The Salt Specificity of an NADH-Oxidase Isolated From an Extreme Halophile[1]

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ABSTRACT

Extracts prepared from an extreme halophile contained an NADH oxidase active at high solute concentrations. The cation requirement was nonspecific since KCl, RbCl, and CsCl replaced NaCl with little or no loss of activity while NH_4Cl was partially effective. Only LiCl failed to replace NaCl. No specific chloride requirement was observed although not all anions replaced chloride. Bromide, nitrate, and iodide were essentially ineffective while acetate, formate, citrate, and sulfate proved suitable. The presence of sulfate affected the ability of a cation to satisfy the solute requirement. It enhanced the rate of NADH oxidation compared to the rate observed in the presence of chloride, and cations which, as chlorides, were inactive (LiCl and $MgCl_2$ at high concentrations) satisfied the cation requirement when added as sulfate salts. While magnesium satisfied the cation requirement, a concentration as well as an anion effect was observed. In the presence of $MgCl_2$, little oxidation was observed at concentrations greater than 1 M. At lower concentrations, the rate of oxidation increased, reaching a maximum value at 0.1 M and remaining constant to 0.05 M $MgCl_2$. Magnesium acetate and $MgSO_4$ also replaced NaCl and the maximum rate of oxidation occurred at 0.05 M with respect to magnesium. There was no change in the rate of oxidation at high magnesium acetate concentrations, whereas with $MgSO_4$ a second region of maximum activity was observed.

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INTRODUCTION

Enzymes isolated from extreme halophiles are not only functional at high solute concentrations but in many instances require a relatively high cation concentration for maximum activity (7). While the cation requirement of the lactic (1), glycerol (2), and malic dehydrogenases (6) isolated from Halobacterium salinarium can be satisfied by various alkali metals, potassium chloride is generally most effective in activating various enzymes isolated from extreme halophiles (3, 6, 7). This is consistent with the observation that the intracellular potassium ion concentration in extreme halophiles is unusually high (4). Baxter (1) proposed that halophilic enzymes were unstable as a consequence of intramolecular electrostatic charges and that cations functioned by neutralizing these charges so that the enzymes could assume the conformation in which they were most active as catalysts.

During studies on a salt-dependent NADH oxidase isolated from an extreme halophile, certain anomalies were observed which suggested that the cation requirement for NADH oxidation could not be solely explained by the neutralization of electrostatic charges. The data described in this paper report the effects of certain ions on the oxidation of NADH by crude extracts isolated from the extreme halophile, AR-1.

MATERIALS AND METHODS

Organisms. The organism employed in this study was a red chromogenic rod isolated from a local saltern and designated as strain AR-1. It was judged an extreme halophile on the basis of its failure to grow in media containing less than 15% NaCl as well as its conversion from rods to spheres, and eventual lysis, when the cells were placed in medium containing less than 15% NaCl. *Micrococcus halodenitrificans* (ATCC 13511) was obtained from the American Type Culture Collection and *Escherichia coli*, K-12, was obtained from Mr. Herbert Ginoza.

Media and growth conditions. Strain AR-1 was grown in a medium containing the following additions (in grams) in a final volume of 1 liter: Hy-Case SF (Sheffield Chemical Company), 5; yeast extract (Difco), 5; glycerol, 1; $MgCl_2 \cdot 6H_2O$, 20; KCl, 2; $CaCl_2 \cdot 2H_2O$, 0.2; NaCl, 200; tris (hydroxymethyl) aminomethane (Tris), 12.1. *M. halodenitrificans* was grown in the same medium except that the NaCl concentration was decreased to 60 g. *E. coli* was grown in nutrient broth. The pH of the media was adjusted to 7.4 with a Beckman E2 electrode.

AR-1 was grown at 37 C in 2-liter flasks containing 350 ml of medium and aerated by incubating the flasks on a Gyrotory shaker. After 48 hr, at which time the cells were in the maximum stationary phase, the cells were harvested and washed once with 0.1 M Tris-chloride buffer (pH 7.4) containing the following salts: 3.4 M sodium chloride, 2.5×10^{-2} M KCl, 9.8×10^{-2} M $MgCl_2$, and 9×10^{-4} M $CaCl_2$. M. halodenitrificans was washed once with 0.1 M Tris-chloride buffer (pH 7.4) containing 1.0 M NaCl, 0.13 M KCl, and 1×10^{-3} M $MgSO_4 \cdot 7H_2O$. E. coli was washed once in 0.1 M Tris-chloride buffer (pH 7.4).

Preparation of cell-free extracts. AR-1 was suspended in 0.1 M Tris-chloride-3.4 M NaCl-0.025 M KCl buffer, pH 7.4 (ST buffer) to a concentration of 200 mg cells (wet weight) per ml of buffer and the cell suspension was passed through a French Pressure Cell operated at 25,000 psi. The disrupted cells were centrifuged at 10,000 × g for 30 min at 4 C and the supernatant was used as the source of NADH oxidase activity. Such extracts could be stored at -70 C for at least 3 months with little loss of activity. The NADH oxidases from M. halodenitrificans and E. coli were prepared in an analogous fashion except that M. halodenitrificans was suspended in 0.1 M Tris-chloride buffer-1.0 M NaCl (pH 7.4) and E. coli was suspended in 0.1 M Tris-chloride buffer (pH 7.4) prior to disruption.

Enzyme assay. NADH oxidase activity was determined spectrophotometrically at 30 C in reaction mixtures containing the following additions in a total volume of 1 ml: Tris-chloride (containing the appropriate salt and at the desired concentration), pH 7.4 (100 μmoles); NADH (0.1 μmole); and enzyme. The enzyme was preincubated with the buffer for 2 min at 30 C and NADH was added to initiate the reaction. The amount of enzyme employed was adjusted so that the change in absorbance at 340 mμ was no greater than 0.150 per min. When measured in this fashion, the initial rate of NADH disappearance was proportional to the amount of extract added to the reaction mixture.

Protein determination. Protein was determined by the method of Lowry et al. (8) using crystalline bovine serum albumin (Sigma) as the standard. Appropriate blanks were employed to correct for the interference caused by the presence of Tris.

Preparation of buffers. Salt-containing Tris buffers were prepared at room temperature (22-24 C) using a Beckman E2 glass electrode. The electrode was assumed to be equally insensitive to all monovalent and divalent cations.

THE EFFECT OF SODIUM CHLORIDE

As shown in Fig. 1, NADH oxidase activity in extracts from AR-1 was dependent upon the concentration of NaCl. At 0.085 M NaCl (the lowest concentration of salt which could be tested because of the carry-over of NaCl with the enzyme), the rate of oxidation was 5% of the maximum rate. As the concentration of NaCl was increased, the rate of NADH oxidation increased, reaching a maximum at approximately 3.5 M sodium chloride and remaining essentially constant at the higher concentrations tested. For purposes of comparison, the effect of NaCl on the NADH oxidases from M. halodenitrificans and E. coli are also included. Extracts from both organisms oxidized NADH at markedly reduced rates when assayed at concentrations of NaCl in which extracts from AR-1 were maximally active. While the oxidation of NADH by extracts from E. coli was inhibited at all concentrations of NaCl tested, extracts from M. halodenitrificans were maximally active at from 0.4 to 1.0 M NaCl. Furthermore, while extracts from E. coli were essentially inactive at 3 M NaCl, extracts from M. halodenitrificans exhibited about 40% of maximum activity.

THE SPECIFICITY OF THE CATION REQUIREMENT

As shown in Fig. 2, various alkali metal chlorides satisfied the cation requirement for NADH oxidation. At low concentrations differences of questionable significance were observed. The maximum activities observed in the presence of NaCl, KCl, and CsCl were very nearly identical while the maximum activity observed in the presence of RbCl was approximately 30% greater than that observed in NaCl. In all four cases, the dose response curves exhibited a sigmoid-like response to solute concentration, and passed through a maximum at a concentration of 4 M (with the possible exception of KCl). NH_4Cl partially satisfied the cation requirement. However, the dose response curve was neither sigmoid in shape nor did it attain a saturating value. Furthermore, NADH oxidation in the presence of NH_4Cl was significantly lower than in the presence of NaCl. LiCl was ineffective in replacing sodium chloride.

THE EFFECT OF ANIONS ON NADH OXIDASE ACTIVITY

As shown in Fig. 3, the oxidation of NADH was relatively nonspecific with respect to anions when they were added as sodium salts. However, not all anions replaced chloride: bromide and iodide were without effect, the latter even inhibited the basal rate of oxidation. Nitrate proved barely stimulatory, and only at the higher concentrations tested.

Formate partially replaced chloride. The maximum rate of oxidation occurred at 2 M sodium formate and was approximately 55% of the rate observed at 4 M NaCl. Acetate and citrate also replaced chloride. As in the case of formate, maximum oxidation occurred at a much lower sodium ion concentration than when sodium was added as the chloride salt. While the maximum rate of oxidation in the presence of sodium acetate was identical to that observed with NaCl, the maximum rate of oxidation in the presence of sodium citrate was some 43% greater. Furthermore, the rate of NADH oxidation was greater in the presence of acetate and citrate than in the presence of chloride at all but the highest concentrations tested.

Sulfate, when added as Na_2SO_4, also satisfied the anion requirement. At the highest concentration tested (1.67 M), the rate of NADH oxidation was approximately 50% greater than the rate of oxidation observed in 4 M NaCl. The stimulatory effect of sulfate was also observed in the presence of other monovalent cations (Table 1). When the solutes were added so that the cation concentrations were essentially identical, the rate of oxidation was greater when the cation was supplied as the sulfate rather than as the chloride salt. The extent of enhancement ranged from 27% for Rb_2SO_4 to 650% for Li_2SO_4. The rate of NADH oxidation at 1.33 M sulfate was greater even when compared to the activity observed at a chloride concentration of 4 M. At this concentration, all the monovalent cations, with the exception of ammonium and possibly potassium, oxidized NADH at a maximum rate when added as chlorides (Fig. 2). The ability of sulfate to enhance NADH oxidation is more clearly shown in Fig. 4. Two effects are apparent: Sulfate can alter the intrinsic efficacy of a cation (cf. Figs. 2 and 4); sulfate can also determine the efficacy of a cation in satisfying the solute requirements for NADH oxidation. This is clearly demonstrated in the case of lithium, which, when supplied as LiCl, was relatively ineffective (Fig. 2), whereas Li_2SO_4 proved satisfactory. As indicated in Fig. 4, maximum oxidation of NADH occurred at a lithium ion concentration of 2.5 M, considerably lower than the concentration at which maximum oxidation occurred in the presence of NaCl. Furthermore, the rate of NADH oxidation at 1.25 M Li_2SO_4 was 87% of the rate observed at 4 M NaCl.

<u>The effect of magnesium</u>. As shown in Fig. 5, magnesium under certain conditions replaced sodium. However, a pronounced concentration effect as well as an anion effect was observed. Thus $MgCl_2$, which at concentrations greater than 1.0 M failed to

substitute for sodium, proved effective at lower concentrations with the maximum rate of oxidation occurring at 0.1 M $MgCl_2$ and remaining unchanged to approximately 0.05 M $MgCl_2$. At still lower concentrations, NADH oxidase activity was proportional to the magnesium concentration resulting in a rate 50% of the maximum at 0.01 M $MgCl_2$. Magnesium acetate and $MgSO_4$ also satisfied the cation requirement. At low concentrations of these salts, the rate was dependent on the magnesium concentration, with maximum activity occurring at approximately 0.05 M with respect to magnesium. At higher concentrations, the response was dependent on the nature of the accompanying anion. In the case of magnesium acetate, increasing the concentration of the salt did not change the rate of oxidation until the concentration of magnesium acetate reached 2 M, at which point a slight inhibition was observed. Increasing the concentration of $MgSO_4$ at first had little effect upon the rate of NADH oxidase activity. However, at approximately 1 M, the rate increased, resulting in a second maximum at 1.5 M $MgSO_4$. At still higher concentrations, an inhibition was observed.

Extracts prepared in ST buffer and subsequently dialyzed against 0.07 M $MgCl_2$-0.1 M Tris.HCl buffer oxidized NADH when assayed either in NaCl or $MgCl_2$. However, the rate of oxidation was greater in the presence of the former salt. Extracts could also be prepared directly in 0.07 M $MgCl_2$-0.1 M Tris-chloride buffer. Such extracts also oxidized NADH. However, the NADH oxidase activity was lower when assayed in NaCl. While the rate of oxidation in the presence of $MgCl_2$ was apparently independent of the manner in which the enzyme was prepared, the activity observed in the presence of NaCl was significantly greater in extracts initially prepared in ST buffer but subsequently dialyzed against 0.07 M $MgCl_2$-0.1 M Tris-chloride buffer (Table 2). Furthermore, both types of preparations retained NADH oxidizing activity for considerable periods of time in the frozen state as well as at 4 C.

DISCUSSION

The observations reported in this paper are not consistent with the proposal that the ionic requirements for NADH oxidation are related solely to the ability of cations to neutralize intramolecular electrostatic charges. It is difficult to reconcile the inability of lithium when supplied as LiCl to satisfy the cation requirement when cations of greater radii (such as rubidium and cesium) or lesser radius such as magnesium (9) are able to satisfy the cation requirement. It is also clear that the suitability of a cation is related in some fashion to the nature of the accompanying anion in both the amount of activity observed at a given cation concentration and the concentration of cation required to attain maximum activity. In addition, cations which are inactive in the presence of certain anions may exhibit considerable activity when supplied with another anion. This was reflected in the inability of NaI, NaBr, or $NaNO_3$ to satisfy the cation requirement, and more clearly demonstrated in the case of lithium, which was virtually ineffective when added as LiCl yet adequately replaced sodium when added as Li_2SO_4. Furthermore, the anion may alter the dose response curve of a cation as witnessed by the sigmoid response observed when $(NH_4)_2SO_4$ replaced NH_4Cl. In a more complicated case, the accompanying anion profoundly affected the ability of magnesium to satisfy the cation requirement although in this case a concentration effect was also present. The ability of magnesium to substitute for monovalent cations was the most inconsistent of the observations. Aside from the anion effects alluded to earlier, two concentration effects were observed. First, $MgCl_2$ at a concentration greater than 1.0 M did not satisfy the cation requirement whereas lower concentrations were effective. Secondly, all the magnesium salts tested satisfied the cation requirement at the rather low concentrations of 0.05 M, a decidedly nonhalophilic concentration relative to the concentration of monovalent cations required for maximum activity. These results were not unique to strain AR-1. We have obtained analogous data (in preparation) using NADH oxidases isolated from H. cutirubrum and H. halobium, both strains obtained from Dr. N. E. Gibbons.

The ability of magnesium to substitute for sodium at concentrations approximately 1/80 the cation concentration and about 1/25 the ionic strength is difficult to reconcile with the high monovalent cation requirements. That there may be factors other than those involving the neutralization of electrostatic charges is suggested by an observation of Holmes and Halvorson (5) who reported that several enzymes isolated from the extreme halophile H. salinarium can exist in an inactive salt-free form and are reactivated when appropriately treated with NaCl.

The complexity of the NADH oxidase system presented in this paper makes any facile interpretation of the data difficult. Operationally, one might conclude that the unusually high monovalent cation requirement is an artifact of isolation and represents the poor molar efficiency with which sodium (and other monovalent cations) replace magnesium. Or it might reflect the existence of two pathways of NADH oxidation: one active at high sodium concentrations while the other is operative at low sodium and magnesium concentrations. At the present time, studies are under way in an attempt to distinguish between these possibilities.

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TABLE 1. The effect of sulfate on the oxidation of NADH by various cations

Cation	$-\Delta A_{340} \cdot min^{-1}$		
	SO_4^-	2.5 M Cl^-	4 M Cl^-
Na^+	0.128	0.063	0.098
Cs^+	0.130	0.048	0.093
Rb^+	0.075	0.055	0.128
NH_4^+	0.105	0.030	0.053
Li^+	0.085	0.013	0.010
Tris	0.005	-	0.010

TABLE 2. The NADH oxidase activity of extracts suspended in $MgCl_2$ containing buffers

Experiment	Conditions	Specific Activity	
		Assayed in 4 M NaCl	Assayed in 0.07 M $MgCl_2$
1	Extract dialyzed against $MgCl_2$	0.074	0.059
2	Extract prepared in $MgCl_2$	0.029	0.048

TABLE LEGENDS

Table 1. The effect of sulfate on the oxidation of NADH by various cations. NADH oxidase activity was measured as described in the text at an extract concentration of 0.76 mg of protein. The sulfate salts were present at a concentration of 1.33 M.

Table 2. The NADH oxidase activity of extracts prepared in $MgCl_2$-containing buffers. In experiment no. 1, extracts were prepared as described in the methods section and then dialyzed for 18 hr against 3 changes of 0.07 M $MgCl_2$-0.1 M Tris-chloride, pH 7.4. In experiment no. 2, the extracts were prepared by suspending and disrupting cells in 0.07 M $MgCl_2$-0.1 M Tris-chloride buffer, pH 7.4. The extracts were subsequently assayed in 4 M NaCl-0.1 M Tris-chloride (pH 7.4) or in 0.07 M $MgCl_2$-0.1 M Tris-chloride (pH 7.4) as indicated. The results are expressed as specific activity (μmoles NADH oxidized × min^{-1} × mg $protein^{-1}$).

FIGURE LEGENDS

Fig. 1. The effect of sodium chloride on NADH oxidase activity. NADH oxidase from the extreme halophile AR-1 (⊙), the moderate halophile M. halodenitrificans (⊡), and E. coli (◇). The maximum activity observed in each case (expressed as μmoles NADH oxidized × min^{-1} × mg protein^{-1}) was AR-1 (0.041), M. halodenitrificans (0.085), and E. coli (0.17).

Fig. 2. The cation specificity of the NADH oxidase from AR-1. CsCl (●), RbCl (⊙), LiCl (▽), NH_4Cl (◇), KCl (⊡), and NaCl (△). Reaction mixtures contained 0.76 mg of protein.

Fig. 3. The anion specificity of the NADH oxidase from AR-1. The following anions were added as the sodium salts: acetate (●), citrate (▽), formate (⊙), sulfate (◇), chloride (△), nitrate (⊡), bromide (◺), and iodide (⌂). Reaction mixtures contained 0.76 mg of protein.

Fig. 4. The effect of various sulfate salts on the NADH oxidase activity from AR-1. Rb_2SO_4 (▽), Cs_2SO_4 (⊙), $(NH_4)_2SO_4$ (⊡), Li_2SO_4 (⊿), Na_2SO_4 (△), and NaCl (◇). Reaction mixtures contained 0.76 mg of protein.

Fig. 5. The effect of magnesium salts on the NADH oxidase activity from AR-1. $MgCl_2$ (⊙), Magnesium acetate (◇), and $MgSO_4$ (⊡). Reaction mixtures contained 0.76 mg of protein.

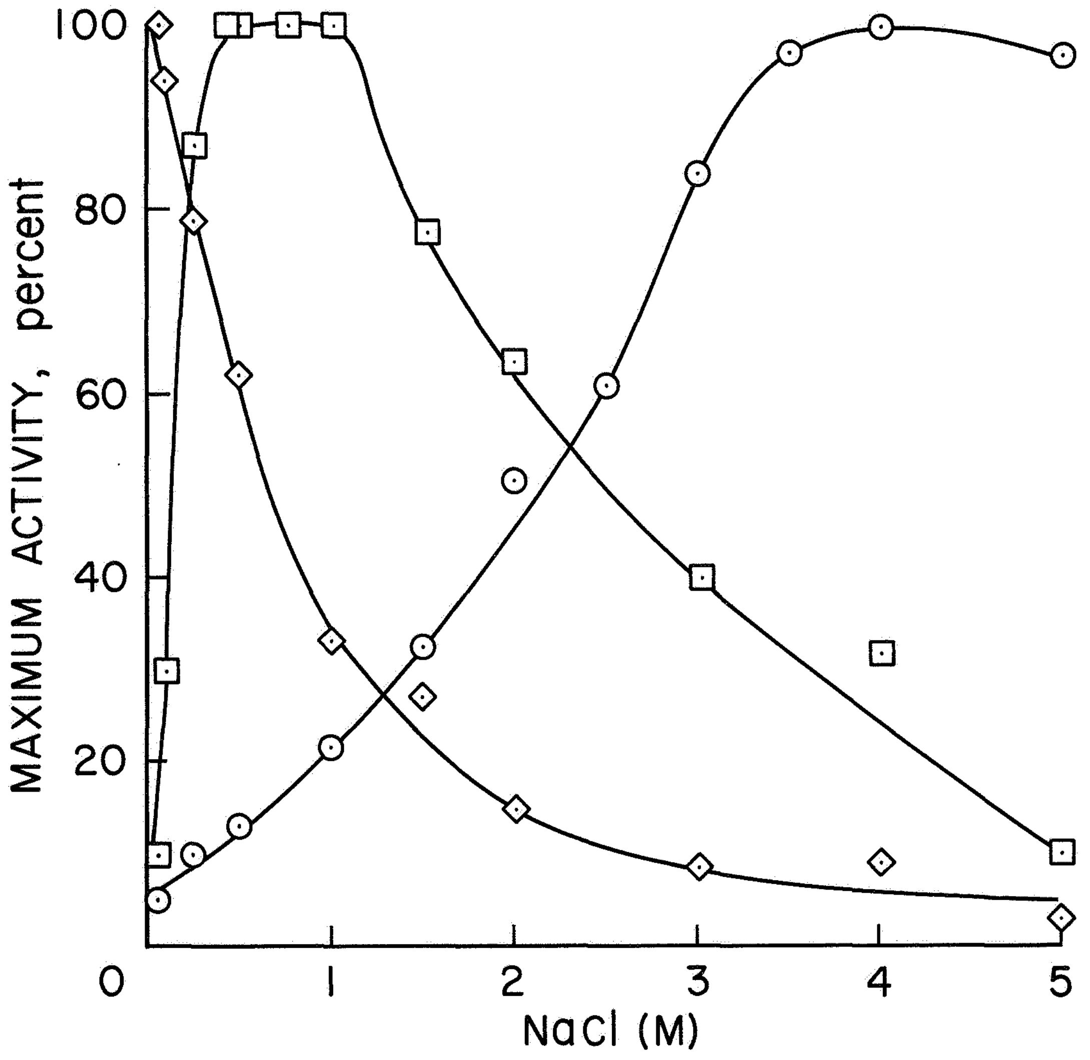

Figure 1.

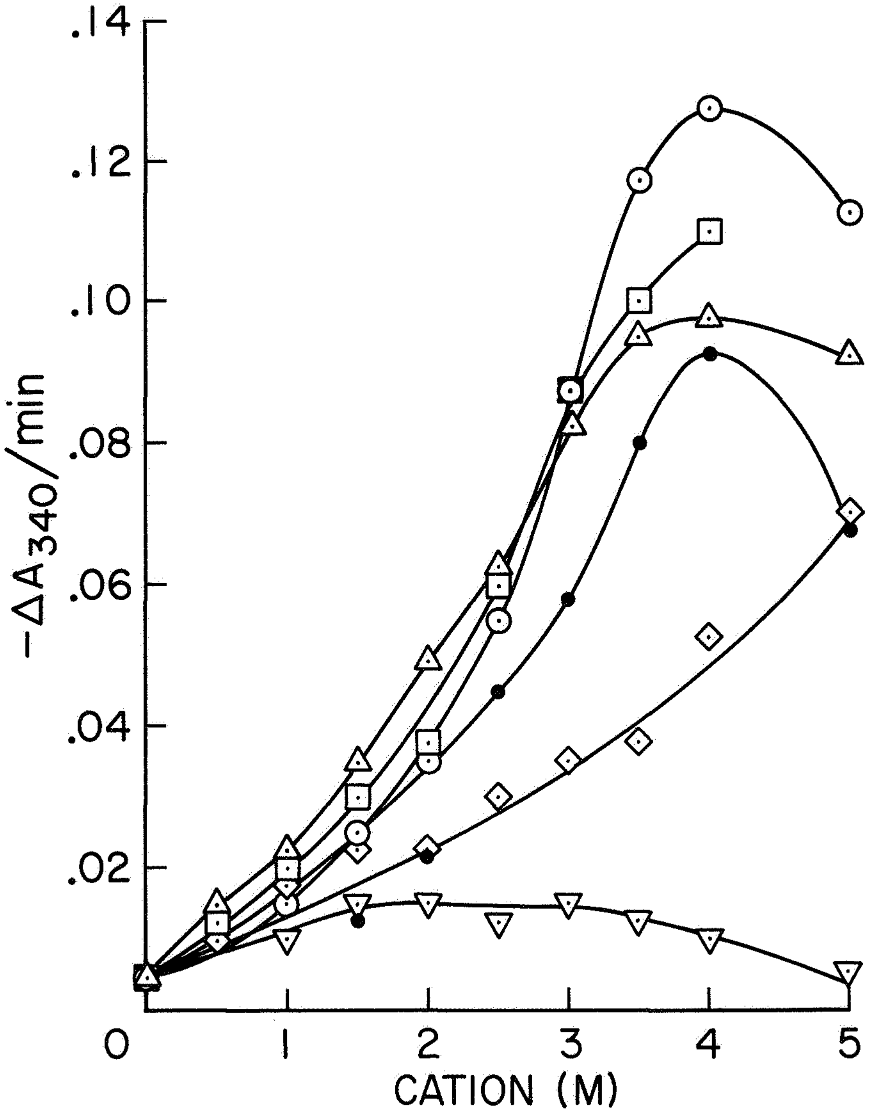

Figure 2.

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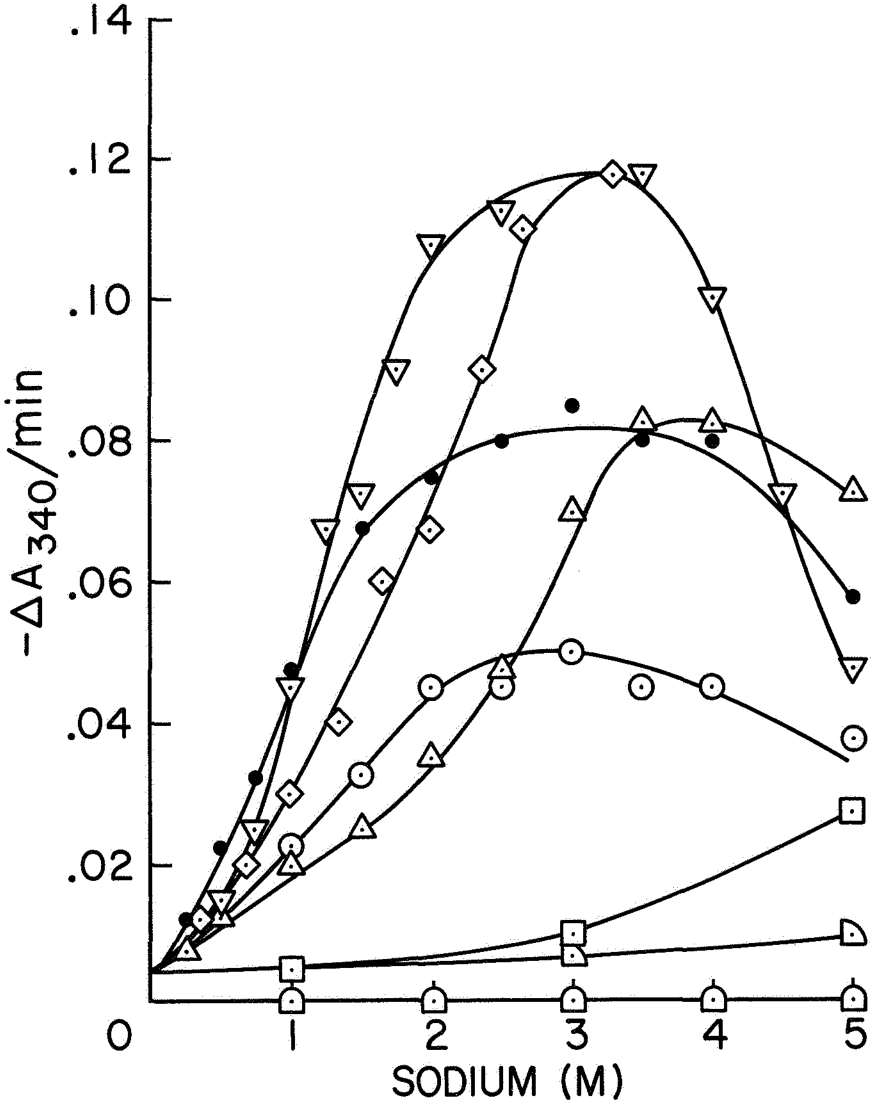

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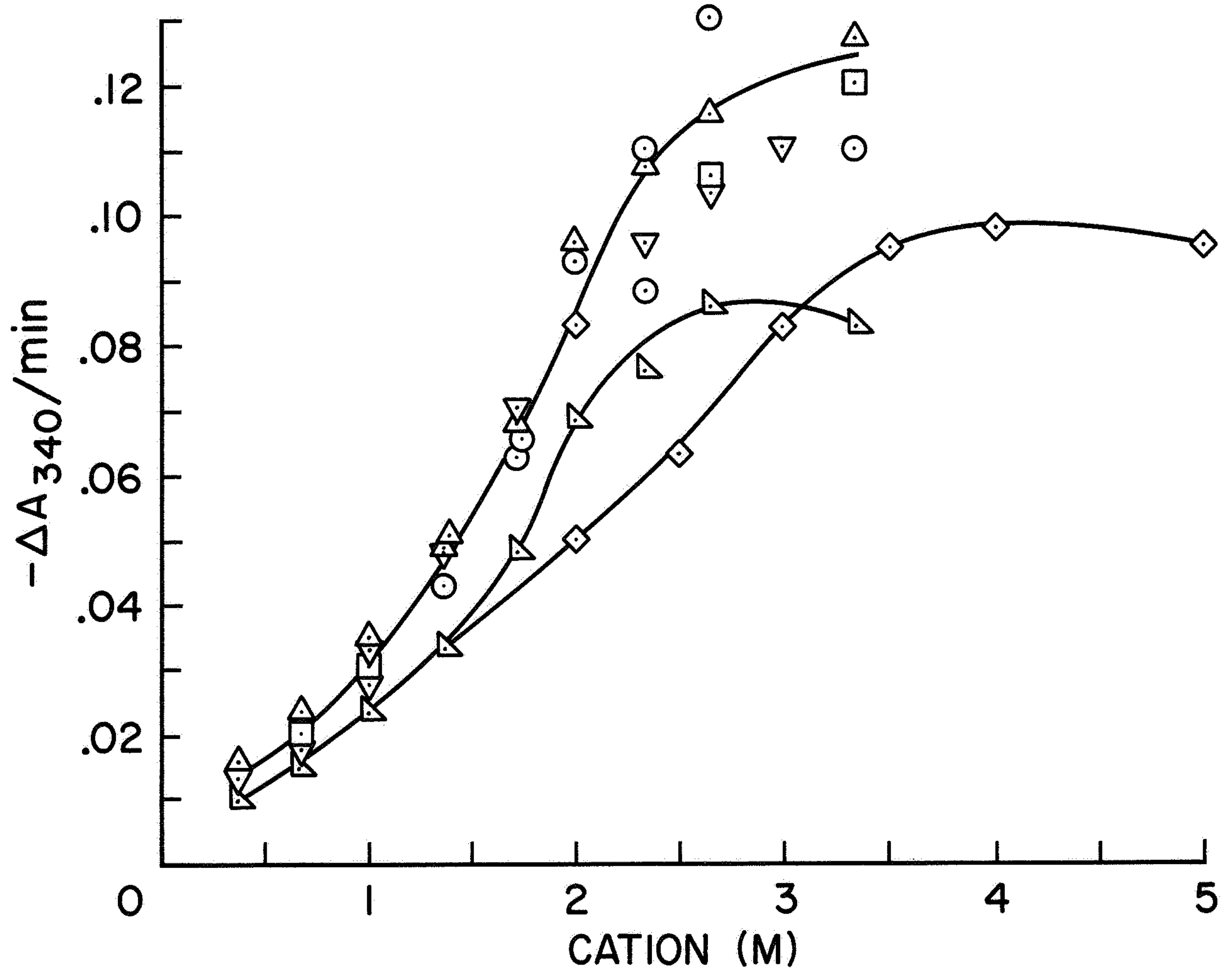

Figure 4.

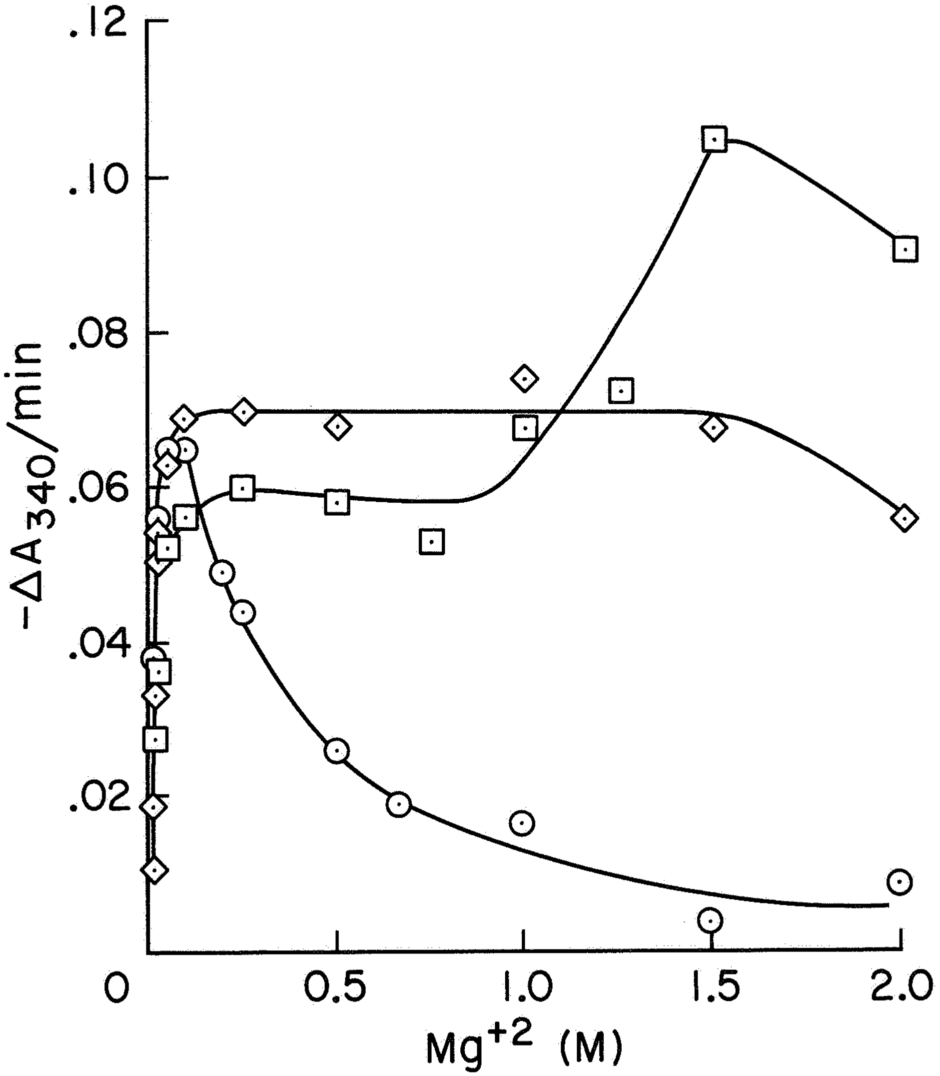

Figure 5.