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Salt Specificity of a Reduced Nicotinamide Adenine Dinucleotide Oxidase Prepared from a Halophilic Bacterium[1]

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Extracts prepared from a halophilic bacterium contained a reduced nicotinamide adenine dinucleotide ($NADH_2$) 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, and NH_4Cl was only 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, whereas acetate, formate, citrate, and sulfate proved suitable. The presence of sulfate affected the ability of a cation to satisfy the solute requirement. Sulfate enhanced the rate of $NADH_2$ oxidation when compared with the rate observed in the presence of chloride. Cations which were inactive as chlorides (LiCl and $MgCl_2$ at high concentrations) satisfied the cation requirement when added as sulfate salts. Although magnesium satisfied the cation requirement, a concentration effect, as well as an anion effect, was observed. In the presence of $MgCl_2$, little $NADH_2$ oxidation was observed at concentrations greater than 1 M. At lower concentrations, the rate of oxidation increased, reaching a maximal value at 0.1 M and remaining constant up to a concentration of 0.05 M $MgCl_2$. Magnesium acetate and $MgSO_4$ also replaced NaCl, and the maximal 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 the rate of $NADH_2$ oxidation increased at higher concentrations of $MgSO_4$.

Enzymes obtained from extreme halophiles not only function at high solute concentrations, but in many instances require a relatively high cation concentration for maximal activity (7). Although 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.

[1]This paper was presented in part at the 67th Annual Meeting of the American Society for Microbiology, New York, N.Y., May 1967.

During studies on a salt-dependent reduced nicotinamide adenine dinucleotide ($NADH_2$) oxidase prepared from an extreme halophilic bacterium, certain anomalies were observed which suggested that the cation requirement for $NADH_2$ 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 $NADH_2$ by crude extracts from the extreme halophile, AR-1.

MATERIALS AND METHODS

Organisms. The bacterium 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 2.6 M NaCl, as well as on the basis of its conversion from rods to spheres, and eventual lysis, when the cells were placed in medium containing less than 2.6 M NaCl. *Micrococcus halodenitrificans* (ATCC 13511) was obtained from the American Type Culture Collection and *Escherichia coli* K-12 was obtained from 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 Co., Norwich, N.Y.), 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 was decreased to 60 g. *E. coli* was grown in Nutrient Broth. The *p*H 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 for 48 hr. The cells, which were in the maximal stationary phase, were harvested and washed once with 0.1 M Tris chloride buffer (*p*H 7.4) containing 3.4 M sodium chloride, 2.5×10^{-3} 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 (*p*H 7.4) containing 1.0 M NaCl, 0.13 M KCl, and 10^{-3} M $MgSO_4 \cdot 7H_2O$. *E. coli* was washed once in 0.1 M Tris chloride buffer (*p*H 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, *p*H 7.4 (ST buffer), at a concentration of 200 mg of cells (wet weight) per ml of buffer, and the cell suspension was passed through a French Pressure Cell operated at 1,700 atm. The disrupted cells were centrifuged at 10,000 $\times$ *g* for 30 min at 4 C, and the supernatant fluid was used as the source of $NADH_2$ oxidase activity. Such extracts could be stored at −70 C, for at least 3 months, with little loss of activity. The extracts from *M. halodenitrificans* and *E. coli* were prepared in a similar fashion, except that, prior to disruption, *M. halodenitrificans* was suspended in 0.1 M Tris chloride buffer-1.0 M NaCl (*p*H 7.4), and *E. coli* was suspended in 0.1 M Tris chloride buffer (*p*H 7.4).

Enzyme assay. $NADH_2$ 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), *p*H 7.4 (100 μmoles); $NADH_2$ (0.1 μmole); and enzyme. The enzyme was preincubated with the buffer for 2 min at 30 C, and $NADH_2$ 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 $NADH_2$ 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), with crystalline bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) 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 to 24 C) by use of a Beckman E2 glass electrode. The electrode was assumed to be equally insensitive to all monovalent and divalent cations.

RESULTS

Effect of sodium chloride. Figure 1 shows that, in extracts from AR-1, $NADH_2$ oxidase activity 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 maximal rate. As the concentration of NaCl was increased, the rate of $NADH_2$ oxidation increased to a maximum at approximately 3.5 M sodium chloride and remained essentially constant at the higher concentrations tested. For comparison, the effects of NaCl on the $NADH_2$ oxidases from *M. halodenitrificans* and *E. coli* are also included. Extracts from both organisms, when assayed at concentrations of NaCl in which extracts from AR-1 were maximally active, oxidized $NADH_2$ at markedly reduced rates. Although the oxidation of $NADH_2$ by extracts from *E. coli* was inhibited at all concentrations of NaCl tested, extracts from *M. halodenitrificans* were maximally active at concentrations of 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 maximal activity at this salt concentration.

Specificity of the cation requirement. As shown in Fig. 2, various alkali metal chlorides satisfied the cation requirement for $NADH_2$ oxidation. At

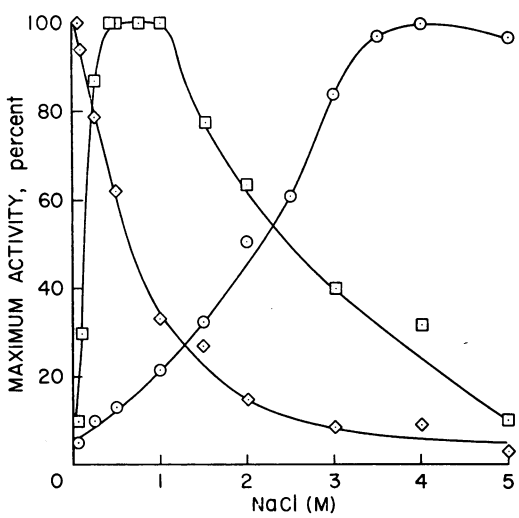

FIG. 1. *Effect of sodium chloride on $NADH_2$ oxidase activity. $NADH_2$ oxidase from the extreme halophile AR-1 (⊙), the moderate halophile Micrococcus halodenitrificans (⊡), and Escherichia coli (◈). The maximal activity observed in each case (expressed as μmoles of $NADH_2$ oxidized $\times$ min^{-1} $\times$ mg of protein^{-1}) was: AR-1 (0.041), M. halodenitrificans (0.085), and E. coli (0.17).*

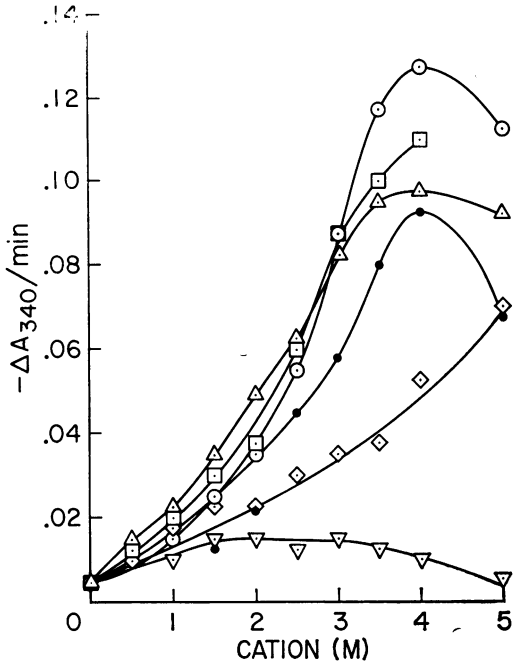

FIG. 2. *Cation specificity of the $NADH_2$ oxidase from AR-1. CsCl (●), RbCl (⊙), LiCl (▽), NH_4Cl (◇), KCl (⊡), and NaCl (△). Reaction mixtures contained 0.76 mg of protein.*

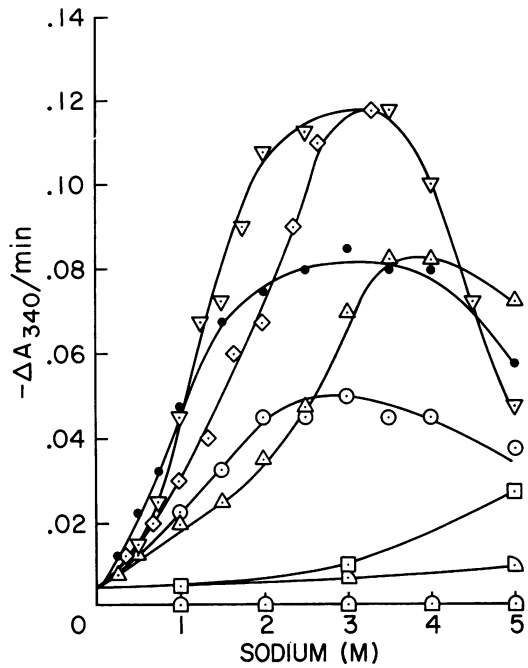

FIG. 3. *Anion specificity of the $NADH_2$ oxidase from AR-1. The following anions were added as sodium salts: acetate (●), citrate (▽), formate (⊙), sulfate (◇), chloride (△), nitrate (⊡), bromide (◺), and iodide (⊡). Reaction mixtures contained 0.76 mg of protein.*

low concentrations, differences of questionable significance were observed. The maximal activities observed in the presence of NaCl, KCl, and CsCl were very nearly identical, whereas maximal 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 sigmoidlike 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, its dose response curve was neither sigmoid in shape nor did it attain a saturating value. Furthermore, $NADH_2$ oxidation in the presence of NH_4Cl was significantly lower than in the presence of NaCl. LiCl was relatively ineffective in replacing sodium chloride.

Effect of anions on $NADH_2$ oxidase activity. As shown in Fig. 3, the oxidation of $NADH_2$ 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 was barely stimulatory, and only at the higher concentrations tested.

Formate partially replaced chloride. The maximal 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, maximal oxidation occurred at a much lower sodium ion concentration than when sodium was added as the chloride salt. Although the maximal rate of oxidation in the presence of sodium acetate was identical to that observed with NaCl, the maximal rate of oxidation in the presence of sodium citrate was some 43% greater. Furthermore, the rate of $NADH_2$ oxidation was greater, at all but the highest concentrations tested, in the presence of acetate and citrate than in the presence of chloride.

Sulfate, when added as Na_2SO_4, also satisfied the anion requirement. At the highest sulfate concentration tested (1.67 M), the rate of $NADH_2$ 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 sup-

TABLE 1. *Effect of sulfate on the oxidation of $NADH_2$ by various cations*[a]

Cation	$-\Delta A_{340} \times min^{-1}$		
	1.33 M SO_4^{-2}	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

[a] $NADH_2$ oxidase activity was measured, as described in the text, at an extract concentration of 0.76 mg of protein/ml.

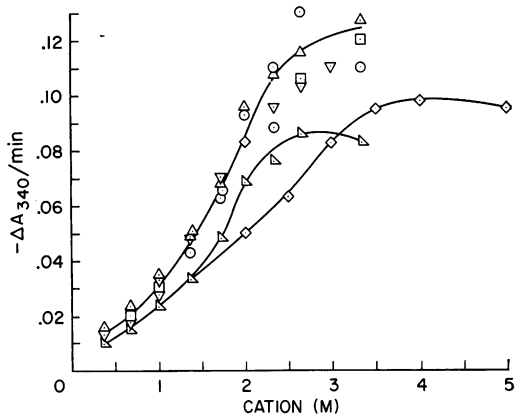

FIG. 4. *Effect of various sulfate salts on the $NADH_2$ oxidase activity from AR-1. Rb_2SO_4 (▽), Cs_2SO_4 (⊙), $(NH_4)_2SO_4$ (⊡), Li_2SO_4 (◺), Na_2SO_4 (△), and NaCl (◇). Reaction mixtures contained 0.76 mg of protein.*

plied as the sulfate rather than as the chloride salt. The extent of the increase ranged from 27% for Rb_2SO_4 to 650% for Li_2SO_4. The rate of $NADH_2$ oxidation at 1.33 M sulfate was greater than that observed at a chloride concentration of 4 M. At a concentration of 4 M, all the monovalent cations, with the exception of ammonium and possibly potassium, when added as chlorides, promoted $NADH_2$ oxidation at a maximal rate (Fig. 2). The ability of sulfate to enhance $NADH_2$ oxidation is more clearly shown in Fig. 4. Sulfate can alter the existing efficacy of a cation (cf. Fig. 2 and 4) and can also determine the efficacy of a cation in satisfying the solute requirements for $NADH_2$ oxidation. These effects of sulfate are clearly demonstrated in the case of lithium. When lithium was supplied as LiCl, it was relatively ineffective (Fig. 2), whereas when it was supplied as Li_2SO_4 it proved satisfactory. As indicated in Fig. 4, maximal oxidation of $NADH_2$ occurred at a lithium ion concentration of 2.5 M, considerably lower than the concentration at which maximal oxidation occurred in the presence of NaCl. Furthermore, the rate of $NADH_2$ oxidation at 1.25 M Li_2SO_4 was 87% of the rate observed at 4 M NaCl.

Effect of magnesium. As shown in Fig. 5, magnesium replaced sodium under certain conditions. 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 maximal rate of oxidation occurring at 0.1 M $MgCl_2$ and remaining unchanged up to a concentration of approximately 0.05 M $MgCl_2$. At still lower concentrations, $NADH_2$ 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 maximal 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. Increasing the concentration of magnesium acetate did not change the

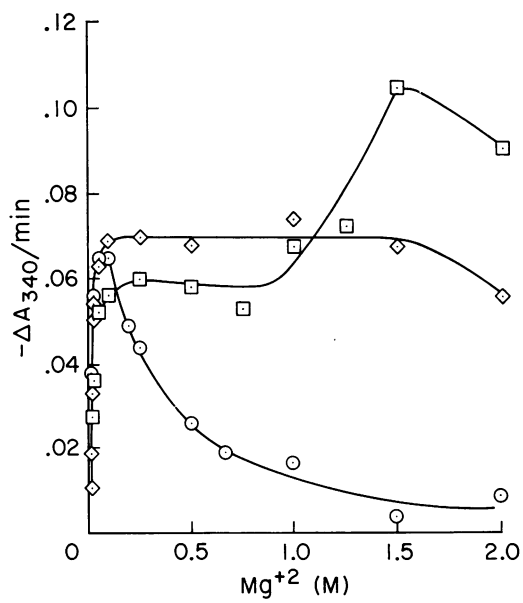

FIG. 5. *Effect of magnesium salts on the $NADH_2$ oxidase activity from AR-1. $MgCl_2$ (⊙), magnesium acetate (◇), and $MgSO_4$ (⊡). Reaction mixtures contained 0.76 mg of protein.*

rate of oxidation until the concentration of magnesium acetate reached 2 M, at which point a slight inhibition was observed. Concentrations of $MgSO_4$ greater than 0.05 M initially had little effect upon the rate of $NADH_2$ oxidase activity. However, at approximately 1 M, the rate increased, resulting in a maximal rate of oxidation at 1.5 M $MgSO_4$. At still higher concentrations, inhibition was observed.

Extracts prepared in ST buffer, and subsequently dialyzed against 0.07 M $MgCl_2$–0.1 M Tris chloride buffer, oxidized $NADH_2$ 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 $NADH_2$. However, the $NADH_2$ oxidase activity was lower when assayed in NaCl. Although 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 $NADH_2$ oxidizing activity for considerable periods of time in the frozen state, as well as at 4 C.

TABLE 2. *$NADH_2$ oxidase activity of extracts suspended in $MgCl_2$-containing buffers*[a]

Expt	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

[a] In experiment no. 1, extracts were prepared as described in the Materials and Methods section, and then dialyzed for 18 hr against three changes of 0.07 M $MgCl_2$–0.1 M Tris chloride, *p*H 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, *p*H 7.4. The extracts were subsequently assayed, as indicated, in 4 M NaCl–0.1 M Tris chloride (*p*H 7.4) or in 0.07 M $MgCl_2$–0.1 M Tris chloride (*p*H 7.4). The results are expressed as specific activity (μmoles of $NADH_2$ oxidized $\times$ min^{-1} $\times$ mg of $protein^{-1}$).

DISCUSSION

The observations reported in this paper are not consistent with the hypothesis that the ionic requirements for $NADH_2$ 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 of nearly identical radius, such as magnesium (9), are able to satisfy the cation requirement. It is also clear that the suitability of a cation is in some way related, both in the amount of activity observed at a given cation concentration and in the concentration of cation required to attain maximal activity, to the nature of the accompanying anion. 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 our 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. Second, all the magnesium salts tested satisfied the cation requirement at the rather low concentration of 0.05 M, a decidedly nonhalophilic concentration relative to the concentration of monovalent cations required for maximal activity. These results were not unique to strain AR-1. We have obtained analogous data (*in preparation*) by use of $NADH_2$ oxidases isolated from *Halobacterium cutirubrum* and *Halobacterium halobium*, both strains obtained from N. E. Gibbons.

The ability of magnesium to substitute for sodium, at concentrations approximately $\frac{1}{80}$ of the cation concentration and about $\frac{1}{25}$ of 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 $NADH_2$ oxidase system presented in this paper makes any facile interpretation of the data difficult. Operationally, one may 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) replaces magnesium. Or, it may reflect the existence of two pathways of $NADH_2$ oxidation, one active at high sodium concentrations, while the other is operative at low sodium and magnesium concentrations. At the present time, studies are being carried out in an attempt to distinguish between these possibilities.

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