

Lysis of *Streptococcus mutans* by Hen Egg White Lysozyme and Inorganic Sodium Salts

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Streptococcus mutans BHT was grown in a synthetic medium containing radioactive thymidine to monitor deoxyribonucleic acid release. Kinetic experiments demonstrated that although lysozyme alone could not liberate deoxyribonucleic acid, cellular deoxyribonucleic acid was liberated from lysozyme-treated cells by addition of low concentrations of inorganic sodium salts. When the salts were tested for their ability to dislodge cell-bound tritiated lysozyme, the extent of the initial release of enzyme by individual anions correlated with the anion potency for deoxyribonucleic acid liberation ($\text{SCN}^- > \text{ClO}_4^- > \text{I}^- > \text{Br}^- > \text{NO}_3^- > \text{Cl}^- > \text{F}^-$), although the total amount of lysozyme dislodged did not correspond directly with cell lysis. Differences in the effectivenesses of anions (SCN^- , HCO_3^- , Cl^- , and F^-) in potentiating cell lysis could be enhanced or minimized by varying the lysozyme, anion, and bacterial cell concentrations. As the anion concentration was increased for each enzyme concentration and cell concentration, the lysis increased, in some cases markedly, until maximum levels of released deoxyribonucleic acid were attained. The maximum levels of lysis for SCN^- and HCO_3^- were similar and were greater than those for Cl^- and F^- . In addition, the maximum levels were observed to increase for each of the anions as the concentration of lysozyme increased.

Several investigators have reported that certain gram-positive microorganisms which are normally resistant to the lytic action of hen egg white lysozyme (EC 3.2.1.17) (HEWL) undergo gross cellular lysis in the presence of HEWL and either an inorganic salt, usually NaCl, or sodium dodecyl sulfate (4, 8, 13, 14, 16, 22, 25, 26). In two of these reports, the sodium salts of fluoride, chloride, bromide, and iodide were compared for their effects on bacteriolysis (8, 22). Each of the studies demonstrated that $\text{I}^- > \text{Br}^- > \text{Cl}^- > \text{F}^-$ in effecting cell lysis. The investigators concluded that the radius of the anionic moiety appeared to be of cardinal importance in the lytic reaction. These pioneering studies (8, 22) emphasized the importance of electrostatic effects, and their conclusions were based on the presumption that the order of salt effectiveness reflected the ability of HEWL to gain access to its substrate, the cell wall peptidoglycan, and lyse the cells. Preliminary investigations with additional anions, other than halides, have suggested a consideration of cell membrane interactions in that the sequence of anion potencies in the lysozyme lytic system follows a lyotropic series (25). The lysis effects noted with halides

by previous investigators may therefore be due to both electrostatic and hydrophobic interactions.

Detailed studies of the bacteriolyses of a variety of microorganisms exhibiting different degrees of susceptibility to lysozyme and inorganic salts are required to ultimately understand the mechanism of cell lysis. The main purpose of this study was to determine the effects of anion, HEWL, and cell concentrations on the lysis of *Streptococcus mutans* BHT in order to begin to acquire information on the lytic sensitivity of oral bacteria. Since some of the anions, such as chloride, fluoride, thiocyanate, and bicarbonate, are readily detectable in saliva where lysozyme is also present (15, 20), a study of the effects of anion concentration on the lysis of oral bacteria would be physiologically relevant in terms of possible in vivo antibacterial effects. A second purpose of our studies was to provide preliminary information concerning the degree of involvement of lysozyme in the lytic process. The enzyme may be required only to bind to the cell surface and thereby set in motion the initial preparatory stages of salt lysis. Alternatively, lysozyme may play an integral part in the ensu-

ing lysis, as its dislodgment from the cell by the salt may be a mandatory requirement for the lytic event to occur. To study the role of lysozyme in this lytic phenomenon, the kinetics of release of [^3H]HEWL and of [^{14}C]thymidine as DNA were examined in radiolabeled *S. mutans* BHT which had been treated sequentially with [^3H]HEWL and inorganic anions. Data are presented which demonstrate that the initial kinetics of DNA release and the dislodgment of HEWL followed the same anion order, although the total release of HEWL could not account for the differences in lysis observed among the anions. Alteration of the concentrations of HEWL, anions, or bacterial cells was observed to markedly influence the degree of cell lysis.

MATERIALS AND METHODS

Sodium salts. All salts were of the highest purity available. Sodium bromide, sodium iodide, sodium chloride, sodium perchlorate, sodium bicarbonate, and sodium thiocyanate were purchased from Fisher Scientific Co., Pittsburgh, Pa. Sodium nitrate was obtained from General Biochemicals, Chagrin Falls, Ohio, and sodium fluoride was obtained from Baker Laboratories, Phillipsburg, N.J.

Chemicals. HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) was obtained from Calbiochem, La Jolla, Calif. [$\text{methyl-}^{14}\text{C}$]thymidine and ACS were purchased from Amersham Corp., Arlington Heights, Ill. [$\text{methyl-}^3\text{H}$]thymidine was a product of New England Nuclear Corp., Boston, Mass., and Filter-Solv was acquired from Beckman Corp., Somerset, N.J.

Tritiated HEWL. HEWL (3 \times crystallized; Sigma Chemical Co., St. Louis, Mo.) was subjected to tritium gas, and the labeling reaction was initiated by microwave or electric discharge activation (Vega Biochemicals, Tucson, Ariz.). After lyophilization to remove labile tritium, HEWL was purified to a specific activity of 11 $\mu\text{Ci}/\text{mg}$ by a combination of gel filtration and ion-exchange chromatography (26). When compared at identical concentrations, [^3H]HEWL was as active in hydrolysis of *Micrococcus lysodeikticus* as similarly purified unlabeled HEWL (26). The concentrations of unlabeled HEWL and labeled HEWL were determined from the extinction of $E_{1\text{cm}}^{1\%} = 26.9$ at 280 nm for HEWL (17).

Bacterial cultures. *S. mutans* BHT (serotype b strain) (2) was obtained from Harold Jordan, Forsyth Dental Center, Boston, Mass. Cultures were grown in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) to late log phase (optical density of 0.80 at 675 nm, 1-cm light path). Freshly grown cells were then inoculated as a 1:400 dilution into FMC synthetic medium (29), and at the late log phase of growth (optical density of 0.90 at 675 nm), bacteria were harvested and washed three times in ice-cold distilled water. Cells were suspended in 0.025 M ammonium acetate, pH 6.8, or in 0.025 M HEPES buffer, pH 7.3, to an optical density at 700 nm of 0.40 (1×10^9 cells per ml, Petroff-Hausser counting chamber) or 0.14 (3.5

$\times 10^8$ cells per ml) immediately before lytic or aggregation assays.

Radioisotopes were incorporated into *S. mutans* BHT by supplementing the FMC synthetic medium (10 ml) with either 1 μCi of [$\text{methyl-}^{14}\text{C}$]thymidine (specific activity, 50 $\mu\text{Ci}/\text{ml}$, 53 mCi/mmol) per ml or 10 μCi of [$\text{methyl-}^3\text{H}$]thymidine (specific activity 1 mCi/ml, 20 Ci/mmol) per ml.

Assays. HEWL-mediated aggregation of *S. mutans* was performed as previously described (26). Cellular lysis was assayed by measurement of the liberation of DNA as macromolecular [^3H]- or [^{14}C]thymidine from *S. mutans* BHT treated sequentially with HEWL and inorganic salts. The presence of macromolecular thymidine was determined by applying supernatants containing the released thymidine label to Sephadex G-25 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) columns to separate free thymidine from thymidine incorporated into DNA. Soluble DNA released into supernatants of lysates of cell suspensions and total DNA of control and lysed pellets were also precipitated with trichloroacetic acid according to Coleman et al. (5). DNA was then quantitated by the colorimetric procedure of Burton (3).

In kinetic experiments, [^{14}C]thymidine-labeled cell suspensions (10^9 cells per ml) of *S. mutans* BHT in 0.025 M ammonium acetate, pH 6.8, were preincubated with stirring at 37°C. After 1 h, [^3H]HEWL (specific activity 11 $\mu\text{Ci}/\text{mg}$, 10.74 mg/ml) and unlabeled HEWL were added to a total final concentration of 143 $\mu\text{g}/\text{ml}$, and samples (1 ml) were withdrawn for radioactivity determination. Incubation was continued for an additional 3 h, after which 1-ml samples of the cell suspension were distributed into screw cap tubes (13 by 100 mm) containing 140 μl of sodium salts. Immediately upon addition, the tubes' contents were blended vigorously in a Vortex mixer for 30 s and then incubated in blood tube rotators with end-over-end mixing. During the subsequent 3-h incubation, the tubes' contents were blended in a Vortex mixer every hour. At designated times over the entire course of the incubation, samples were processed by first removing 0.1 ml from each tube for total counts of ^3H and ^{14}C radioactivity (mark III liquid scintillation counter; Tractor Analytic, Austin, Tex.) and then centrifuging the remaining sample at 2,100 $\times g$ for 20 min at 4°C (PR-6000 centrifuge; International Equipment Co., Needham Heights, Mass.). Resultant supernatants (0.1-ml aliquots) were similarly quantitated by double-channel counting, using a cocktail consisting of 1 ml of Filter-Solv and 10 ml of ACS, and the percentages of [^3H]HEWL bound and of [^{14}C]thymidine released were calculated (25).

In experiments to determine the effects of anion, bacterial cell, and HEWL concentrations on the lysis process, cell suspensions (1×10^9 or 3.5×10^8 cells per ml) labeled with [^3H]thymidine were preincubated for 1 h in 0.025 M HEPES buffer, pH 7.3, with stirring at 37°C. Various concentrations of unlabeled HEWL were then added, and incubation continued for an additional 3 h. Samples (2 ml) of cell suspension were then distributed into screw cap tubes (13 by 100 mm) containing 280 μl of stock salt solutions such that the final salt concentrations were 0.036, 0.054, 0.073, 0.085, 0.097, and 0.115 M. Sodium salts stock solutions of

fluoride, chloride, and thiocyanate were made up in 0.045 M Tris-hydrochloride buffer, pH 8.5, and sodium bicarbonate was added as an aqueous solution so that the final pH, upon addition of bacterial cells, was 7.9 for all reaction mixtures. After 2.5 h of incubation, 0.1 ml was removed from each sample for total tritium counts and the remainder was centrifuged at $2,100 \times g$ for 20 min at 4°C . Resultant supernatants (0.1 ml) were similarly quantitated for radioactivity, and the percentage of [^3H]thymidine released was calculated.

Electron microscopy. After sequential incubation of *S. mutans* BHT with HEWL for 3 h and 0.1 M NaSCN for 1 h, 9 ml of each cell suspension (10^9 cells per ml) was mixed with 1 ml of 50% glutaraldehyde (Polysciences, Inc., Warrington, Pa.). Fixation was carried out for 2 h at 4°C , and suspensions were then centrifuged at $27,500 \times g$ for 1 h in an HB-4 rotor (RC-5 refrigerated centrifuge; Ivan Sorvall, Inc., Norwalk, Conn.). Excess fixative was removed, and membrane pellets were sliced into approximately 2-mm² sections. After washing for 15 min (3 times, 5 min each) in 0.1 M sodium cacodylate buffer, pH 7.4, postfixation was accomplished by incubation with 1% osmium tetroxide in cacodylate buffer for 1 h at room temperature. Pellets were then washed (3 times, 5 min each) with 0.1 M maleate buffer, pH 5.15, and subjected to en bloc staining for 1 h at room temperature, using 1% uranyl acetate in 0.1 M maleate buffer, pH 6.0. After washing in pH 5.15 maleate buffer, preparations were dehydrated, using a graduated series of cold ethanol (50 to 100%) and propylene oxide treatments. Dehydrated specimens were then embedded in an Epon mixture (19).

For ultrastructural studies, ultrathin sections (60 nm in thickness) were cut on a Porter-Blum MT2 ultramicrotome equipped with a Dupont diamond knife. Sections were placed on 200-mesh grids coated with 0.3% Formvar film for low-magnification work and on 300-mesh uncoated grids for high-magnifica-

tion observations. Sections were sequentially stained for 25 min with freshly saturated uranyl acetate in 50% ethanol and for 7 min with lead citrate (28). The stained sections were then examined and photographed with a JEOL 100B electron microscope.

RESULTS

Kinetics of binding and dissociation of [^3H]HEWL and of release of [^{14}C]thymidine from *S. mutans* BHT treated sequentially with [^3H]HEWL and either NaSCN or NaCl. Cellular lysis was assayed by measurement of the liberation of [^{14}C]thymidine in DNA. Control reaction mixtures incubated for a period of 7 h retained essentially all of their previously incorporated radioactivity (Fig. 1 and 2). Addition of [^3H]HEWL alone did not seem to effect cell lysis; however, subsequent treatment with 0.097 M NaSCN resulted in >90% release of [^{14}C]thymidine from the cells (Fig. 1). The thymidine was released only to a minimal extent in the free form or as a small-molecular-weight nucleotide derivative. More than 90% of the [^{14}C]thymidine label was isolated in the void volume Sephadex G-25 columns and was precipitable with 10% trichloroacetic acid. When cell suspensions were quantitated for DNA by trichloroacetic acid precipitation followed by the diphenylamine colorimetric assay, identical results were obtained. If 0.097 M NaCl was added to HEWL reaction mixtures, efflux of DNA was barely apparent (Fig. 2). At the concentration of HEWL (143 $\mu\text{g}/\text{ml}$) used, 75 to 80% of the enzyme was observed to be maximally bound to *S. mutans* BHT. When either NaCl or NaSCN was

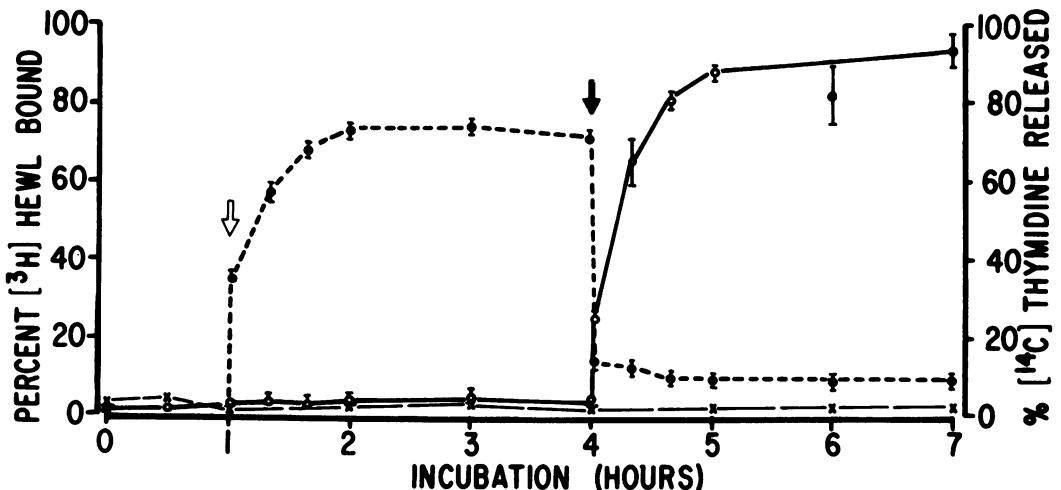


FIG. 1. [^{14}C]thymidine release from HEWL-treated *S. mutans* BHT by NaSCN. Symbols: ●, [^3H]HEWL bound; ○, [^{14}C]thymidine released in the [^3H]HEWL reaction mixture; ×, [^{14}C]thymidine released in the absence of HEWL (control reaction mixture); open arrow, HEWL addition; solid arrow, 0.097 M NaSCN addition; vertical bars, mean values of triplicate samples \pm the standard errors.

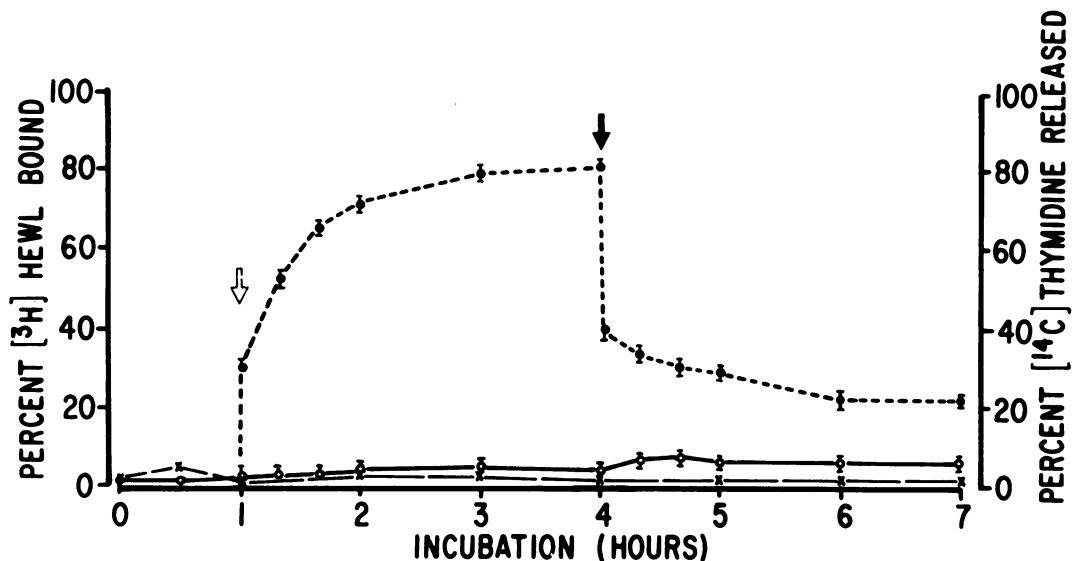


FIG. 2. $[^{14}\text{C}]$ thymidine release from HEWL-treated *S. mutans* BHT by NaCl. Symbols: ●, $[^3\text{H}]$ HEWL bound; ○, $[^{14}\text{C}]$ thymidine released in the $[^3\text{H}]$ HEWL reaction mixture; ×, $[^{14}\text{C}]$ thymidine released in the control (no HEWL); open arrow, HEWL addition; solid arrow, 0.097 M NaCl addition; vertical bars, mean values of triplicate samples \pm the standard errors.

added to reaction mixtures under these experimental conditions, there was an immediate dislodgment of HEWL from the bacterial cell. NaSCN proved to be more effective than NaCl at 0.097 M in the dislodgment process, as loss of cell-bound HEWL was greater in the presence of the thiocyanate anion (Fig. 1 and 2).

Demonstration of gross cellular lysis by electron microscopy. To further confirm that gross cellular lysis with release of intracellular macromolecules had actually occurred, cells treated with HEWL and sodium thiocyanate were examined by electron microscopy. Figure 3 illustrates a virtually complete absence of normal, intact cells in the HEWL-NaSCN preparation. A large number of cells appeared to have completely lysed and had exploded their cytoplasmic contents, yielding plasma membrane ghosts, cell wall fragments, and cytoplasmic debris (Fig. 3). The outer surfaces of other cells were extensively damaged and appeared to have lysed partially (Fig. 3). In the presence of HEWL but in the absence of salt, the large majority of bacterial cells appeared to have maintained their cell shape (2), as cell lysis was dependent upon NaSCN addition (Fig. 3).

Kinetics of $[^{14}\text{C}]$ thymidine and $[^3\text{H}]$ HEWL release by various inorganic anions. In an effort to study the mechanism of salt-induced lysis, a number of sodium salts were added at 0.097 M final concentration to reaction mixtures containing HEWL and *S. mutans* BHT. In comparison with all inorganic anions examined at

this concentration, thiocyanate released the greatest amount of $[^{14}\text{C}]$ thymidine (approximately 90% after 3 h of incubation) (Fig. 4). Although thiocyanate was superior, addition of perchlorate or iodide to HEWL reaction mixtures resulted in a significant release (approximately 65 or 55%, respectively) of the DNA from the cells (Fig. 4). In contrast, both chloride and fluoride were apparently ineffective at the concentration tested (Fig. 4), whereas bromide and nitrate exhibited intermediate lytic capabilities (approximately 20%, data not shown). Cell lysis under these experimental conditions was therefore dependent upon the nature of the anion, and, in order of effectiveness, a series emerged with $\text{SCN}^- > \text{ClO}_4^- > \text{I}^- > \text{Br}^- > \text{NO}_3^- > \text{Cl}^- > \text{F}^-$.

The identical series was also observed for dislodgment of $[^3\text{H}]$ HEWL (data not shown). During the first minutes of incubation, $[^3\text{H}]$ HEWL release was greatest with SCN^- , followed in order of effectiveness by ClO_4^- , I^- , Cl^- , and F^- . Sodium salts of bromide and nitrate gave virtually identical lysis curves slightly greater than chloride. Upon further incubation, HEWL release gradually leveled off for all anions tested except fluoride, which showed increasing liberation of $[^3\text{H}]$ HEWL for approximately 2 h. With the exception of fluoride, the total amount of HEWL dislodged from the cells obeyed the anion series $\text{SCN}^- > \text{ClO}_4^- > \text{I}^- > \text{Br}^-$, $\text{NO}_3^- > \text{Cl}^-$.

Kinetics of dependence of $[^{14}\text{C}]$ thymidine

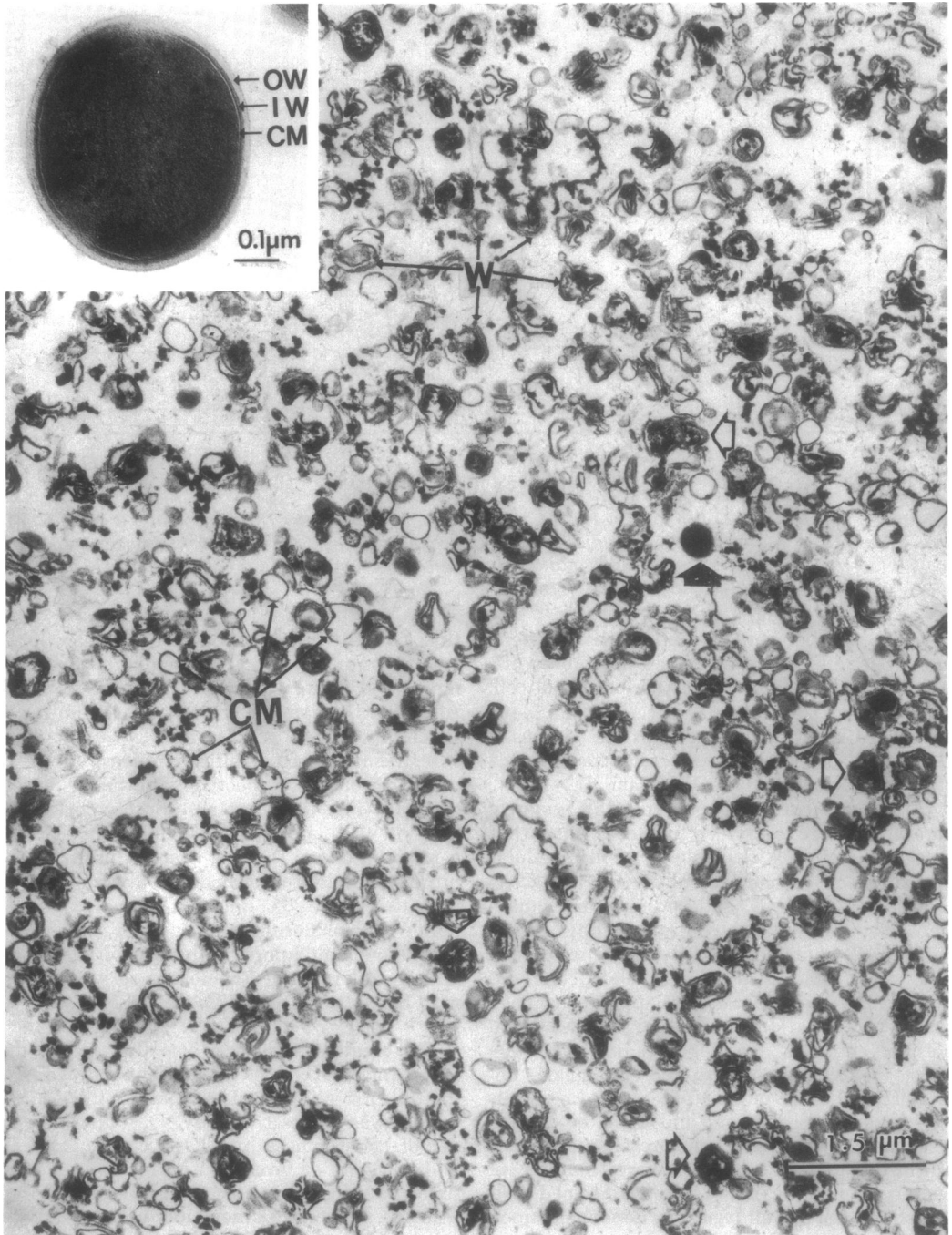


FIG. 3. Electron micrographs of *S. mutans* BHT control cells and cells lysed with HEWL and NaSCN. Cell membrane ghosts (CM), cell walls (W) attached to lysed cells, and cytoplasmic debris resulted from the complete lysis of cells. Partially lysed cells (open arrow) were also noted. Intact cells (solid arrow) were rarely observed ($\times 12,000$). (Inset) Control cell incubated in the absence of HEWL and NaSCN, showing well-defined outer cell wall (OW), dense inner cell wall (IW), and cell membrane (CM) ($\times 67,000$).

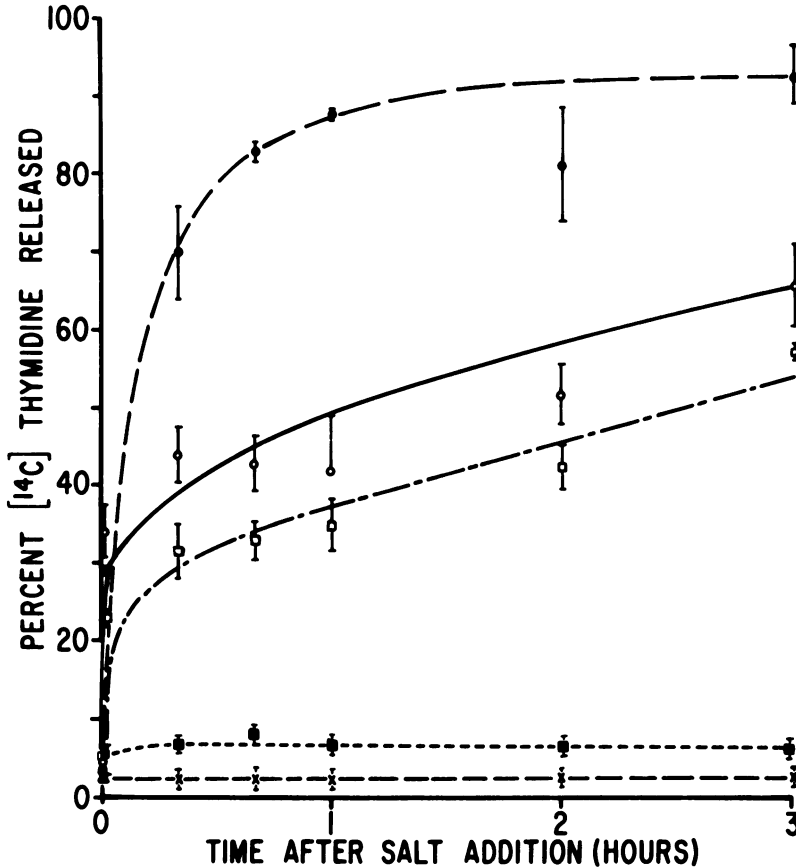


FIG. 4. Kinetics of release of [^{14}C]thymidine from HEWL-treated *S. mutans* BHT by inorganic salts. Each sodium salt was used at a final concentration of 0.097 M. At zero time before the addition of salts, cell suspensions were incubated with HEWL for a period of 3 h (see text for details). Symbols: ●, NaSCN; ○, NaClO₄; □, NaI; ■, NaCl; ×, NaF; vertical bars, mean values of triplicate samples \pm the standard errors.

and [^3H]HEWL release on anion concentration. The kinetics of lysis were observed to depend on the concentration of anion tested. At 0.097 M NaCl, [^{14}C]thymidine release was minimal (Fig. 5). When the concentration of NaCl was changed to 0.115 M, lysis approached 20% after 3 h of incubation. A further increase in NaCl concentration (0.130 M) gave rise to significant release of thymidine (approximately 55%) (Fig. 5), comparable to the lysis values obtained with lower concentrations of iodide (0.097 M NaI, Fig. 4). [^3H]HEWL dislodgment was also dependent on the NaCl concentration. Figure 5 illustrates that higher concentrations of NaCl caused more HEWL to be released from the cells during the first minute of incubation. At the end of the incubation period, the levels of released HEWL varied directly with the NaCl concentration (Fig. 5).

Turbidimetric assays for lysis and dependence on the nature of the anion. Pre-

vious studies have demonstrated that *S. mutans* BHT aggregates in the presence of lysozyme (26). Aggregation was monitored spectrophotometrically, and HEWL addition resulted in increased bacterial suspension turbidity as reflected by an increase in optical density (data not shown). In the absence of HEWL, the optical density remained constant. Previous work also demonstrated that lysozyme-aggregated cells could be clarified by NaCl addition (26). Figure 6 indicates that other anions could behave similarly to NaCl. Moreover, the decrease in turbidity varied directly with the anion, and, in order of effectiveness, we observed the series to be $\text{SCN}^- > \text{ClO}_4^- > \text{I}^- > \text{Cl}^- > \text{F}^-$ (Fig. 6).

Dependence of bacteriolysis on HEWL, anion, and cell concentrations. A more detailed examination of the effects of varying the concentrations of HEWL, anions, and cells on cell lysis was examined by monitoring [^3H]thymidine release after 2.5 h of incubation with four

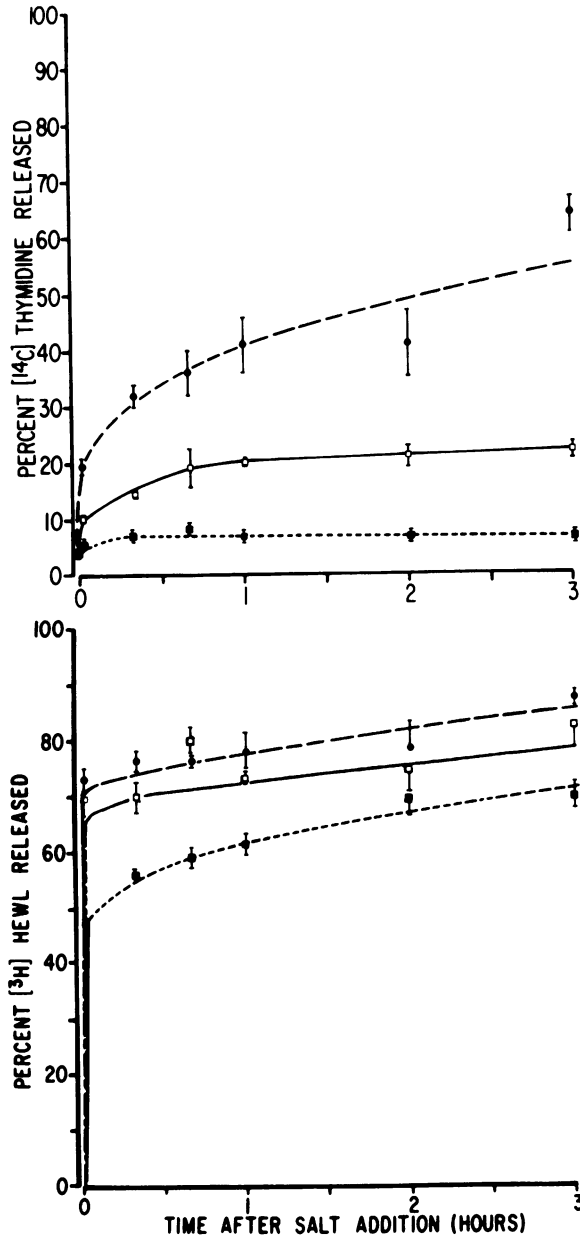


FIG. 5. Effect of NaCl concentration on release of [¹⁴C]thymidine and [³H]HEWL from HEWL-treated *S. mutans* BHT. Details of procedures are given in the text. NaCl concentrations were 0.097 (■), 0.115 (□), 0.130 (●) M.

sodium salts: NaSCN, NaHCO₃, NaCl, and NaF. The results are depicted in Fig. 7 and 8 for the two bacterial cell concentrations tested. In agreement with the results of the kinetic experiments (Fig. 4 and 5), lysis was noted with high concentrations of HEWL in the presence of 0.097 M SCN⁻ or 0.115 M Cl⁻ but not with 0.097 M

Cl⁻ or F⁻ (Fig. 7D). However, both F⁻ and Cl⁻ could significantly influence cell lysis by varying the concentrations of anions, HEWL, and bacterial cells (Fig. 7 and 8). As a general phenomenon, lysis was observed to increase with anion concentration until maximum levels of lysis were attained. This was true for SCN⁻ and HCO₃⁻;

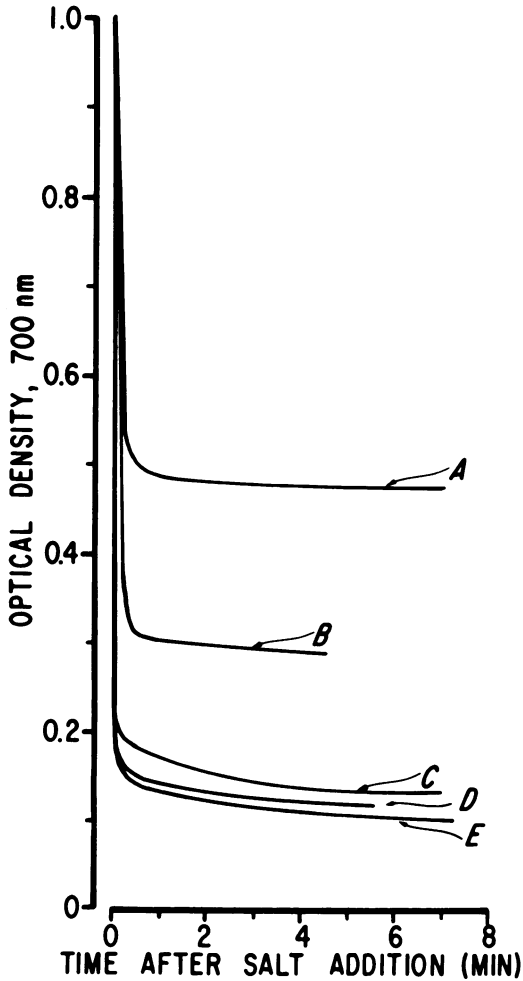


FIG. 6. Effects of inorganic salts on light absorbance properties of HEWL-aggregated *S. mutans* BHT. After 3 h of incubation with unlabeled HEWL (final concentration, 143 $\mu\text{g}/\text{ml}$), aggregated bacterial cells (2 ml) were placed in stirred cuvettes (Spectrostar setting of 7.5) at 37°C in a Gilford spectrophotometer. Optical density decreases were recorded at 700 nm upon addition of 280 μl of NaF (A), NaCl (B), NaI (C), NaClO₄ (D), or NaSCN (E) at a final concentration of 0.097 M.

however, maximum lysis was not observed for Cl⁻ and F⁻ at higher HEWL concentrations (Fig. 7C and D and 8C). Maximum levels of lysis also varied for the four salts tests, with Cl⁻ and F⁻ always found to be less effective than SCN⁻ and HCO₃⁻ (Fig. 7 and 8). Marked changes in the lysis patterns were noted as the salt concentrations were varied from 0.036 to 0.115 M. For a given cell concentration, this large change in the amount of lysis with anion concentration oc-

curred at higher (Fig. 7C and D and 8C) as compared with lower (Fig. 7A and B and 8A and B) HEWL concentrations. Higher HEWL concentrations yielded more lysis than did lower HEWL concentrations at concentrations of anions resulting in maximum or plateau levels of cell lysis; however, less or no lysis was observed with higher HEWL concentrations at 0.036 M salt (for a particular cell concentration), whereas lysis was achieved at this salt concentration with lower HEWL concentrations (all four anions behaved similarly) (Fig. 7 and 8). When concentrations of salts greater than 0.036 M and less than plateau concentrations were used, the nature of the anion and the concentrations of HEWL and bacterial cells were important in the determination of whether a lower HEWL concentration would yield greater lysis than a higher HEWL concentration (Figs. 7 and 8).

DISCUSSION

The selective effects of anions in biological systems have been explored extensively by numerous investigators (31). Anion potency varies according to the particular system under study and is dependent upon the effects of the anion on macromolecular structure (30). When *S. mutans* BHT was reacted with high concentrations of lysozyme and 0.097 M sodium salts, cell lysis was found to be dependent upon the nature of the anion, and, in order of effectiveness, a Hofmeister, or lyotropic, series emerged, with SCN⁻ > ClO₄⁻ > I⁻ > Br⁻ > NO₃⁻ > Cl⁻ > F⁻ (Fig. 4 and Results). However, as the anion, HEWL, and cell concentrations were varied, it was found that the observed differences in lysis elicited by chloride and fluoride and by thiocyanate and bicarbonate could be minimized (Fig. 7 and 8), suggesting that the anion series order would change, depending upon experimental conditions. Alterations in anion potency with variations in anion concentration would in fact be expected based on what is currently known about the general patterns of anion selectivity in biological systems (31).

The effective salt concentrations used to elicit bacteriolysis in the present experiments were observed to be distinctly lower than the concentrations of lyotropic salts required to partially solubilize bacterial membranes (12). However, these results are consistent with reported studies in the literature where the biological effects of anions have been observed in other experimental systems at concentrations of anions within the same range as that used in the HEWL lysis of *S. mutans* BHT (1, 6, 7). Typically, saturation achieved at low additive salt concentrations is characteristic of specific-site-binding interac-

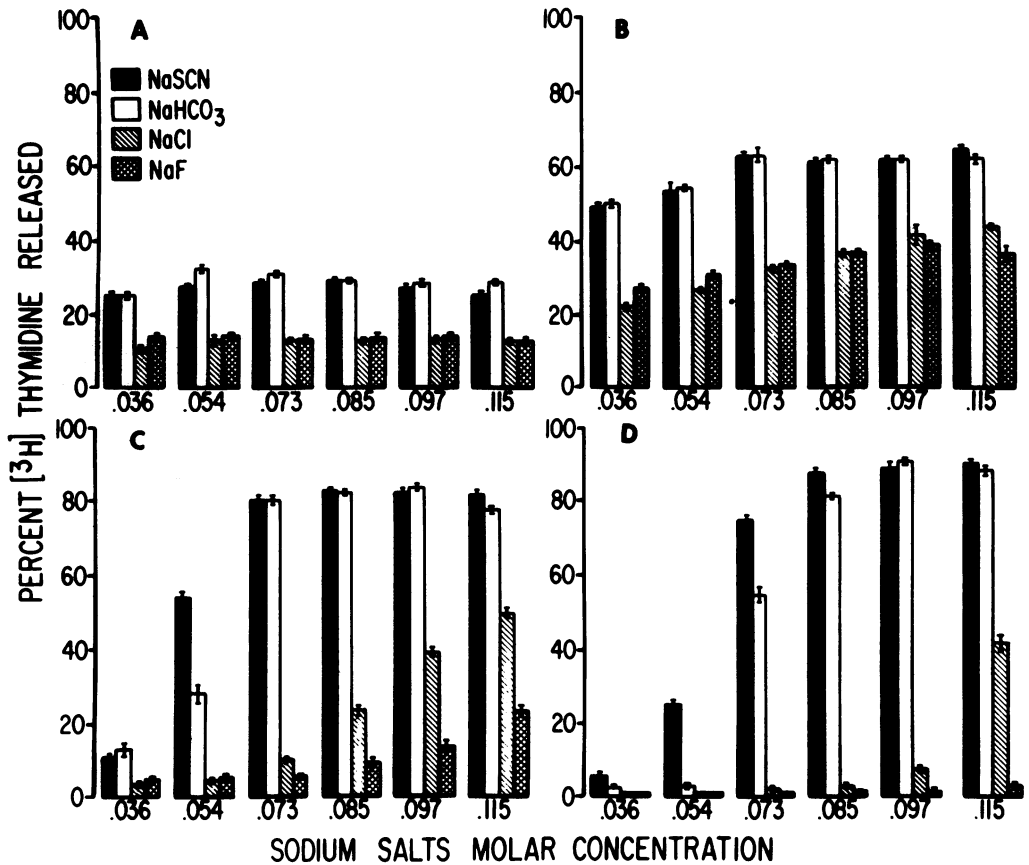


FIG. 7. Effects of inorganic salts on release of [^3H]thymidine from *S. mutans* BHT (10^9 cells per ml) treated with HEWL. HEWL was used at the following final concentrations: 10 $\mu\text{g/ml}$ (A), 22.8 $\mu\text{g/ml}$ (B), 50.2 $\mu\text{g/ml}$ (C), 164 $\mu\text{g/ml}$ (D). Bar graphs represent the mean values of triplicate samples \pm the standard errors.

tions (12, 30). In the *S. mutans* BHT lytic system, the anions are observed to dislodge bound HEWL (Results and Fig. 5) and therefore could influence the interaction of lysozyme with wall- or membrane-specific sites (25) and conceivably could dissociate other structures, e.g., autolysins, to activate the lytic process. Activation of autolytic enzymes by high concentration of inorganic salts alone has been proposed previously (9), and cell-bound HEWL may in some way lower the salt concentration required for autolysis in the *S. mutans* BHT lytic system. A discussion of the effects of HEWL on the hydrolysis of cell wall peptidoglycan and of the possible role of endogenous autolytic peptidoglycan hydrolase enzymes is given in the companion paper (11). Interactions of the anions at a specific site(s) likely involve electrostatic interactions, as suggested by Metcalf and Deibel (22). The sequence of anion potency results obtained in these studies indicates also that hydrophobic interactions

may be involved (30, 31). Future experiments designed to study the interactions of lysozyme and inorganic anions with protoplasts which have recently been prepared for *S. mutans* BHT (24) may provide an understanding of the mechanism(s) involved in the lysis process.

Dislodgment of lysozyme apparently precedes the loss of cellular DNA (25) (Fig. 1). Although initial HEWL dislodgment and lysis appear to be related in these experiments, it is possible that lysis takes place simply by physical shock through salt-induced shrinkage of the cell wall (21, 23). It is noteworthy that Kruse and Hurst (16) previously observed that the liberation of lysozyme from *Streptococcus lactis* cells by high NaCl concentrations or sodium dodecyl sulfate corresponded to cellular lysis. At present, it is not clear whether the release of lysozyme is a requirement for DNA quantitation. With the exception of the fluoride reaction mixtures, the released HEWL quickly reached plateau levels

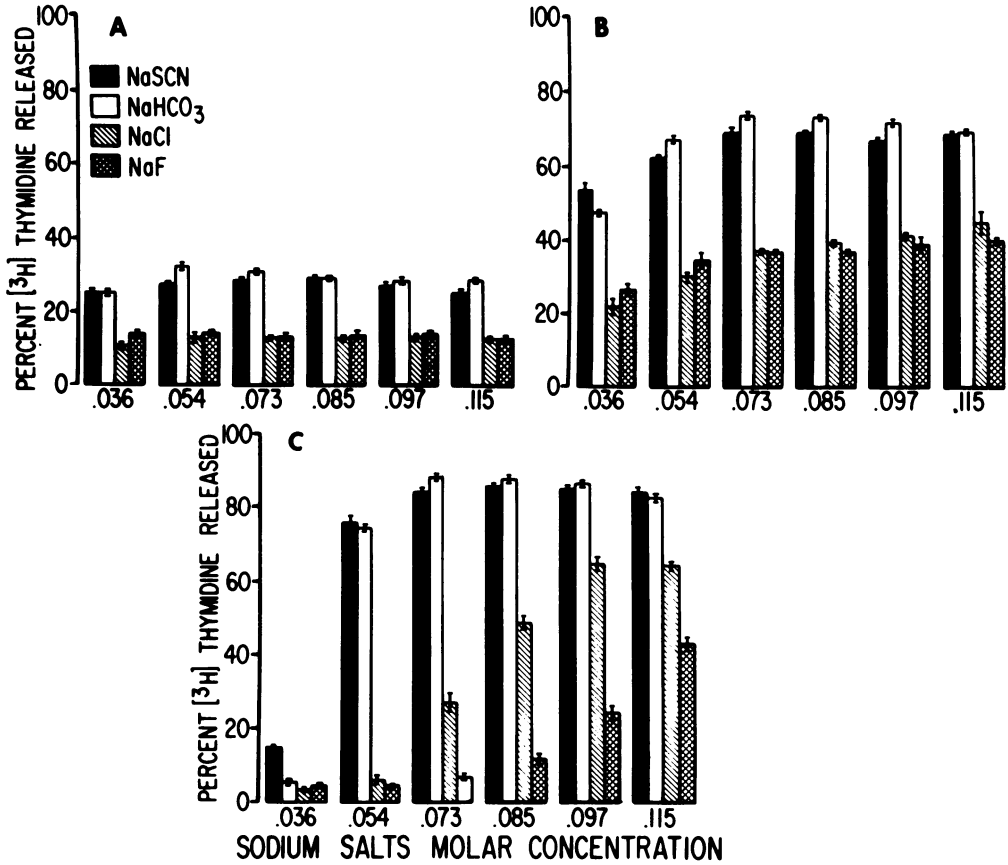


FIG. 8. Effects of inorganic salts on release of [³H]thymidine from lower cell concentration of *S. mutans* BHT (3.5×10^8 cells per ml) treated with HEWL. HEWL was used at the following final concentrations: 2.7 µg/ml (A), 10 µg/ml (B), 22.8 µg/ml (C). Bar graphs represent the mean values of triplicate samples ± the standard errors.

in the expected anion series order. Interestingly, sodium fluoride caused a decrease in cell turbidity under the experimental conditions (Fig. 6). This decrease in optical density was more dramatic with sodium chloride (Fig. 6), and yet the amounts of DNA that could be measured in the assay at either 0.097 M NaF or 0.097 M NaCl and 143 µg of HEWL per ml were minimal (Fig. 2 and 4). The relationship of turbidimetric reductions and DNA quantitation to the lytic process therefore requires further clarification. Furthermore, the results with fluoride and chloride would suggest that although a certain percentage of the HEWL can be either rapidly released or maximally released over time, lysis does not necessarily ensue, despite the apparent correlation of initial HEWL dislodgment with cell lysis. One possible explanation for these results is that HEWL interacts with various cell membrane components with different affinities in such a way that very-high-affinity HEWL-membrane

binding sites are not altered satisfactorily to release critical HEWL molecules, stabilizing the cell against lysis.

An interesting observation noted in our experiments was that low salt concentrations (for example, 0.036 M) could liberate more DNA from *S. mutans* BHT treated with lower as compared with higher HEWL concentrations (Fig. 7 and 8). This presumably reflected the amount of HEWL bound per cell. At higher HEWL concentrations, in comparison with lower concentrations, more enzyme is bound to the cell (25, 26). Therefore, upon addition of a low salt concentration, a greater absolute amount of HEWL may remain still attached to critical sites on the cells, thus preventing or decreasing cell lysis. At lower HEWL concentrations, the absolute quantity of HEWL bound to the critical sites, e.g., the cell membrane, could be much smaller and therefore more readily released by the lower salt concentration (J. J.

Pollock, L. I. Katona, H. Goodman, M. I. Cho, and V. J. Iacono, Arch. Oral Biol., in press). Such an interpretation might also explain how slight changes in anion concentration effect marked changes in bacteriolysis (Fig. 7 and 8), as lysis may rise sharply when a critical amount of lysozyme has been dislodged from the cell by an appropriate concentration of anion, which in turn would permit the efflux of DNA.

Damage to both the cell wall and the cell membrane is likely required for cell lysis to occur, although it is as yet by no means clear where and what on the cell surface must be "digested" or "destabilized" before lysis ensues. This suggestion has been proposed previously by other investigators for penicillin-lytic bacterial systems (10, 18, 27) and is discussed in light of recent results obtained from our laboratory (11). Further investigations of the mechanism of lysozyme-inorganic salt lysis should prove fruitful in understanding lysozyme's potential in vivo antibacterial role.

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