demonstrated in this work.

Enzyme Stability Studies

Holoenzyme Stability

The rate of release of manganese ion from the holoenzyme surface was studied as a function of pH in 0.05 M and 4 M sodium chloride. Sufficient EDTA was added to remove all manganous ion from equilibrium with the enzyme. The results shown in Figure 19 demonstrated that the release of manganese from the enzyme was exceedingly pH dependent in 4 M sodium chloride, and that the apoenzyme generated could be reactivated to very nearly the original activity simply by adding manganese back to the enzyme in the assay solution. Similar studies in 0.05 M sodium chloride

demonstrated that upon release of manganese the generated apoenzyme denatured spontaneously and could not be reactivated as before. The rapid rate of denaturation of the apoenzyme in low salt concentrations was supported by work on the stability of this species. This work will be discussed later. These results suggest that bound manganese does exert a strong influence on the stability of the enzyme in low salt concentrations, and that the release of manganese is the rate determining step in the low salt denaturation of the enzyme.

The rate of release of manganese from the holoenzyme was first-order as shown in Figures 20 and 21. The half-life of the holoenzyme at each pH was then determined from the slope of each line. The data obtained were then re-plotted as shown in Figure 22. The general features of the curves agree very well with the curves of K_a versus pH determined kinetically. The affinity of the enzyme for manganous ion was very low below pH 8 and increased dramatically up to a pH of 10.5. The titration nature of the curves suggests that the removal of one or several hydrogen protons from a group or groups having a pK of 9.2-9.5 dramatically changes the enzyme's affinity for manganese. The nature and location of these groups have not yet been determined.

Apoenzyme Stability

The stability of the apoenzyme was studied under a number of conditions. The primary objective was to correlate salt stabilization of the alkaline phosphatase with the nature of the stabilizing salt in the absence of bound manganese. Stability under these conditions would then be solely a measure of the strength of the intramolecular forces of the protein.

The apoenzyme was generated very rapidly at pH 7 in the presence of high salt concentrations and EDTA. Its level was then determined by reactivation in the presence of manganese under the assay conditions described. Denaturation was initiated by diluting the salt concentration to the desired concentration and the apoenzyme level determined as a function of time. Preliminary studies on the denaturation kinetics of the apoenzyme suggest a complex denaturation process. However, the initial rates were first-order and were used as an index of the stability of the apoenzyme under the conditions described.

As shown in Figure 24, the apoenzyme exhibited sharp transitions in stability over very narrow ranges in salt concentration. The position of each transition was a strong function of the anion present. Sulfate ion had the greatest stabilizing effect followed by chloride, bromide, and iodide. Cations were also examined for

their ability to stabilize the apoenzyme. Sodium and potassium chlorides were found to give comparable stabilization. Lithium and ammonium chlorides did not stabilize the apoenzyme. Ammonium sulfate gave stabilization comparable to sodium chloride which demonstrated again the stronger stabilizing features of sulfate ion over chloride.

The general features of the apoenzyme stability curves do not appear similar to those expected for pure electrostatic shielding as observed for the binding of manganese. The strong dependence of apoenzyme stability on the nature of the anion also suggested stabilization by salt effects other than electrostatic. The stabilizing ability of the anions studied paralleled very nicely the lyotropic or Hofmeister anion series shown in Table 5. With the exception of ammonium ion, the stabilizing ability of the cations also paralleled the lyotropic cation series. It then appeared that lyotropic salt effects were of major importance in the salt stabilization of halophilic alkaline phosphatase. To interpret lyotropic stabilization, the data of Schrier and Schrier (27) have been examined and are presented here to demonstrate the basic features of such stabilization.

Schrier and Schrier studied the effects of salt solutions on the activity coefficients of N-methyl acetamide and N-methyl propionamide as models for salt effects on proteins. The amide portion was considered analogous to the peptide linkage and the aliphatic portion

Table 5. Lyotropic or Hofmeister Series of the Ions.

	——— Increasing Disruptive Power ——→				
Cations	Na^+	Li^+			Ca^{++}
Anions	$SO_4^=$	Cl^-	Br^-	I^-	SCN^-
	←—— Increasing Stabilizing Power ———				

Table 6. Salting-Out Constants*.

Salt	k_{CH_3}	k_a	$k_{CH_3} + k_a$	$k_\phi + k_a$
NaCl	0.10	-0.11	-0.01	+0.005
NaBr	0.085	-0.11	-0.025	-0.01
NaI	0.061	-0.10	-0.04	-0.03
LiCl	0.07	-0.11	-0.04	-0.03
LiBr	0.046	-0.096	-0.05	-0.043
$CaCl_2$	0.14	-0.21	-0.07	-0.05
$CaBr_2$	0.091	-0.18	-0.09	-0.075
NaSCN	0.077	-0.18	-0.10	-0.09
LiSCN	0.048	-0.18	-0.13	-1.25
Na_2SO_4	0.182	0.008	+0.19	+ .21

*Data of Schrier and Schrier (27).

analogous to the hydrophobic amino acid side chains. The activity coefficients obtained gave the linear relationship

$$\log f_i/f_i^o = k_s c$$

where f_i and f_i^o are the activity coefficients in the presence and absence of salt, respectively, c is the molar concentration of the salt, and k_s is the salting-in constant if negative or the salting-out constant if positive. Assuming the effects on the aliphatic and amide portions of these compounds to be independent, k_s could be separated into k_a for the amide portion, and k_{CH_3} and k_{CH_2} for the aliphatic portion. The constants obtained are shown in Table 6. K_{CH_2} is omitted, for it equals $1/1.6\ k_{CH_3}$. It should be noted that all of the salts studied salt-out the hydrophobic portion of these model compounds and, with the exception of sulfate, salt-in the amide portion. If extended to the effects on proteins, lyotropic effects would then arise from the sum of salt effects on the hydrophobic amino acid side chains and the peptide linkages. It would then be the sum of k_a and k_{CH_3} which would determine the magnitude and direction of lyotropic salt effects as shown in Table 6. For lyotropic effects to be stabilizing, the magnitude of the salting-out term must exceed that of the salting-in term. An interesting prediction of this data then is that the mean-residue hydrophobicity of the amino acid

residues (3) must exceed that of the single methyl group of alanine (750) for sodium chloride to stabilize protein structure. There is no evidence to support this prediction. However, it is interesting to note that the calculated hydrophobicity of halophilic alkaline phosphatase, being 860, is within the predicted range for lyotropic stabilization. The prediction may be extended further by setting the salting-out term, k_ϕ, equal to 860/750 or 1.15 k_{CH_3}. This gives the mean-residue hydrophobicity of the alkaline phosphate in terms of equivalent methyl groups, and allows each salt to be ordered in regard to its stabilizing ability toward the enzyme. The more positive the sum, $k_\phi + k_a$, the stronger will be lyotropic stabilization, whereas the more negative the sum the stronger will be lyotropic disruption. As shown in Table 6, sodium sulfate would then be the most efficient in stabilization of the enzyme followed by sodium chloride. Sodium bromide, and iodide would disrupt the enzyme structure. In the cation series, sodium would be the most efficient in stabilizing the enzyme. Lithium and calcium would disrupt the enzyme. The series predicted from the work of Schrier and Schrier follow very well the observed stabilizing effects of salts on the halophilic alkaline phosphatase.

Several preliminary studies on enzyme stability during the purification work demonstrated that 20% sorbital, sucrose, and glucose did impart considerable stabilization to the enzyme in low salt.

This work was not pursued in any great detail. It is mentioned here only as a suggestion for future work on the mechanism of salt stabilization of halophilic systems. Stabilization by these compounds has been observed for a number of proteins and has been interpreted as the result of indirect effects on hydrophobic interactions and hydrogen bonding within the protein (9). It is interesting that such effects are in some respects very similar to lyotropic salt effects.

The picture which emerges from this work is that the intramolecular forces within the halophilic enzyme are not sufficient to maintain the integrity of the enzyme in a solvent environment in which extensive solvent-protein interactions can take place. The function of salt is then to lower solvent-protein interactions thereby increasing intramolecular interactions within the protein required for the stability of the enzyme. The binding of manganese gives additional stabilization to the enzyme. Binding is strongest above pH 8 and in the presence of sufficient salt to shield the electrostatic component on the enzyme surface. The model presented in Figure 25 incorporates the basic features of electrostatic, hydrophobic, and manganese-enzyme interactions which appear to influence the integrity of the halophilic alkaline phosphatase.

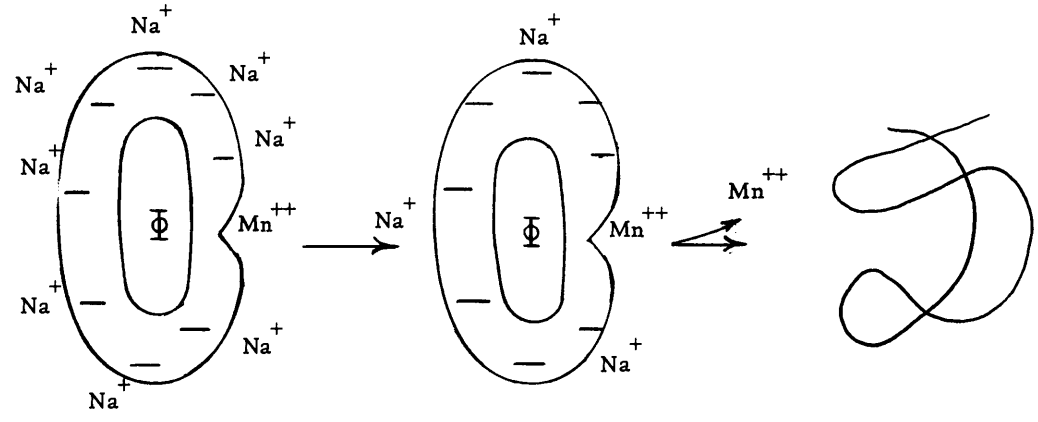

Figure 25. Model for Salt Stabilization of Halophilic Alkaline Phosphatase.

The area designated by the symbol Φ represents the sum total of all hydrophobic pockets at the interior of the protein salted-out from the solvent environment and surrounded by an electrostatic shell of negative charge. When the enzyme is transferred to low salt, it would be expected to swell resulting in greater distances between negative charges and strain in the conformational features of the manganese binding site. This would be associated with a decrease in K_a. Upon loss of manganese, the enzyme could then undergo considerable conformational change leading to extensive solvent-protein interactions and disruption of the hydrophobic interior.

BIBLIOGRAPHY

1. Bayley, S. T. Composition of ribosomes of an extremely halophilic bacterium. Journal of Molecular Biology 15:420-427. 1966.

2. Bayley, S. T. and E. Griffths. A cell-free amino acid incorporating system from an extremely halophilic bacterium. Biochemistry 7:2249-2256. 1968.

3. Bigelow, Charles C. On the average hydrophobicity of proteins and the relation between it and protein structure. Journal of Theoretical Biology 16:187-211. 1967.

4. Breed, Robert S., E. G. D. Murray and Nathan R. Smith (eds.) Bergy's manual of determinative bacteriology. 7th ed. Baltimore, Williams and Wilkins Company, 1957. 1095p.

5. Canal Industrial Corporation. Chemical formulation for disc electrophoresis. Bethesda, Maryland, 1962. 5p.

6. Canal Industrial Corporation. Model 12 disc electrophoresis instructions. Bethesda, Maryland, 1962. 7p.

7. Christian, J. H. B. and M. Ingram. The freezing points of bacterial cells in relation to halophilism. Journal of General Microbiology 20:27-31. 1959.

8. Dorn, Gordon L. Purification and characterization of phosphatase I from Aspergillus nidulans. Journal of Biological Chemistry 243:3500-3506. 1968.

9. Gerlsma, S. Y. Reversible denaturation of ribonuclease in aqueous solutions as influenced by polyhydric alcohols and some other additives. Journal of Biological Chemistry 243: 957-961. 1968.

10. Green, Arda Alden and Walter L. Hughes. Protein fractionation on the bases of solubility in aqueous solutions of salts and organic solvents. In: Methods in enzymology, ed. by Sidney P. Colowich and Nathan O. Kaplan. Vol. I. New York, Academic Press, 1955. p. 67-90.

11. Holmes, P. K. and H. Orin Halvorson. The inactivation reactivation of salt-requiring enzymes from an extreme obligate halophile. Canadian Journal of Microbiology 9:904-906. 1963.

12. Holmes, P. K. and H. Orin Halvorson. Properties of a purified halophilic malic dehydrogenase. Journal of Bacteriology 90:316-326. 1965.

13. Holmes, P. K. and H. Orin Halvorson. Purification of a salt-requiring enzyme from an obligately halophilic bacterium. Journal of Bacteriology 90:312-315. 1965.

14. Kadner, Robert J., Joseph F. Nyc and Douglas M. Brown. A repressible alkaline phosphatase in Neurospora crassa. II. Isolation and chemical properties. Journal of Biological Chemistry 243:3076-3082. 1968.

15. Kushner, D. J. Lysis and dissolution of cells and envelopes of an extremely halophilic bacterium. Journal of Bacteriology 87:1147-1156. 1964.

16. Kushner, D. J. Mass culture of red halophilic bacteria. Biotechnology and Bioengineering 8:237-245. 1966.

17. Kushner, D. J. et al. Morphological and chemical properties of cell envelopes of the extreme halophile, Halobacterium cutirubrum. Canadian Journal of Microbiology 10:483-497. 1964.

18. Larsen, Helge. Biochemical aspects of extreme halophilism. Advances in microbial physiology 1:97-132. 1967.

19. Larsen, Helge, Halophilism. In: The bacteria, ed. by I. C. Gunsalus and Roger Y. Stanier. Vol. 4. New York, Academic Press, 1962. p. 297-342.

20. Layne, Ennis. Spectrophotometric and turbidimetric methods for measuring protein. II. Protein estimation with the Folin-Ciocalteu reagent. In: Methods in enzymology, ed. by Sidney P. Colowich and Nathan O. Kaplan. Vol. 3. New York, Academic Press, 1957. p. 448-450.

21. Moore, Stanford and W. H. Stein. Chromatographic determination of amino acids by use of automatic recording

equipment. In: Methods in enzymology, ed. by Sidney P. Colowich and Nathan O. Kaplan. Vol. 6. New York, Academic Press, 1963. p. 819-831.

22. Onishi, H., Margaret E. McCance and N. E. Gibbons. A synthetic medium for extremely halophilic bacteria. Canadian Journal of Microbiology 11:365-373. 1965.

23. Pocker, J. and J. T. Stone. The catalytic versatility of erythrocyte carbonic anhydrase. III. Kinetic studies of the enzyme-catalysed hydrolysis of p-nitrophenyl acetate. Biochemistry 6:668-678. 1967.

24. Reiner, John M. Behavior of enzyme systems. Minneapolis, Burgess Publishing Company, 1959. 317p.

25. Rothman, Frank and Raymond Byrne. Fingerprint analysis of alkaline phosphatase of Escherichia coli K12. Journal of Molecular Biology 6:330-340. 1963.

26. Schlesinger, Milton J. Activation of a mutationally altered form of the Escherichia coli alkaline phosphatase by zinc. Journal of Biological Chemistry 241:3181-3188. 1966.

27. Schrier, Eugene E. and Evelyn B. Schrier. The salting-out behavior of amides and its relation to the denaturation of proteins by salts. Journal of Physical Chemistry 71:1851-1860. 1967.

28. Spackman, D. H. Accelerated methods. In: Methods in enzymology, ed. by C. H. W. Hirs. Vol. 11. New York, Academic Press, 1967. p. 3-15.

29. Torriani, Annamaria. Influence of inorganic phosphate in the formation of phosphatases by E. coli. Biochimica et Biophysica Acta 38:460-469. 1960.

30. Von Hippel, Peter H. and Thomas Scheich. The effects of neutral salts on the conformational stability of macromolecules in solution. In: Biological macromolecules, ed. by G. Fasman and S. Timasheff. Vol. 2. New York, Marcel Lekker. (In press)

31. Webb, John Leyden. Enzymes and metabolic inhibitors. Vol 1. New York, Academic Press, 1963. 949p.

APPENDIX

APPENDIX

Medium of Halvorson (11)

	g/l
NaCl	250
$MgCl_2 \cdot 6H_2O$	30
Trypticase	2.5
Yeast Extract	2

Medium of Kushner (16)

	g/l
Casamino Acids	7.5
Yeast Extract	10
Trisodium Citrate	3
KCl	2
$MgSO_4$	10
NaCl	250
	mg/l
$FeSO_4 \cdot 7H_2O$	50
*$MnCl_2 \cdot 4H_2O$	20

*Added as a 1 M solution just after inoculation.

Defined Medium of Gibbons (22)

DL-Alanine	43	Glycerol	0.1
L-Arginine	40	NH_4Cl	0.5
L-Cysteine	5	NaCl	25
L-Glutamic Acid	130	$MgSO_4 \cdot 7H_2O$	2
or DL-Aspartic Acid	45	KNO_3	mg/100 ml
Glycine	6	Trisodium citrate	50
DL-Isoleucine	44	$MnSO_4 \cdot H_2O$	0.03
L-Leucine	80	$CaCl_2 \cdot 7H_2O$	0.7
L-Lysine	85	$ZnSO_4 \cdot 7H_2O$	0.044
DL-Methionine	37	$FeCl_2$	0.23
DL-Phenylalanine	26	$CuSO_4 \cdot 5H_2O$	5μg
L-Proline	5		
DL-Serine	61		
DL-Threonine	50		
L-Tyrosine	20		
DL-Valine	100		
(Adenosine-5'PO_4)	(10)		
(Uridine-5'PO_4)	(10)		

200 mM KH_2PO_4 was added for P_i-limiting growth.

Nucleotides were excluded for P-free growth.

```
00001:          PROGRAM LSQRMCP
00002:          DIMENSION X(20), Y(20), YEVAL (20),  RESID (20), COE (2), STD (2)
00003:       1  I = 0
00004:          PRINT 2
00005:       2  FORMAT (1HO, 'X', 10X, 'Y'//)
00006:       3  I = I + 1
00007:          X (I) = TTYIN (0)
00008:          IF (X (I). LE. 0. 0) GO TO 5
00009:          Y (I) = TTYIN (0)
00010:          GO TO 3
00011:       5  N = I - 1
00012:          CALL LSQR (X, Y, N, COE, STD, RMS)
00013:          DO 10 I = 1, N
00014:          YEVAL (I) = COE (1) + COE (2) * X (I)
00015:      10  RESID (I) = YEVAL (I) - Y (I)
00016: C
00017:          PRINT 20
00018:      20  FORMAT (1HO, 'I', 9X, 'X', 12X, 'Y', 8X, 'YEVAL', 5X, 'RESID')
00019:          PRINT 30, (I, X (I), Y (I), YEVAL (I), RESID (I), I = 1, N)
00020:      30  FORMAT (1H , 2X, I2, 2X, 4F12. 4)
00021:          PRINT 40, COE (1), STD (1), COE (2), STD (2), RMS
00022:      40  FORMAT (1HO, 'COE (1) = ', F12. 4, 6X, 'STD (1) = 1, F12. 4//
00023:          11X, 'COE (2) = ', F12. 4, 6X, 'STD (2) = ', F12. 4// 1X, 'RMS = ', F12. 4/
///)
00024:          GO TO 1
00025:          END
00026:
00027:          SUBROUTINE LSQR (X, Y, N, COE, STD, RMS)
00028:          DIMENSION X (20), Y (20), COE (2), STD (2)
00029:          FN = N
00030:          SUMX = 0. 0
00031:          SUMY = 0. 0
00032:          SUMXY = 0. 0
00033:          SUMXSQ = 0. 0
00034:          SUMYSQ = 0. 0
00035:          DO 10 I = 1, N
00036:          SUMX = SUMX + X (I)
00037:          SUMXSQ = SUMXSQ + X (I)**2
00038:          SUMXY = SUMXY + X (I)*Y (I)
00039:          SUMY = SUMY + Y (I)
00040:      10  SUMYSQ = SUMYSQ + Y (I)**2
00041:          CONST = SUMXSQ*FN - SUMX**2
00042:          COE (2) = (SUMXY*FN - SUMX*SUMY)/CONST
00043:          COE (1) = (SUMY - COE (2)*SUMX)/FN
00044:          RMS = SUMYSQ - FN*COE (1)**2 - 2. 0*COE (2)*SUMXY + COE (2)**2
*SUMXSQ
00045:          CONST = RMS/(CONST*(FN - 2. 0))
00046:          STD (1) = SQRT (SUMXSQ*CONST)
00047:          STD (2) = SQRT (FN*CONST)
00048:          RMS = SQRT (RMS/FN)
00049:          RETURN
00050:          END
```