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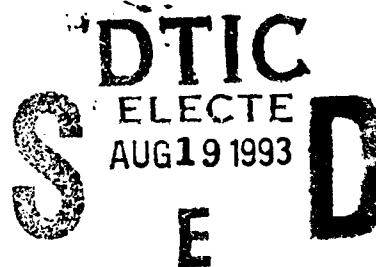


**SODIUM IONS AFFECT THE pH BEHAVIOR OF THE SOLUBLE
HYDROGENASE OF ALCALIGENES EUTROPHUS H16**

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The experiments reported herein were conducted according to the principles set forth in the current edition of the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This technical report has been reviewed by the NMRI scientific and public affairs staff and is approved for publication. It is releasable to the National Technical Information Service where it will be available to the general public, including foreign nations.

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REPORT DOCUMENTATION PAGE

1a. REPORT SECURITY CLASSIFICATION UNCL			1b. RESTRICTIVE MARKINGS	
2a. SECURITY CLASSIFICATION AUTHORITY			3. DISTRIBUTION/AVAILABILITY OF REPORT Approved for public release; distribution is unlimited	
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE				
4. PERFORMING ORGANIZATION REPORT NUMBER(S) NMRI 93-04			5. MONITORING ORGANIZATION REPORT NUMBER(S)	
6a. NAME OF PERFORMING ORGANIZATION Naval Medical Research Institute		6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION Bureau of Medicine & Surgery	
6c. ADDRESS (City, State, and ZIP Code) 8901 Wisconsin Avenue Bethesda, MD 20814-5055			7b. ADDRESS (City, State, and ZIP Code) Department of the Navy Washington, DC 20372-5120	
8a. NAME OF FUNDING/SPONSORING ORGANIZATION Naval Medical Research & Development Command		8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER	
8c. ADDRESS (City, State, and ZIP Code) 8901 Wisconsin Avenue Bethesda, MD 20814-5044			10. SOURCE OF FUNDING NUMBERS	
			PROGRAM ELEMENT NO.	PROJECT NO.
			61153N	MR04101
			TASK NO.	WORK UNIT ACCESSION NO.
			.00D	1103
11. TITLE (Include Security Classification) SODIUM IONS AFFECT THE pH BEHAVIOR OF THE SOLUBLE HYDROGENASE OF ALCALIGENES EUTROPHUS H16				
12. PERSONAL AUTHOR(S) Tyree, B., A.L. Harabin, M.C. Falk, J.R. DeJesus, and K.K. Kumaroo				
13a. TYPE OF REPORT Technical Report		13b. TIME COVERED FROM 1/91 TO 10/91	14. DATE OF REPORT (Year, Month, Day) January 1993	15. PAGE COUNT
16. SUPPLEMENTARY NOTATION				
17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number) Alcaligenes eutrophus hydrogenase, hydrogenase, hydrogenase inhibitors, hydrogen metabolism	
FIELD	GROUP	SUB-GROUP		
19. ABSTRACT (Continue on reverse if necessary and identify by block number) Hydrogenase activity of the soluble hydrogenase from <i>Alcaligenes eutrophus</i> H16 was inhibited by monovalent cations in the order $\text{Na}^+ > \text{Li}^+ > \text{K}^+ > \text{NH}_4^+ > \text{Cs}^+$. Hydrogenase activity was also slightly inhibited by monovalent anions. The Na^+-induced inhibition was not altered with varying NAD^+ or H_2 concentrations, suggesting that Na^+ ions do not interfere with substrate binding. The inhibition was lowered when the H^+ concentration was increased over a range of pH 8.5 to 7.0. Examination of the pH-velocity curve shows that NaCl lowers and shifts the pH optimum in the acidic direction. These data suggest that Na^+ ions bind to the enzyme, perhaps displacing protons.				
20. DISTRIBUTION/AVAILABILITY OF ABSTRACT ☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT. ☐ DTIC USERS			21. ABSTRACT SECURITY CLASSIFICATION Unclassified	
22a. NAME OF RESPONSIBLE INDIVIDUAL Regina E. Hunt, Command Editor			22b. TELEPHONE (Include Area Code) (202) 295-0198	22c. OFFICE SYMBOL SD/RSD/NMRI

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ACKNOWLEDGEMENTS

The authors are grateful to Drs. Barbel Friedrich and Bernd Lorenz for guidance on enzyme purification and assay. The authors also wish to thank Drs. Karoly Meszaros and Taffy Williams for helpful discussions throughout this investigation, and Ms. Tracy Lester for assistance in the preparation of this manuscript.

This work was supported by Naval Medical Research and Development Command Work Unit No. MR04101.00D-1103. The opinions and assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the U.S. Navy or the naval service at large.

INTRODUCTION

The soluble hydrogenase of *Alcaligenes eutrophus* H16 (E.C.1.12.1.2.) catalyzes the reversible reduction of NAD^+ with molecular hydrogen. The enzyme is a complex heterotetramer with a molecular weight of about 200 kD (1), and contains Ni, Fe-S centers, and FMN as prosthetic groups (2,3). In addition to hydrogenase activity the enzyme also exhibits diaphorase (NADH oxidase) activity, and can reduce a wide variety of physiological and artificial electron acceptors (4). Factors involved in the regulation of catalytic activity are largely unknown. Early reports suggested that the enzyme is sensitive to simple electrolytes in solution, and particularly to sodium salts, but the mechanism of this inhibition is not known (4). Sensitivity to sodium salts was also demonstrated for the *E. coli* enzyme (5). Since sodium is ubiquitous in biological systems it seems plausible that sodium might represent a natural inhibitor. In this report we describe the effects of sodium salts on the kinetic parameters of hydrogenase purified from *A. eutrophus* H16.

MATERIALS AND METHODS

Chemicals

Puratronic NaCl (99.999% pure) was obtained from Specialty Products. Chelex-100 was obtained from Bio-Rad. NAD, NADH, protamine sulfate, and egg white lysozyme (44,000 U/mg) were from Sigma. [2-(N-morpholino)ethanesulfonic acid] was from Calbiochem-Behring. All other chemicals were of the highest purity available.

Enzyme Purification

A. eutrophus H16 was grown heterotrophically as previously described (6). The cells were harvested by centrifugation, suspended in 0.05 M tris-HCl pH 8.0, and stirred 30 min at 23 °C with 12,000 U/ml lysozyme. All other procedures were carried out at 5 °C. The cells were broken by a passage through a pre-chilled French pressure cell, and the suspension centrifuged at 100,000 x g 1 h. To the supernatant was added $K_3Fe(CN)_6$ to a concentration of 0.1 mM. The same concentration of $K_3Fe(CN)_6$ was maintained throughout the purification and added to all column buffers to enhance stability of the enzyme (personal communication, B. Friedrich). Protamine sulfate (10 mg/g cell paste) was added, the sample stirred 30 min, and centrifuged at 10,000 x g 30 min. Ammonium sulfate fractionation and DEAE-Sephacel and procion red-agarose chromatographies were performed as previously described (7). The enzyme preparation was approximately 90% pure as judged by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (8), and had a specific activity of 40-75 U/mg.

Enzyme Assays

Assays were performed in 0.05 M tris-HCl pH 8.0 saturated with O_2 -free H_2 (4). Reagents were added through a rubber septum into a 1.5 ml quartz cuvette using gas-tight syringes. Typically, the enzyme was activated by the addition of 0.1 mM NADH in H_2 saturated buffer, and the sample assayed within 5 min. Using enzyme isolated by the methods described here, the prolonged activation times were not required in contrast to previous investigations (9). The reaction was started by the addition of 0.8 M NAD^+ and the absorbance at 340 nm was monitored on a Shimadzu UV-160

spectrophotometer. The pH was varied by addition of 2-(N-morpholino)ethanesulfonic acid to the buffer. Diaphorase activity (NADH-ferricyanide reductase) was determined as previously described in an argon atmosphere (4). The evolution of hydrogen by NADH was monitored using a hydrogen electrode.

RESULTS AND DISCUSSION

The effectiveness of different types of salts on hydrogenase activity is summarized in Fig. 1. When assayed in 0.05 M tris-HCl, pH 8.0 in 0.1 M salt, LiCl, NaCl, and KCl reduced enzymatic activity to 66%, 37%, and 88%, respectively. The same concentrations of NH_4Cl , CsCl, and NH_4OAc had little or no effect on activity. Inclusion of NaBr, NaF or Na_2SO_4 in the assay gave similar results as with NaCl; KI reduced activity to about 65%. From these data it can be concluded that $\text{Na}^+ > \text{Li}^+ > \text{K}^+ > \text{NH}_4^+ > \text{Cs}^+$ as enzyme inhibitors and that I^- was the most effective anion inhibitor at these concentrations. These results were in agreement with previous studies which indicated that sodium salts were quite effective as hydrogenase inhibitors (4).

When H_2 -dependent NAD^+ reduction was measured in the presence of 0.02 to 0.5 M NaCl a biphasic inhibition curve was obtained, possibly indicating more than one mode of inhibition or multiple inhibitory binding sites (Fig. 2a). A sharp loss of activity was seen as the salt concentration increased from 0.02 to 0.1M followed by a more gradual decline at higher salt concentrations (Fig. 2a). Approximately 0.05M NaCl was required for 50% inhibition of activity, and no time dependence was observed. The curve was unchanged if the NaCl was repurified on a Chelex column suggesting that the

inhibition was not due to contamination by heavy metals. The reverse reaction, the generation of H_2 by NADH, was also inhibited by NaCl, but to a lesser degree than the forward reaction (Fig. 2b). At NaCl concentrations below 0.1 M diaphorase activity (NADH-ferricyanide reductase) was not affected, however, at higher salt concentrations the activity was enhanced up to 1.6 fold (Fig. 2c).

To examine the reversibility of NaCl inhibition, the ferricyanide-oxidized enzyme was first incubated with 0.1 M NaCl, then diluted into 9 volumes of salt-free buffer, reduced with NADH and assayed. No inhibition was noted (compare the top curve of Fig. 3b with the curves of Fig. 3b). In contrast, when this experiment was repeated except the enzyme was first reduced with NADH in the presence of 0.1M NaCl then diluted and assayed as above, the level of NaCl-induced inhibition remained high (compare bottom curve of Fig. 3b with the curves of Fig. 3a). This suggests that either NaCl does not inhibit the oxidized enzyme or that the effect is fully reversible. Further, the NaCl-induced inhibition which occurs in the reduced enzyme are not readily reversible by dilution.

The inhibition by NaCl did not change with varying NAD^+ concentration (Fig. 4a) or with varying H_2 concentration (Fig. 4b), suggesting that NaCl isn't competitive with either substrate at the substrate levels tested. However, the degree of inhibition was lower as the H^+ concentration was increased from 3×10^{-9} to 1×10^{-7} , suggesting that Na^+ ions are displacing protons. As shown in table I relatively higher concentrations of LiCl and KCl were required for 50% inhibition at pH 8.5. At the lower pH, however, these were relatively ineffective as inhibitors. Similar results were observed with $MgCl_2$,

CaCl_2 , and BaCl_2 , possibly suggesting a common binding site on the enzyme for these cations. Examination of the cation effect at various pHs shows that 0.1 M NaCl produced an apparent change in the ionization constants which determine the pH-velocity curve (Fig. 5). The pH optimum was both lowered and shifted toward the acidic side, possibly as the result of combination of the enzyme with Na^+ ions. The same concentration of KCl also displaced the pH-velocity curve, resulting in an inhibition on the alkaline side and an apparent activation on the acidic side of the curve. The results obtained with 0.1 M LiCl were similar to those obtained with KCl.

In this study we have examined the effects of NaCl on various catalytic activities of the soluble hydrogenase from *Alcaligenes eutrophus* H16. This enzyme is thought to be composed of two different types of subunit dimers; a small dimer that catalyzes the hydrogenase reactions and a somewhat larger diaphorase dimer that catalyzes the diaphorase partial electron transfer reactions (1,10). The results indicate that Na^+ (in physiologically relevant concentrations) is a potent inhibitor of the NAD^+ -dependent hydrogenase reaction. The rate of NADH-dependent reduction of ferricyanide, however, was increased over the same concentration range. Thus, it seems likely that the sodium ion binds to and inhibits the hydrogenase subunits and these subunits do not participate at all in the NADH-dependent reduction of ferricyanide. Sodium ions appear not to inhibit the binding of the substrates, NAD^+ and H_2 to the enzyme, since changing substrate concentrations does not alter the degree of inhibition. Instead, the data suggests that Na^+ ions inhibit enzymatic activity by displacing H^+ ions. It is not clear why this process should not be readily reversible unless the displaced protons have

limited access to the site or subsequent, irreversible reactions occur in the Na^+ -bound form of the enzyme. While the precise location of the site(s) of inhibition cannot be deduced from the results of these studies, it is possible that Na^+ ions bind near an acid or base in the active site and cause a downward shift in its pK_a . It would be interesting to know the mechanisms by which the cell protects a soluble cytoplasmic enzyme from inactivation in a sea of sodium.

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TABLE I
EFFECT OF pH ON CATION INHIBITION OF HYDROGENASE

The reaction mixture contained 0.2 U hydrogenase activated with 0.1 mM NADH in H₂-saturated 0.05 M tris-HCl at the indicated pH containing the indicated concentration of salt in a total volume of 1.5 ml. The reaction was started by the addition of 0.8 mM NAD⁺, and the A(340 nm) monitored.

Salt	% Inhibition at pH 8.5	% Inhibition at pH 7.0
100 mM LiCl	50.2	4.7
300 mM KCl	50.1	4.4
30 mM MgCl ₂	43.8	0
30 mM CaCl ₂	54.6	3.4
40 mM BaCl ₂	65.3	7.3

FIGURE LEGENDS

Fig. 1. Effect of salts on hydrogenase activity. The reaction mixture contained 0.2 U hydrogenase activated with 0.1 mM NADH in H_2 -saturated 0.05 M tris-HCl pH 8.0 containing 0.1 M salt (except Na_2SO_4 , which was 0.05 M) in 1.5 ml total volume. The reaction was started by the addition of 0.8 mM NAD^+ , and the $A(340\text{ nm})$ monitored.

Fig. 2. Concentration dependence of NaCl on enzymatic activities of hydrogenase. a) H_2 -dependent- NAD^+ reduction. Hydrogenase activity was assayed as described in Fig. 1, varying the NaCl concentration from 0.02 to 0.5 M. b) H_2 generation by NADH. The enzyme was assayed in Ar-saturated 0.05 M tris-HCl pH 8.0 containing NaCl. Following addition of 5.5 mM $Na_2S_2O_4$ and 2.4 mM NADH, H_2 production was monitored with a hydrogen electrode. c) NADH-ferricyanide reduction. The reaction was performed in the same Ar-saturated buffer containing 0.6 mM $K_3Fe(CN)_6$. Following addition of 0.6 mM NADH the absorbance was monitored at 405 nm.

Fig. 3. Reversibility of NaCl inhibition of hydrogenase activity. a) Hydrogenase activity in the presence of NaCl. The (H_2 -saturated) enzyme, 5 μ l, was added to 1.5 ml H_2 -saturated 0.05 M tris-HCl pH 8.0 containing no NaCl ($\blacktriangle$), 0.01 M NaCl ($\circ$) or 0.1 M NaCl ($\blacktriangledown$); 15 μ l 10 mM NADH was added and after 2 min the reaction started by the addition of 15 μ l 80 mM NAD^+ . b) Preincubation of the oxidized and reduced enzyme with NaCl. Lower curve ($\blacktriangle$) - The enzyme, 5 μ l, was added to 0.15 ml H_2 -saturated 0.05 M tris-HCl pH 8.0 containing 0.1 M NaCl and 0.1 mM NADH to reduce; after 2 min the NaCl was diluted to 0.01 M by the addition of 1.35 ml 0.05 M tris-HCl pH 8.0 containing 0.1 M NADH, and the cuvette inverted to mix; the reaction was started by addition of 15 μ l 80 mM NAD^+ . Upper curve ($\bullet$) - The enzyme, 5 μ l, was added to 0.15 ml H_2 -saturated 0.05 M tris-HCl pH 8.0 containing 0.1 M NaCl; the NaCl was diluted to 0.01 M by the addition of 1.35 ml 0.05 M tris-HCl pH 8.0, and the cuvette inverted to mix; the enzyme was reduced with 15 μ l 10 mM NADH and after 2 min the reaction started by addition of 15 μ l 80 mM NAD^+ .

Fig. 4. Effect of varying substrate concentrations on hydrogenase activity. Hydrogenase was assayed as described in Fig. 1 using: a) 0.70 mM H_2 with varying NAD^+ , 0.80 mM ($\circ$), 0.53 mM ($\blacklozenge$) or 0.32 mM ($\blacktriangle$); b) 0.80 mM NAD^+ with varying H_2 concentrations, 0.70 mM ($\circ$), 0.35 mM ($+$), 0.14 mM ($\blacklozenge$), and 0.05 mM ($\blacktriangle$); c) 1.5 ml H_2 -saturated tris-HCl pH 8.5 ($\square$), pH 8.0 ($\blacktriangledown$), pH 7.5 ($\blacktriangledown$), pH 7.0 ($\blacksquare$).

Fig. 5. pH-velocity curve of hydrogenase. The enzyme was assayed in H_2 -saturated 0.05 M tris-HCl above pH 7; for the lower pH values, the pH was adjusted with [2-(N-morpholino)ethanesulfonic acid]. ($\square$) no salt; ($\blacktriangle$) 0.1 M NaCl; ($\blacktriangle$) 0.1 M KCl.

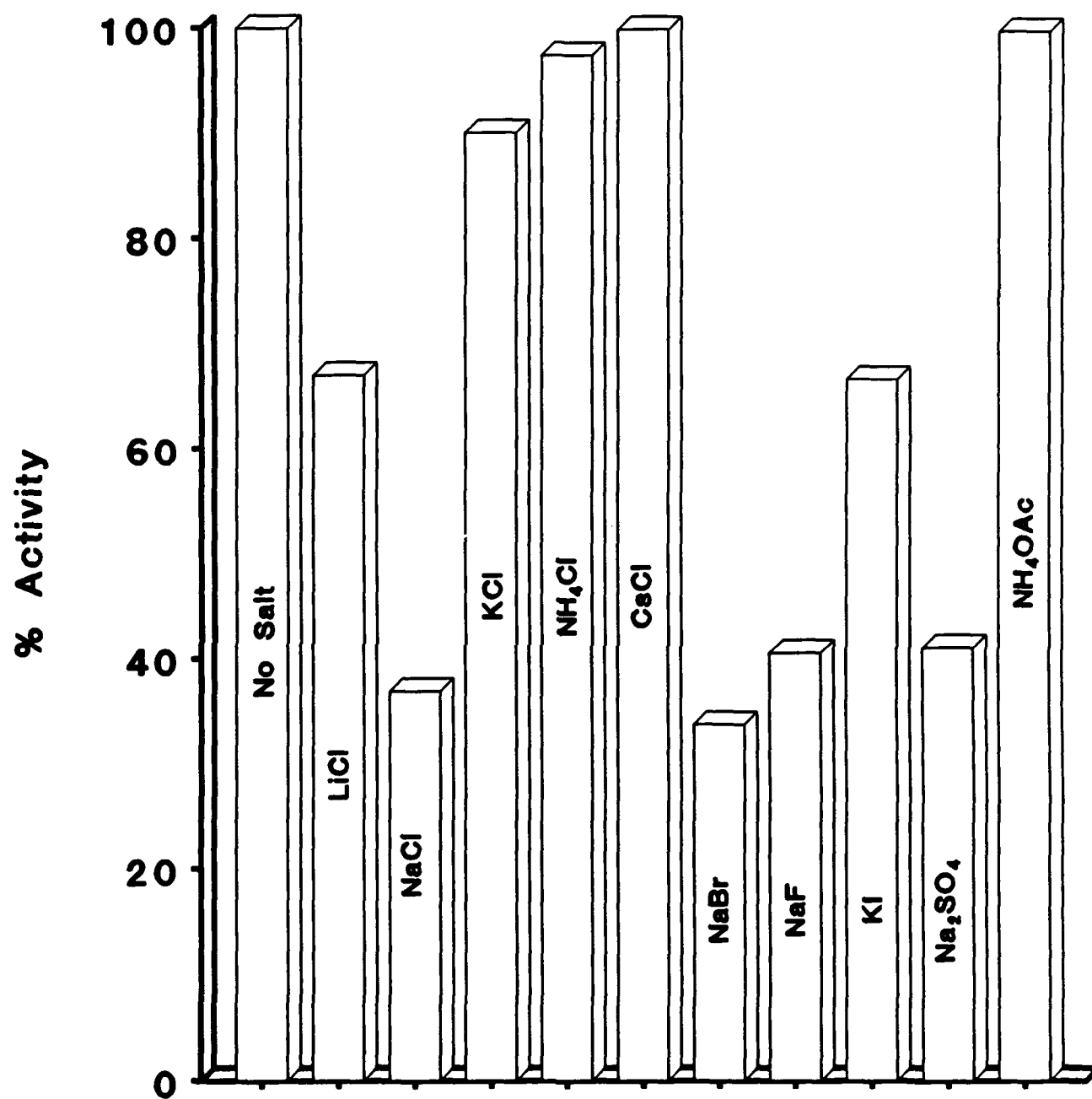
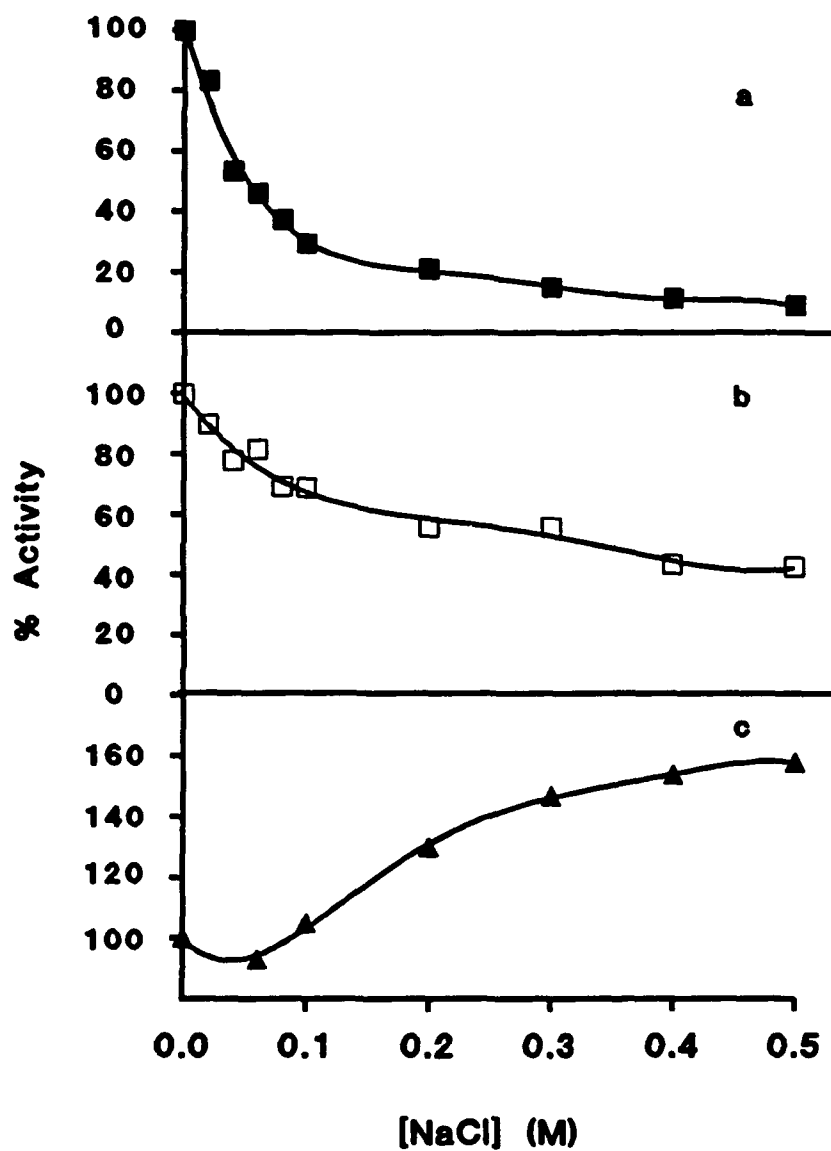


Figure 1

**Figure 2**

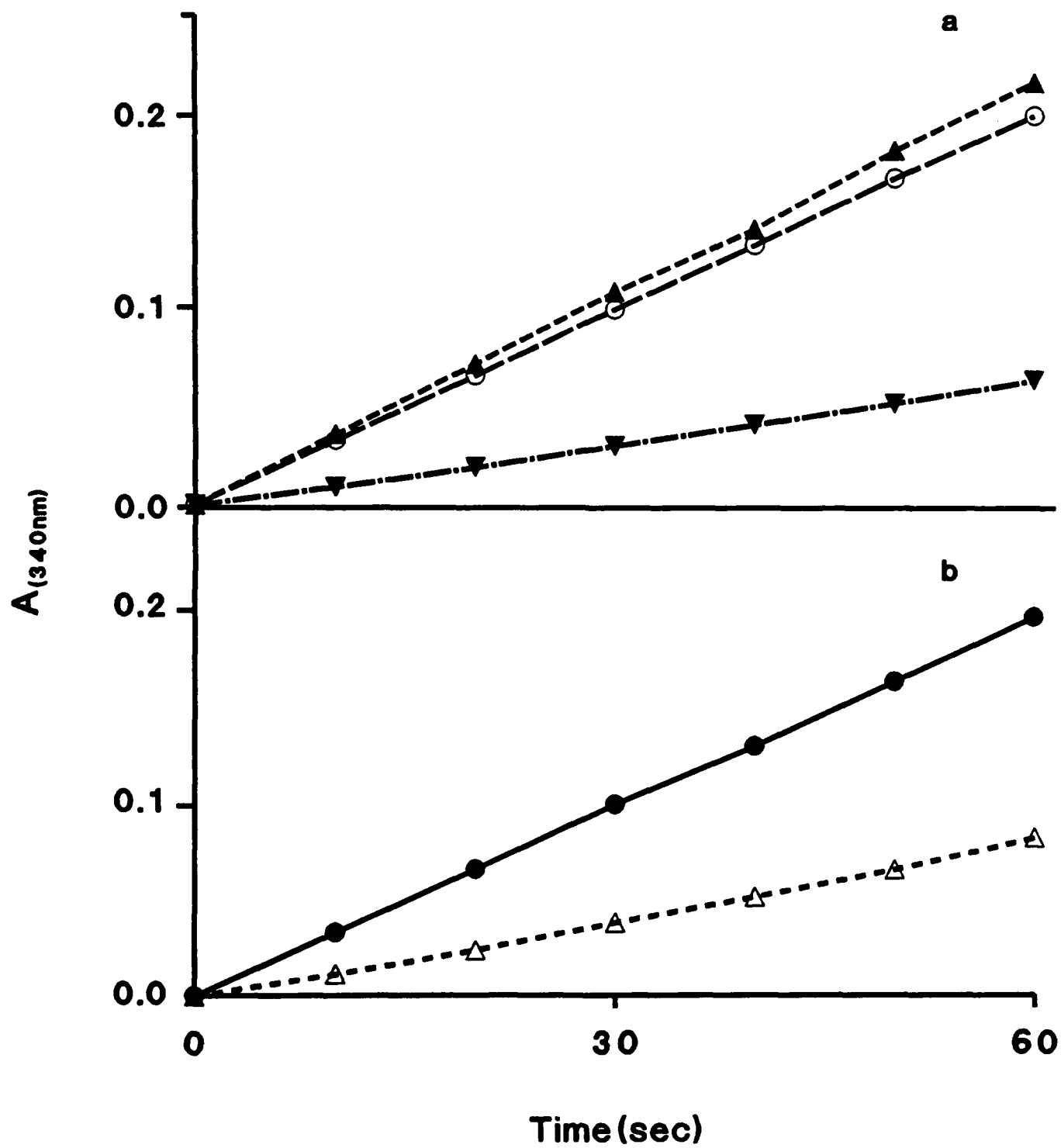


Figure 3

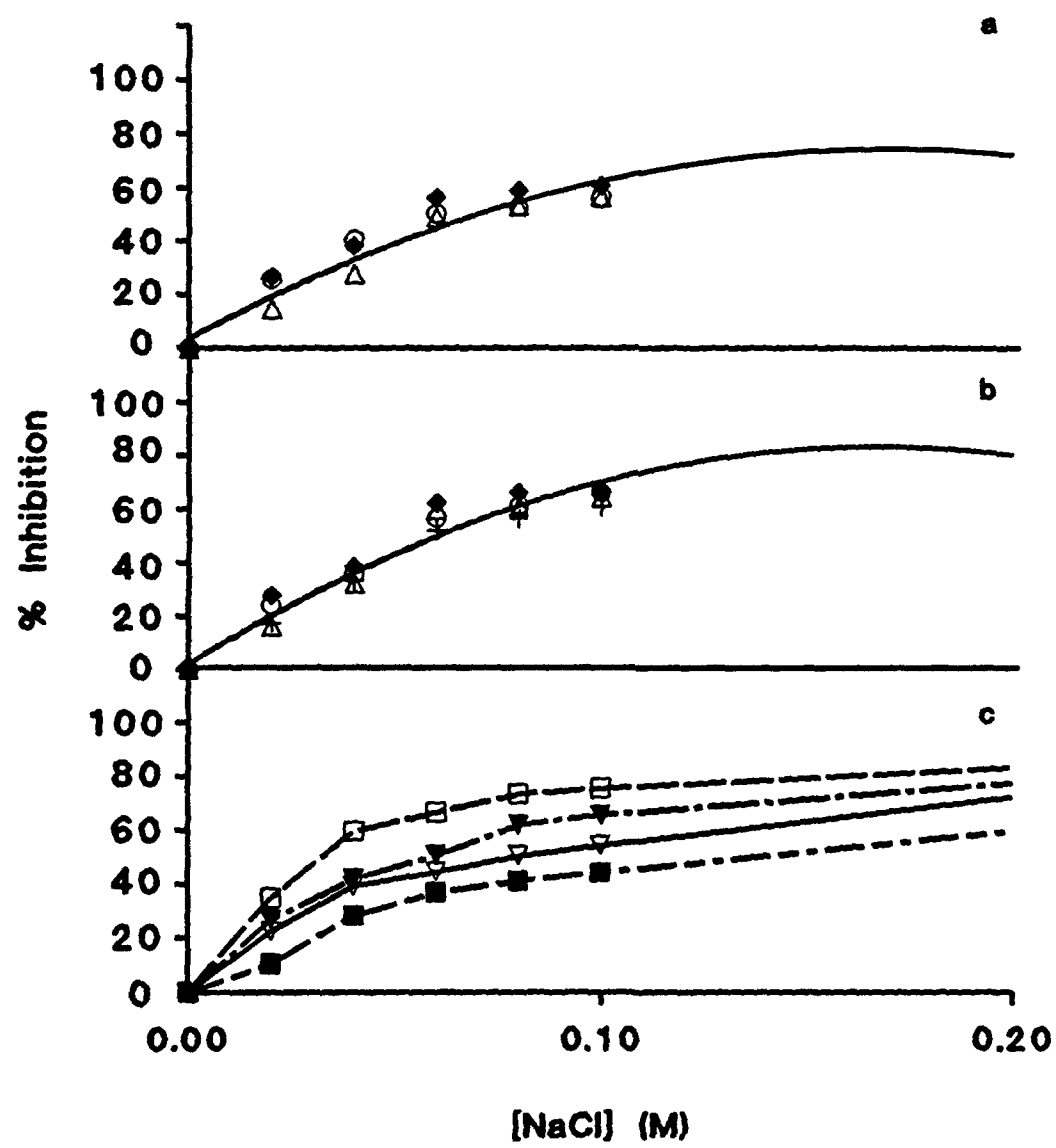


Figure 4

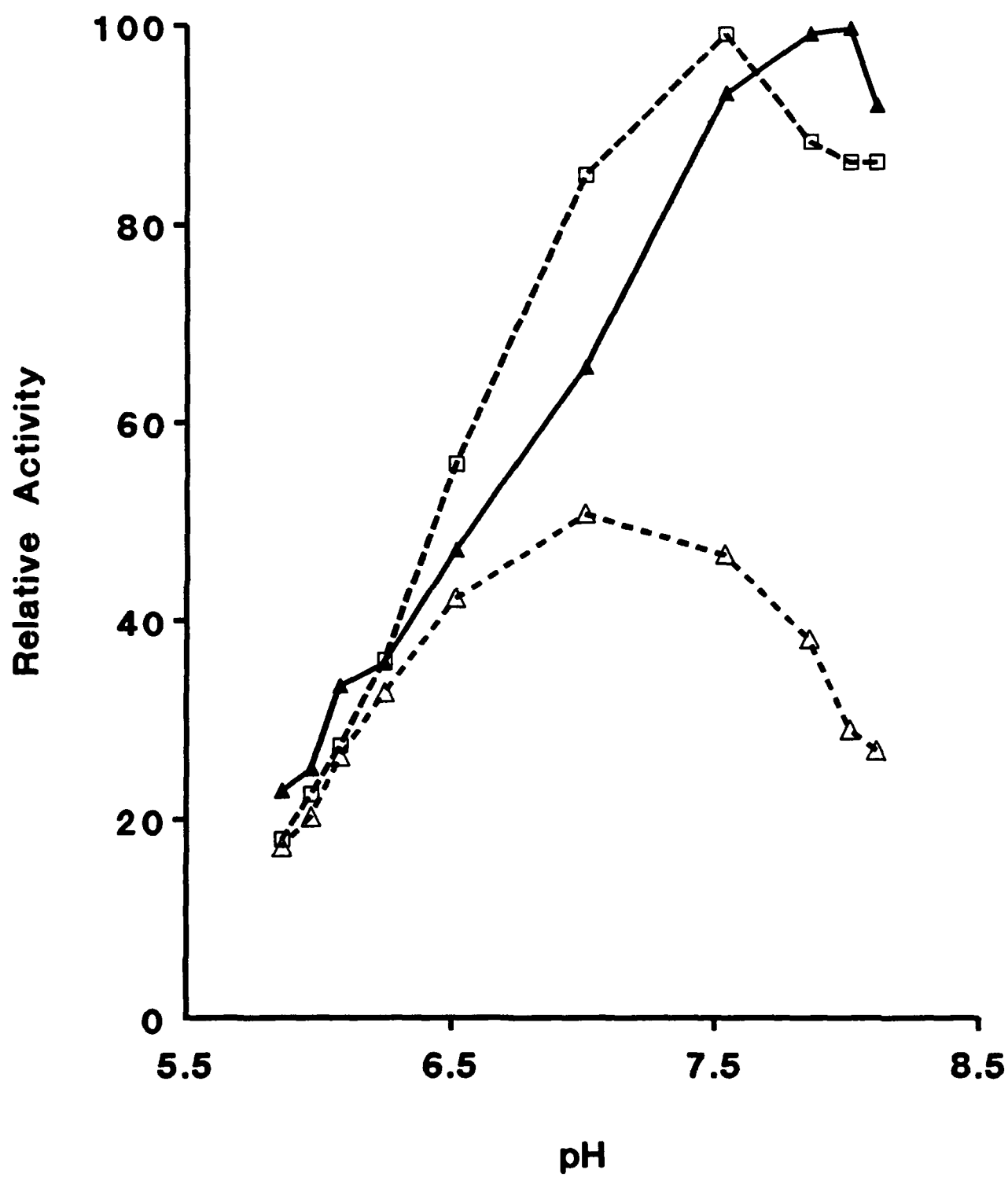


Figure 5