# Effect of Salts and Organic Solvents on the Activity of *Halobacterium cutirubrum* Catalase

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Catalase in extracts of the extreme halophile *Halobacterium cutirubrum* exhibits up to threefold stimulation by 0.5 to 1.5 M monovalent salts and by 0.1 M divalent salts. Above these concentrations, inhibition of enzyme activity is observed. The inhibitory effect, and to some extent the stimulation, is salt-specific; the effectiveness of a salt in inhibiting enzyme activity depends on both cation and anion. Thus, the order of effectiveness is $MgCl_2 > LiCl > NaCl > KCl > NH_4Cl$, and $LiCl > LiNO_3 > Li_2SO_4$. The magnitude of enzyme inhibition for the salts tested is positively correlated with their molar vapor pressure depression in aqueous solution. Stimulation of enzyme activity was observed when one salt was added at its optimal concentration in the presence of inhibiting concentrations of another salt, indicating that the effect on the enzyme is not due to changing water activity but probably to enzyme-salt interaction. Aqueous solutions of ethylene glycol, glycerol, and dimethyl sulfoxide containing no ions influence enzyme activity in the same manner as do salts.

The salt requirement of enzymes isolated from extremely halophilic bacteria has been amply demonstrated. All enzymes isolated from such microorganisms are reported to show optimal activity above 1 M NaCl, a finding consistent with the observed high intracellular salt concentration (9, 10). A large variety of mono- and divalent salts has been shown to enhance the activity of halophilic enzymes (1–3, 6, 7). In some cases, the relative effectiveness of these salts appears to depend on both cation and anion (1, 6). We examined the salt dependence of partially purified catalase from *Halobacterium cutirubrum* and have attempted to explain the differences in the salt effects. The effect of some nonionizing, water-soluble organic solvents on the enzyme activity was also determined.

## MATERIALS AND METHODS

**Preparation of partially purified H. cutirubrum catalase.** Methods for growing *H. cutirubrum*, for harvesting and breaking cells, and for determining protein concentration have been published elsewhere (8). Enzyme purification procedures were carried out at 0 C. The crude extract was diluted fivefold with 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 7.2, containing 0.1 M $MgCl_2$. The resulting buffer, which also contained 0.68 M NaCl, carried over from the crude extract, proved suitable for preserving enzyme activity. The suspension was

centrifuged for 4 hr at 98,600 × g and the pellet was discarded. The supernatant liquid was centrifuged again at 98,600 × g for 64 hr, and the pellet obtained was dissolved in 0.05 M Tris-hydrochloride, pH 7.2, containing 0.1 M $MgCl_2$ and 0.2 M NaCl. Solid ammonium sulfate (enzyme grade, Mann Research Laboratories) was added to 70% saturation, the suspension was stirred for 1 hr, and the precipitated protein was removed by centrifugation and discarded. Adding more $(NH_4)_2SO_4$ to the supernatant liquid to achieve 100% saturation followed by stirring for 1 hr precipitated the enzyme. The precipitate was dissolved in the same buffer used before and was stored at −70 C.

Twice-crystallized beef liver catalase (Sigma Chemical Co.) was used for some of the experiments.

**Catalase assay.** The enzyme was assayed by measuring liberation of oxygen from hydrogen peroxide at 23 C in a Warburg respirometer. The assay mixture consisted of 2.75 ml of buffer and 50 μliters of enzyme preparation to which 0.2 ml of 0.3 M $H_2O_2$ was added from a side-arm within 1 min of the addition of the enzyme. Oxygen liberation was followed for 5 min at 30-sec intervals. All determinations were carried out in duplicate and averages were calculated.

## RESULTS

**Purification and some properties of H. cutirubrum catalase.** In the purification procedure for *H. citurubrum* catalase (Table 1), the first step, centrifugation for 4 hr, served to remove the deep red membrane fraction (9) which sedimented

TABLE 1. *Partial purification of H. cutirubrum catalase*

| Fraction | Vol | Protein | Specific activ- ity[a] | Total activity[b] |
|---|---|---|---|---|
| | ml | mg/ml | | |
| Crude............... | 40 | 56.4 | 11.6 | 26,200 |
| 4-hr Supernatant liquid............. | 215 | 7.8 | 13.8 | 23,160 |
| 64-hr Pellet......... | 150 | 5.8 | 25.2 | 21,900 |
| 70% (NH₄)₂SO₄ supernatant liquid............... | 190 | 2.3 | 45.2 | 19,760 |
| 100% (NH₄)₂SO₄ precipitate........ | 50 | 6.0 | 56.8 | 17,040 |

[a] Expressed in microliters of $O_2$ per minute per milligram of protein.

[b] Starting material: 8 liters of culture.

rapidly in the relatively low-salt $MgCl_2$ buffer. The enzyme was recovered under these conditions from a very slowly sedimenting fraction. The ammonium sulfate fractionation steps yielded a slightly tan-colored preparation used in these studies.

*H. cutirubrum* and beef liver catalase were separately chromatographed on a Biogel P-300 column (2.5 by 35 cm) in 0.05 M Tris-hydrochloride, pH 7.2, containing 0.1 M $MgCl_2$ and 0.2 M NaCl. Both enzymes emerged well after the void volume was collected; the retention constants were 1.70 and 1.60, respectively.

Both enzymes were inhibited by high concentrations of hydrogen peroxide (11). Optimal $H_2O_2$ concentration for *H. cutirbrum* catalase was 20 mM. The halophilic enzyme was 87% inhibited by potassium cyanide and 86% inhibited by sodium azide at 1 mM concentration.

**Salt-dependence of catalase activity.** *H. cutirubrum* catalase activity was determined in the presence of a series of monovalent cations as chloride, nitrate, and sulfate salts. The salt solutions were buffered with 0.05 M Tris-hydrochloride at pH 7.2. The results obtained are plotted as relative enzyme activity versus salt concentration. The reference value was enzyme activity determined in the absence of added salt.

Catalase activities in the presence of KCl, NaCl, NH₄Cl, and LiCl are given in Fig. 1a, in which the salt-dependence curves are similar in shape for the salts tested, exhibiting 30 to 50% of maximal activity without added salt, reaching optimal activity at an intermediate salt concentration, and declining in activity at higher salt concentrations (9). Figure 1a also demonstrates that the effect of salts varied with the cation, both for activation and for inhibition. The most striking

example of this phenomenon was the case of LiCl in which the activation and inhibition parts of the curve had much greater slopes than for the other salts, and the point of optimal enzyme activity was shifted considerably. We found that the parameters that best describe these salt-dependence curves were the slopes of activation and inhibition on either side of the maximal activity. The relative magnitude of the slopes of inhibition at high salt concentrations followed the order: LiCl > NaCl > KCl > NH₄Cl. The same order may apply also to the activation slopes; however, the values of these were less discernible.

The data obtained with Na₂SO₄, (NH₄)₂SO₄, and Li₂SO₄ (Fig. 1b) and with KNO₃, NaNO₃, and LiNO₃ (Fig. 2) gave salt-dependence curves of the same general shape in the presence of anions other than chloride. For sulfate salts, the relative values of the slopes of high-salt inhibition were in the following order: Li₂SO₄ > Na₂SO₄ > (NH₄)₂-SO₄; for nitrate salts, the order was LiNO₃ > NaNO₃ > KNO₃. The order of inhibition for cations thus appears to be unaffected by changing the anion. On the other hand, the absolute magnitude of the slope of inhibition by the salts is affected by both cation and anion. Examples of the influence of anion are seen in the differences in the slopes of inhibition caused by KCl and KNO₃ as well as by LiCl and Li₂SO₄.

*H. cutirubrum* catalase was assayed in the presence of $MgCl_2$, $CaCl_2$, and $MgSO_4$ to test the effect of these divalent cation salts on enzyme activity. For $MgCl_2$ and $CaCl_2$, both activation
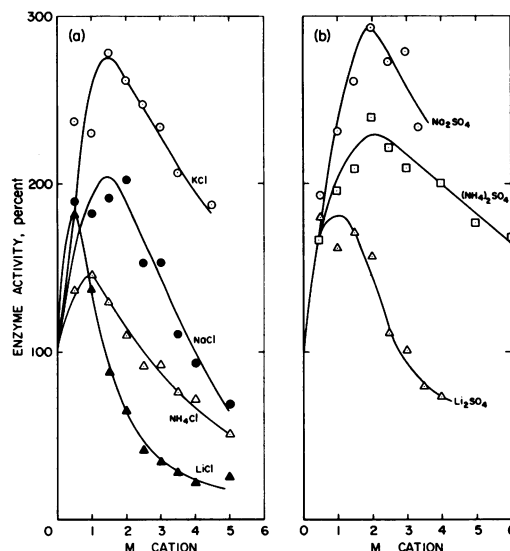


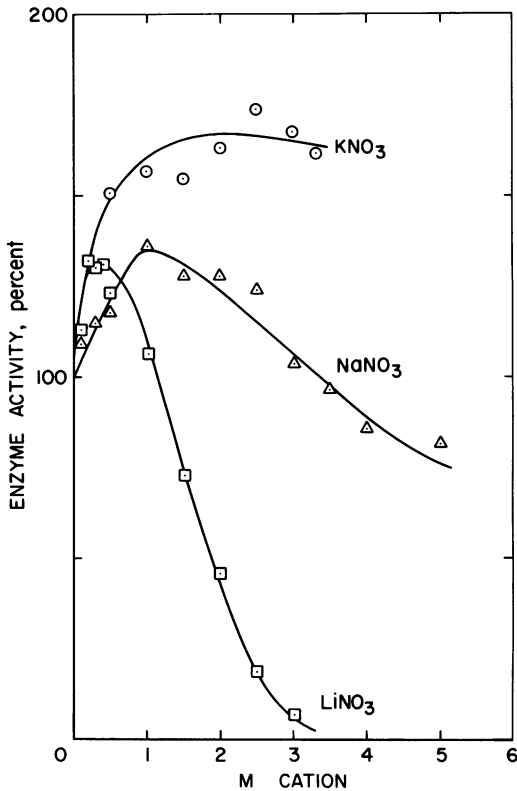FIG. 1. *H. cutirubrum catalase activity in the presence of chloride and sulfate salts.*

FIG. 2. *H. cutirubrum catalase activity in the presence of nitrate salts.*

and inhibition slopes were much greater than for any of the monovalent salts (Fig. 3), thereby lowering the optimal salt concentration to approximately 0.1 M. In the case of MgSO$_4$, however, the activation slope was high but inhibition at higher concentrations was relatively slight.

For comparison, the effect of various salts on beef liver catalase was also tested. The enzyme activity verus salt concentration curves for KCl, LiCl, and MgCl$_2$ (Fig. 4) show that, in contrast to the observations with *H. cutirubrum* catalase, these salts exhibited only inhibition of the beef liver catalase, and salt specificity is not apparent.

**Effect of multiple-salt buffers on H. cutirubrum catalase activity.** Stimulation of enzyme activity by intermediate concentrations of salts was tested in the presence of inhibitory amounts of another salt. Two sets of experiments were carried out. In the first experiment, 4 M KCl served as inhibiting salt, and LiCl and MgCl$_2$ were added separately at their optimal concentrations. The addition of the latter salts caused stimulation of enzyme activity (Table 2). In the second experiment (Table 2), 2 M MgCl$_2$ was used as inhibiting

salt and KCl or MgSO$_4$ was added at 1.5 and 1.0 M concentration, respectively. The stimulating effect of KCl and MgSO$_4$ in the presence of inhibitory concentrations of MgCl$_2$ is evident.

**Effect of organic solvents on catalase activity.** *H. cutirubrum* and beef liver catalase were assayed in aqueous solutions of ethylene glycol, glycerol, and dimethyl sulfoxide (DMSO) containing 0.05 M Tris-hydrochloride at pH 7.2. For the halophilic enzyme (Fig. 5), the activity curves with these substances are similar in shape to the salt-dependence plots in Fig. 1 and 2. Thus, these organic solvents gave both activation at moderate concentrations and inhibition at high concentrations. These effects, however, were much lower in magnitude and occurred at higher concentrations than in the case of the salts. Good correspondence between the slopes of activation and of inhibition by each substance was observed; that is, if a given solvent gave a high rate of activation, it also gave a comparable rate of inhibition. The order for the magnitude of either effect was DMSO > glycerol > ethylene glycol. The results obtained with beef liver catalase (Fig. 5) indicate
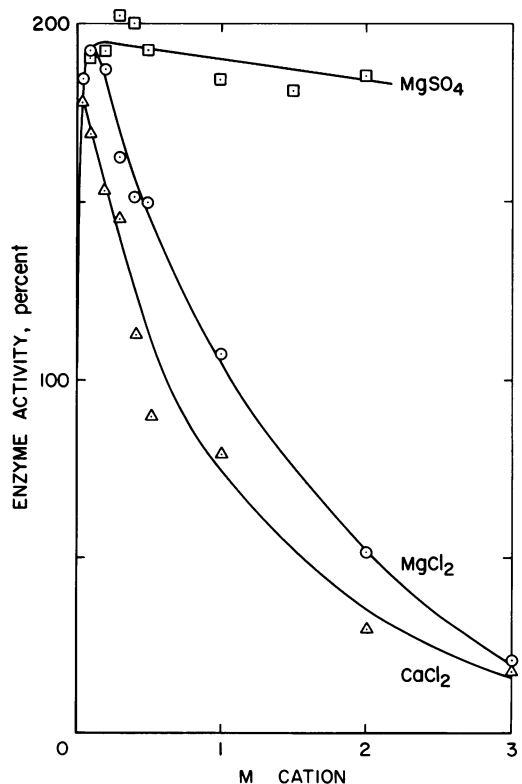


FIG. 3. *H. cutirubrum catalase activity in the presence of divalent salts.*

that this enzyme was not activated, but was inhibited by the organic solvents tested. The inhibitory effects followed the order shown for *H. cutirubrum* catalase.
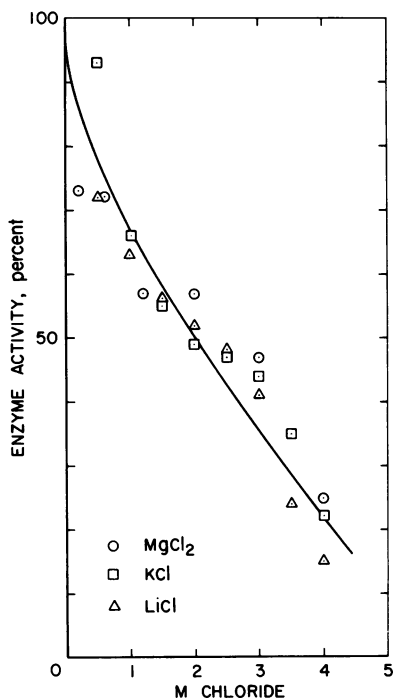


FIG. 4. *Beef liver catalase activity in the presence of salts.*

TABLE 2. *Effect of combined salts on H. cutirubrum catalase activity*

| Expt | Salt | Concn | Enzyme activity[a] |
|------|------|-------|----------------|
|  |  | *M* |  |
| 1 | KCl | 4.0 | 58.7 ± 3.4 |
|  | KCl | 4.0 | 75.9 ± 3.7 |
|  | LiCl | 0.5 |  |
|  | KCl | 4.0 | 85.7 ± 7.7 |
|  | MgCl₂ | 0.1 |  |
| 2 | MgCl₂ | 2.0 | 26.3 ± 3.2 |
|  | MgCl₂ | 2.0 | 53.4 ± 4.9 |
|  | KCl | 1.5 |  |
|  | MgCl₂ | 2.0 | 50.3 ± 4.6 |
|  | MgSO₄ | 1.0 |  |

[a] Enzyme activity in microliters of $O_2$ per minute per milligram of protein; means and standard deviation of the means calculated from five determinations.
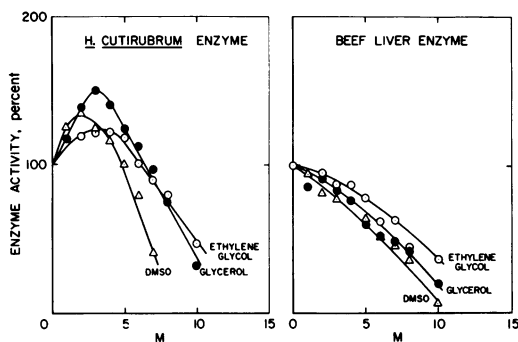


FIG. 5. *H. cutirubrum and beef liver catalase activity in the presence of aqueous solutions of organic solvents. Symbols: ethylene glycol (O), glycerol (●), dimethyl sulfoxide (△).*

## DISCUSSION

Catalase preparations isolated from different sources show striking similarities in molecular weight and kinetic properties (11). Our studies of *H. cutirubrum* catalase show that this halophilic enzyme has some properties in common with beef liver catalase. Thus, gel fractionation experiments demonstrated a close similarity in molecular weights, found to be about 240,000 for the beef liver enzyme (11); the halophilic enzyme was found to be inhibited by cyanide and azide, inhibitors that are known to interact with other catalases (4, 11). The two enzymes are different, however, in their behavior toward various concentrations of salts. Beef liver catalase is inhibited in a nonspecific manner by mono- and divalent salts. *H. cutirubrum* catalase, on the other hand, exhibits up to threefold activation with 0.5 to 1.5 M monovalent salts, with 0.1 M divalent salts it is inhibited by salt concentrations higher than these values, and it demonstrates salt-specific activation and inhibition.

In seeking a unified explanation for the differences observed in the effect of various salts on the enzyme, we found that they became predictable on the basis of a property of salts that is dependent on the physical dimensions and the charges of the ions in aqueous solution. This property is the molar vapor pressure depression as measured in aqueous solution at the boiling point. The values of vapor pressure depression for all the salts tested were calculated from tables (*Handbook of Chemistry and Physics*, 48th ed., 1968). For monovalent salts, the values are independent of concentration; for divalent salts, the values taken were from the 0.5- to 1.0-M concentration interval. When the slopes of inhibition of enzyme activity by salts are plotted against their molar vapor pressure depression, a positive correlation between the two quantities is evident (Fig. 6).
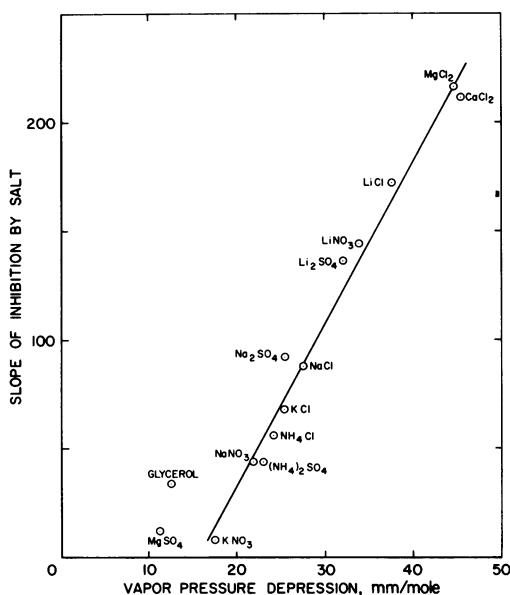
FIG. 6. *Relationship between catalase inhibition and molar vapor pressure depression of various salts and glycerol. The slopes of inhibition were calculated from Fig. 1, 2, 3, and 5 on a valence-equivalence basis and are given in arbitrary units.*

Thus, differences in enzyme inhibition due to the cation, as in the case of KCl, LiCl, and $MgCl_2$, and differences due to the anion, as in the case of $MgCl_2$ and $MgSO_4$, are correlated with differences in the vapor pressure depression caused by these salts. For some of the salts, the activation slopes are also positively correlated with vapor pressure depression, as in the case of KCl, LiCl, and $MgCl_2$ (Fig. 1a and 3). The relationship does not hold, however, for activation by $MgCl_2$ and $MgSO_4$, since these salts are equally effective in activating the enzyme (Fig. 2), but differ greatly in their effect on the vapor pressure of water. The anion thus appears to be less influential in determining the activating effect on the enzyme.

Several explanations are possible for the finding that the salt behavior of enzyme activity, as the lowering of vapor pressure, is determined by both cations and anions.

(i) The influence of salt on the enzyme may be due to binding of water by the salts, which changes the structure of water around the enzyme and thus affects its configuration and activity. Such lowering of water activity by highly concentrated salt solutions would be greater at 23 C than is implied by the relatively small vapor pressure depression values (Fig. 6) of salt solutions at the boiling point. If the effect of salts on the enzyme were exerted through a lowering of water activity, add-

ing a second salt to a concentrated salt solution in which the enzyme is already inhibited should result in even greater inhibition, since water activity is further reduced. The data in Table 2 indicate that, under these conditions, stimulation instead of further inhibition was observed. Therefore, the effect of salts on the enzyme is not due primarily to lowering water activity but rather to the interaction of the salt with the enzyme itself.

(ii) The species acting on the enzyme may be the ion pair rather than free ions. Although ion pairs have not been detected in dilute solutions of monovalent salts of halides (5), these species are expected to be present in concentrated solutions. It has been proposed on the basis of electrostatic theory (5) that, when the distance between the centers of oppositely charged ions is less than a given value, the ions are no longer independent but should be regarded as ion pairs. This critical distance for monovalent ions is 3 to 4 nm, and from 7 to 8 nm for mixed ions such as $MgCl_2$. From purely geometrical considerations, the average distance between the ions of a monovalent salt at 4 M concentration is 7 to 8 nm. Thus, assuming a distribution around this mean value, a non-negligible fraction of the ions would be close enough to be in the associated form. The activation and inactivation of the enzyme may therefore be related to the affinity of the enzyme for specific ion pairs rather than individual cations or anions whose effects would be independent of one another.

(iii) The enzyme may simultaneously bind cations and anions which then form an association whose effectiveness depends on both ionic species. Such interaction is possible if the binding sites for the cations and anions are close and allow the bound ions to resemble ion pairs.

Although we cannot decide at present between possibilities ii and iii, the correlation of the inhibition of the enzyme by the salts and the effectiveness of the salts in binding water molecules, as shown by the lowering of vapor pressure, indicates that the binding of the salts by the protein and the binding of water by the salts may be similar and appear to be influenced by the same properties of the salts. The nature of ion binding and its possible effect on the tertiary structure of halophilic enzymes has been discussed by others (1, 7, 9, 10).

The behavior of the enzyme in the presence of ethylene glycol, glycerol, and dimethyl sulfoxide indicates that activation at lower concentrations and inhibition at higher concentrations occur with nonionic polar molecules as well as with salts. The order of effectiveness of these substances in enzyme activation and inhibition correlates positively with the dipole moments of the molecules.

Although the magnitude of these effects is small, as expected from the low molar vapor pressure depression of glycerol (*International Critical Tables*, vol 13; Fig. 6), it appears that, in contrast to findings with halophilic lactic dehydrogenase (1), *H. cutirubrum* catalase is affected by nonionic molecules in a manner which suggests that the influence of organic solvents and salts may have a common basis.

Hochstein and Dalton (6) reported on the effect of various salts on the activity of halophilic reduced nicotinamide adenine dinucleotide (NADH) oxidase and showed the influence of both cation and anion on enzyme activity. The order of effectiveness in activating this multienzyme system was NaCl > LiCl and the order of inhibition at high concentrations was $MgCl_2$ > $MgSO_4$. Moreover, $Li_2SO_4$ activated this enzyme system well, whereas LiCl was ineffective. Thus, in this case, salts that have strong binding tendencies and high molar vapor pressure depression were unable to activate the enzyme and in some cases appeared inhibitory. On the other hand, some salts, most notably $NaNO_3$ and $NH_4Cl$, did not obey this rule. Thus, NADH oxidase appears to be a more complicated system than catalase in its salt-dependent behavior. Baxter (1) determined the activity of a halophilic lactic dehydrogenase in the presence of a variety of salts at a single cation concentration. The order of effectiveness in activating the enzyme correlated with the molar vapor pressure depression for the following series: NaCl > $Na_2SO_4$ > $NaNO_3$, and KCl > $KNO_3$. However, the effect of some other salts, particularly of KCl, NaCl, LiCl, and $NH_4Cl$, cannot be made to fit the model. Thus, even though the behavior of *H. cutirubrum* catalase

activity is predictable from the molar vapor pressure depression of the salts used to activate the enzyme, the general applicability of this approach to explain the salt effects in halophilic bacteria cannot be assessed because of the limited data available at present.

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