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Lysis of *Vibrio succinogenes* by Ethylenediaminetetraacetic Acid or Lysozyme[1]

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ABSTRACT

WOLIN, M. J. (University of Illinois, Urbana). Lysis of *Vibrio succinogenes* by ethylenediaminetetraacetic acid or lysozyme. J. Bacteriol. **91**:1781–1786. 1966.—Cell suspensions of *Vibrio succinogenes* are lysed by ethylenediaminetetraacetic acid (EDTA) or lysozyme. Lysis occurs at alkaline *p*H and is prevented by 0.15 M NaCl or KCl or 0.3 M sucrose. The addition of 10^{-3} M Mg^{++}, 10^{-3} M spermine, or 10^{-2} M Ca^{++} prevents lysozyme lysis, and 10^{-4} M spermine prevents EDTA lysis. EDTA lysis leads to the formation of a cell ghost, and lysozyme lysis leads to the formation of an empty round body. Freezing and thawing of cells permits lysozyme attack which is not prevented by the protective agents mentioned above. Much of the cell protein, and almost all of the nucleic acids, are released from the cells during EDTA lysis. Treatment of frozen-thawed cells with lysozyme at neutral *p*H does not cause release of more than 50% of the cell protein and 60% of the nucleic acids of the cells.

Ethylenediaminetetraacetic acid (EDTA) sensitized certain bacteria to the action of lysozyme. Repaske (4) first described the EDTA sensitization reaction and also mentioned in a subsequent publication (5) that *Pseudomonas aeruginosa* was lysed to a considerable extent by EDTA alone, and the lytic activity of EDTA was more pronounced with young cultures. Carson and Eagon (Bacteriol. Proc., p. 32, 1964) also reported on the lysis of *P. aeruginosa* by EDTA. Gray and Wilkinson (1) showed that EDTA is bactericidal for *P. aeruginosa* and *Alcaligenes faecalis* and concluded that the action of EDTA is related to its lytic activity. These investigators also demonstrated that EDTA causes the release of lipopolysaccharides from isolated cell walls of *P. aeruginosa* and *A. faecalis* (1*a*).

This report is concerned with the effect of EDTA and lysozyme on the structural integrity of *Vibrio succinogenes* (8). EDTA or lysozyme caused lysis of *V. succinogenes*, but only if the cells were suspended in a hypotonic environment and at alkaline *p*H.

MATERIALS AND METHODS

Growth of organism. V. succinogenes was routinely transferred in a medium which consisted of $(NH_4)_2SO_4$, 0.1%; K_2HPO_4, 0.5%; fumaric acid, 0.3%; sodium formate, 0.3%; yeast extract (Difco), 0.1%; $MgCl_2 \cdot 6H_2O$, 0.02%; and $FeSO_4$, 0.001%. The *p*H was 7.0 to 7.2. Sterile, autoclaved sodium thioglycolate (Difco) was aseptically added before inoculation to a final concentration of 0.05%. Cell suspensions were prepared from 1-liter or 20-liter cultures grown on a different medium than the routine transfer medium; the primary difference was the substitution of NO_3^- for fumarate as the electron acceptor which supported growth. The medium for growth of 1-liter and 20-liter quantities was the same as the routine transfer medium, except for the omission of fumaric acid and the addition of KNO_3, 0.6%; sodium succinate, 0.03%; and, only for the growth of 20-liter cultures, phenol red, 0.00015%. The *p*H of the medium was adjusted to 7.0 to 7.2 before autoclaving. Thioglycolate was added aseptically. $MgCl_2 \cdot 6H_2O$ and $FeSO_4$ were sterilized in a single, separate solution and added aseptically when 20-liter quantities were grown.

The inoculum for 1-liter cultures was 10 ml of a 16-hr culture grown on the routine transfer medium. The inoculum for 20-liter cultures was 1 liter of a 16-hr culture grown on the NO_3^--containing medium. The 1-liter and 20-liter cultures were grown in an atmosphere of N_2. All cultures were incubated at 37 C.

Cells were harvested by centrifugation. They were washed (2- to 20-liter cultures) with a 1,400-ml and twice with 200-ml washes of a solution which contained 0.01 M $MgCl_2$, 0.15 M NaCl, and 0.01% β-mercaptoethanol, and were finally resuspended in 40 ml

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of the same solution. The resuspended cells were flushed with H_2 and stored at 4 C.

Chemical. Cell fractionation with trichloroacetic acid was performed as described by Roberts et al. (6) to obtain the cold and hot trichloroacetic acid-soluble fractions. Protein was estimated directly on cells and cell fractions without using the preliminary trichloroacetic acid-fractionation procedure. Insoluble cell fractions were heated in an Arnold steamer for 15 min in 0.5 N NaOH, and the soluble portion was used for protein determinations. Protein was determined by the method of Lowry (2) with crystalline serum albumin as a standard.

The lysozyme used was crystalline egg white lysozyme from Worthington Biochemical Corp., Freehold, N.J.

RESULTS

Lysis by EDTA or lysozyme. Incubation of a resting cell suspension at *p*H 9.0 with 5×10^{-3} M EDTA or 100 μg/ml of lysozyme resulted in rapid cell lysis which was measured by the decrease in suspension turbidity (Fig. 1). EDTA and lysozyme together produced a slower lytic rate than that observed with lysozyme alone, which suggests that EDTA may inhibit lysozyme action to some extent.

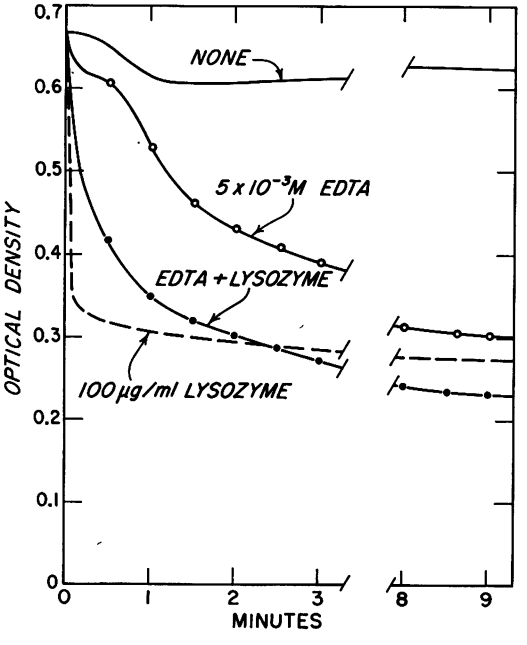

FIG. 1. *Lysis by EDTA or lysozyme at pH 9.0. Resting cells were added to solutions containing 0.09 M tris(hydroxymethyl)aminomethane (Tris), (pH 9.0) and the additions are indicated in the figure. The final volume was 2.0 ml, and the OD was followed in a Cary Model 14 recording spectrophotometer at room temperature. A cuvette containing water was used as a blank.*

The *p*H dependence of EDTA or lysozyme lysis is shown in Fig. 2. Lysis by either lytic agent was slight at *p*H 7.3 and increased with increasing *p*H with an apparent optimum at *p*H 8.6.

Figure 3 shows the effect of concentration of EDTA on lysis at *p*H 9.0.

Protection against lysis. The lysis of *V. succinogenes* at *p*H 9.0 by either EDTA or lysozyme was completely prevented by the inclusion of isotonic NaCl in the incubation medium (Table 1). Lysis was also prevented by the addition of 0.3 M sucrose or 0.15 M KCl but not by the addition of high concentrations of glycerol or mannitol.

Lysozyme lysis at *p*H 9.0 was also prevented by the inclusion of low concentrations of Mg^{++}, Ca^{++}, or spermine to the incubation medium (Fig. 4). Mg^{++} and spermine were effective inhibitors of lysis at approximately the same concentrations; 10^{-3} M of either agent almost completely abolished lysis. Ca^{++} was effective at approximately a 10-fold higher concentration.

Low concentrations of spermine also protected the cells against lysis by EDTA at *p*H 9.0. Figure 5 shows the effect of concentration of spermine on EDTA lysis. Spermine (10^{-4} M) completely abolished lysis. The slight increase in optical density observed with 10^{-4} M and 10^{-3} M spermine was due to a slight aggregation of cells caused by spermine.

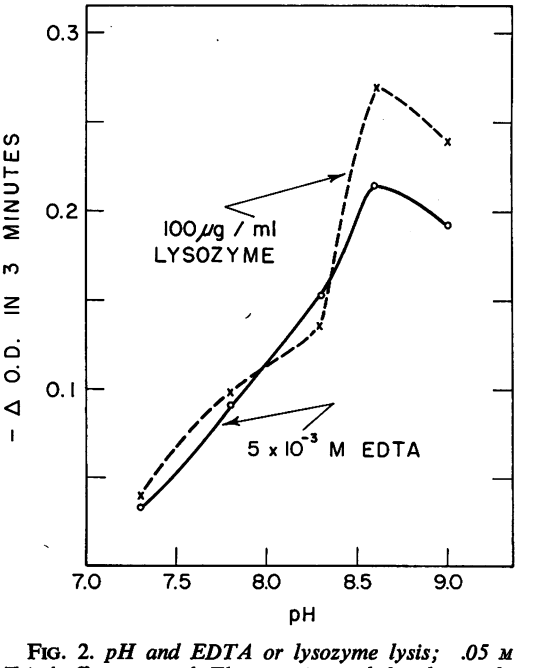

FIG. 2. *pH and EDTA or lysozyme lysis; .05 M Tris buffer was used. The experimental details are the same as in Fig. 1; 5×10^{-3} M EDTA or 500 μg/ml of lysozyme was used.*

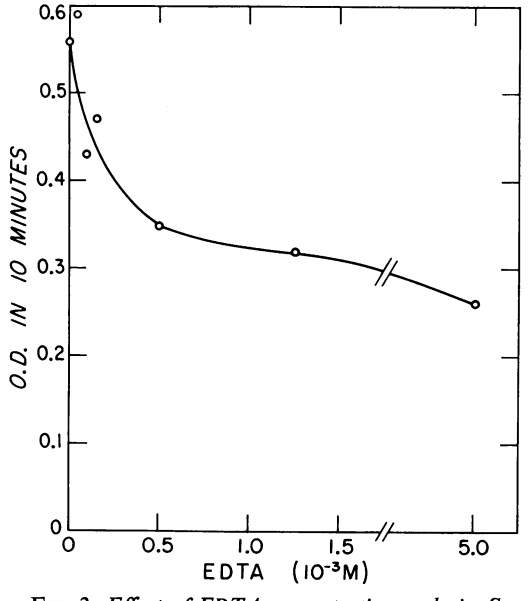

FIG. 3. *Effect of EDTA concentration on lysis. See Fig. 1 for experimental details. The pH was 9.0.*

TABLE 1. *Prevention of lysis by 0.15 M NaCl*

Additions	−Δ OD in 10 min*	
	−NaCl	+NaCl
None	.041	.032
5×10^{-3} M EDTA	.363	.009
100 μg/ml of lysozyme	.387	.013
EDTA + lysozyme	.434	.000

* Incubations at *p*H 9.0, 25 C; other experimental details as in Fig. 1.

Morphological changes accompanying lysis. Phase contrast microscopic observations showed the formation of cell ghosts when cells were lysed with EDTA at *p*H 9.0. When no lysis was obtained with EDTA treatment, either when protective salts or neutral *p*H was used, no morphological change was detected. Lysozyme treatment at *p*H 9.0 resulted in the appearance of empty, round bodies. At neutral *p*H, however, there was a conversion of cells to optically dense round bodies or spheroplastlike bodies which slowly lysed over a period of hours rather than minutes, as occurred at *p*H 9.0. No morphological change was observed when cells were treated with lysozyme at *p*H 7.3 or *p*H 9.0 in the presence of agents which protected against lysis.

Effect of freezing and thawing. Rapid freezing and thawing (three cycles of shell freezing in an acetone-Dry Ice bath followed by thawing in cold

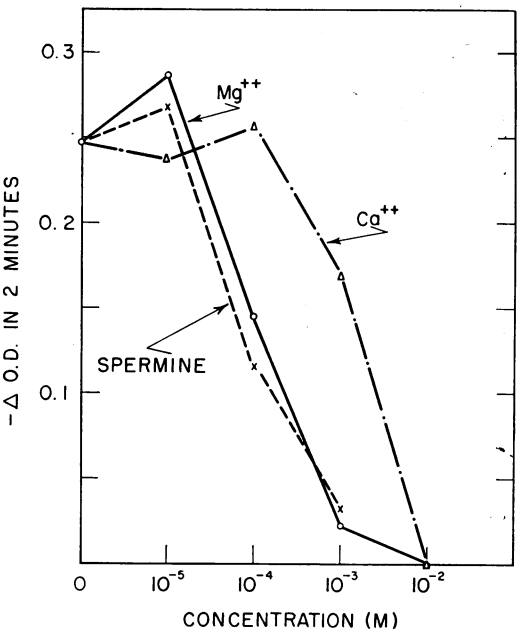

FIG. 4. *Prevention of lysozyme lysis by Mg^{++}, Ca^{++}, and spermine. See Fig. 1 for experimental details. Lysozyme (100 μg/ml) was used at pH 9.0.*

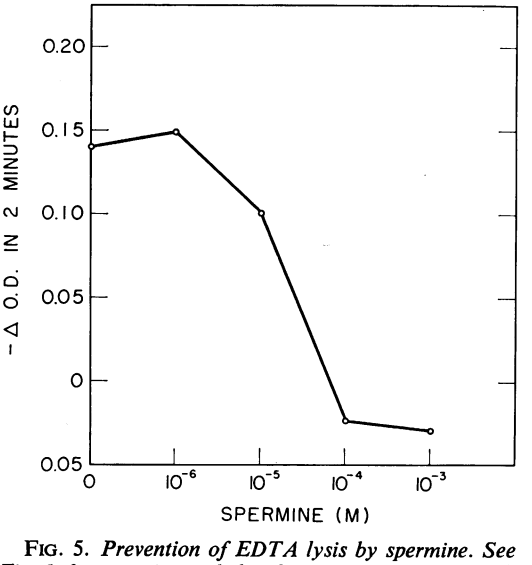

FIG. 5. *Prevention of EDTA lysis by spermine. See Fig. 1 for experimental details. EDTA (5×10^{-3} M) was used at pH 9.0.*

tap water) of *V. succinogenes* suspensions changed their response to EDTA or lysozyme. The major change observed was a lack of protection, by 0.15 M NaCl and Mg^{++} from lysis with lysozyme and by 0.15 M NaCl against alkaline EDTA lysis.

No gross morphological changes were observed in frozen-thawed cells either by phase-contrast or electron microscopy.

Chemical analysis of EDTA-lysed and frozen-thawed cells. Cells lysed with 10^{-3} M EDTA at *p*H 9.0 lost almost all of the hot and cold trichloroacetic acid-soluble, 260 mμ-absorbing material present in the whole cells (Table 2). The residual EDTA pellet, recovered after centrifugation of the EDTA lysate at 14,000 × *g* for 10 min, contained approximately 33% of the initial cell protein. The EDTA pellet is suceptible to lysozyme attack, because a morphological change from cell-shaped ghosts to round ghosts occurred when the EDTA pellet was treated with lysozyme. This treatment did not, however, cause a significant release of protein from the EDTA pellet.

Freezing and thawing three times caused the release of essentially all of the cold trichloroacetic acid-soluble, 260 mμ-absorbing material in the cells (Table 3). The bulk of the hot trichloroacetic acid-soluble, 260 mμ-absorbing material was retained in the pellet recovered after centrifugation at 14,500 × *g* for 10 min, along with most of the cell protein. Lysozyme treatment led to the formation of a round body without the loss of large amounts of protein or hot trichloroacetic acid-soluble, 260 mμ-absorbing material.

Nitrate reduction. Part of the impetus for this work stemmed from previous observations that the reduction of nitrate by hydrogen, which can be demonstrated with resting cell suspensions, cannot be demonstrated in cell-free extracts prepared by sonic oscillation (1*b*). A variety of cofactors and protective agents did not restore or maintain cell-free activity, but the addition of an artificial electron carrier, benzyl viologen, did permit nitrate reduction by hydrogen in extracts. A possible explanation of the requirement for benzyl viologen is that the physical integrity of the electron transport system was disrupted during sonic oscillation which then produced the requirement for an artificial electron carrier to couple between the hydrogenase and nitrate reductase of the organism. It was hoped that less destructive breakage procedures could be found which would permit the demonstration of nitrate reduction by hydrogen without the need for benzyl viologen.

None of the methods described in this paper for disruption of cells, however, lead to an "intact" nitrate-reducing system. EDTA lysis or lysozyme lysis at alkaline *p*H lead to preparations which require benzyl viologen for nitrate reduction. In fact, it was found that merely freezing and thawing of the vibrio leads to a requirement for benzyl viologen for nitrate reduction.

TABLE 2. *Distribution of cell material after lysis with EDTA and lysozyme treatment*

Cell fraction*	Hot trichloroacetic acid†	Cold trichloroacetic acid†	Protein	
			Amt	Per cent
			mg	
Cells	98.5	4.5	26.0	100
EDTA pellet	6.7	0.0	8.6	33
EDTA supernatant fraction + wash	79.5	12.2	15.1	58
Lysozyme-EDTA pellet	—	—	7.3	28
Lysozyme-EDTA supernatant fraction	—	—	1.0	4

* Cells were lysed by treating with 10^{-3} M EDTA at *p*H 9.0 (.05 M tris(hydroxymethyl)aminomethane) for 30 min at 25 C and centrifuged at 14,500 × *g* for 10 min to obtain the EDTA pellet and EDTA supernatant fraction. The EDTA pellet was washed with 0.15 M NaCl, resuspended in 0.15 M NaCl, and treated with 100 μg/ml of lysozyme for 30 min at 25 C and similarly centrifuged to obtain the lysozyme-EDTA pellet and supernatant fraction.

† Total absorbancy at 260 mμ of the cell fraction was analyzed.

TABLE 3. *Distribution of cell material after freezing and thawing and lysozyme treatment*

Cell fraction*	Hot trichloroacetic acid†	Cold trichloroacetic acid†	Protein	
			Amt	Per cent
			mg	
Cells	145.4	5.3	17.4	100
Freeze-thaw pellet	82.0	0.3	9.5	55
Freeze-thaw supernatant fraction + wash	18.8	5.1	4.0	22
Lysozyme pellet	68.7	0.5	8.1	47
Lysozyme supernatant fraction	15.7	2.0	1.1	6

* Cells were subjected to three cycles of freezing (acetone-Dry Ice bath) and thawing (cold water) and centrifuged at 14,500 × g for 10 min to obtain the freeze-thaw pellet and supernatant fraction. The freeze-thaw pellet was washed with 0.15 M NaCl, resuspended in 0.15 M NaCl, and treated with 100 μg/ml of lysozyme for 30 min at 25 C and similarly centrifuged to obtain the lysozyme pellet and supernatant fraction.

† Total absorbancy at 260 mμ of the cell fraction was analyzed.

DISCUSSION

Although EDTA-induced lysis has been described for *P. aeruginosa* (5; Carson and Eagon, Bacteriol. Proc., p. 32, 1964), a specific requirement for hypotonicity has not been demonstrated. The requirement for hypotonicity for attack by EDTA or lysozyme on *V. succinogenes* suggests that a change in the surface structure induced by hypotonicity sensitizes the cells to attack by either agent. The requirement for alkaline *p*H in addition to hypotonicity indicates that the sites of attack of EDTA and lysozyme are not completely labilized by hypotonicity alone. Alkaline *p*H may cause a dissociation of a charged complex as a further requirement for lysis. Alkaline lysis by EDTA and the protection against lysis by the polyamine, spermine, and the protection afforded against lysozyme lysis by spermine, magnesium, and calcium strongly suggests that a divalent cation dissociation caused by alkaline *p*H is a factor in sensitizing the cells to EDTA or lysozyme.

It should be emphasized that the requirement for alkaline *p*H, hypotonicity, and EDTA or lysozyme are simultaneous requirements. All attempts to treat the cells in a stepwise fashion, e.g., exposure to *p*H 9.0 in a hypotonic environment, centrifuging and resuspending the cells in 0.15 M NaCl, and then treating with lysozyme or EDTA, failed to give any indication of lysis or gross morphological changes in the cells. Treatment with the lytic agents in 0.15 M NaCl at *p*H 7.0 or *p*H 9.0, followed by washing and resuspension in a hypotonic environment at *p*H 9.0, also failed to give any indications of attack by the lytic agents.

The exact physical events which lead to the lysis by EDTA and lysozyme have yet to be identified, but a working hypothesis can be set forth based on the available information concerning the structure of cell walls of gram-negative bacteria. The walls of *Escherichia coli* consist of layers of mucopeptide, lipoprotein, and lipopolysaccharide (7). Assuming that the wall of *V. succinogenes* is similar in construction, it seems possible that the nonmucopeptide components are structurally altered in a hypotonic environment. This alteration permits the dissociation of structurally essential divalent cations at alkaline *p*H. EDTA chelation of the cations causes a further alteration of the nonmucopeptide layer, and lysis of the cell results from this alteration. The alkaline dissociation of structurally essential cations could also lead to exposure of mucopeptide to the action of lysozyme. Freezing and thawing could lead to a susceptibility to lysozyme by affecting the nonmucopeptide portion of the wall, which may normally protect the cells against lysozyme action.

Lysozyme action on frozen-thawed cells does not lead to massive escape of macromolecules from the cell. Lysozyme treatment of cells at neutral *p*H in the presence of EDTA also leads to the formation of a round body whch does not lose much of its macromolecular components. It is possible that both freezing and thawing and EDTA treatment at neutral *p*H destroy the association between the nonmucopeptide and mucopeptide layers, making the mucopeptide susceptible to lysozyme without destroying the nonmucopeptide portion itself. Divalent cations may have a dual function: to hold the nonmucopeptide wall material together and to link the mucopeptide to the nonmucopeptide portion of the wall. The latter binding can be disrupted by EDTA at *p*H 7.0 or exposure to alkaline *p*H, but the former only at *p*H 9.0 and with EDTA.

The various lytic treatments described in this paper cannot be used to obtain a fully intact nitrate-reducing system, although the methods of disruption would appear to be reasonably gentle. It is necessary to add an artificial dye to effect a couple between the hydrogenase and nitrate reductase of the organism. All attempts at protection and reactivation have failed. These negative data have been accumulated in vast enough quantities to warrant the suggestion that the particulate electron-transport system cannot interact properly even when the cells are disrupted by the most gentle means available. All of the particulate preparations described in this paper contain almost all of the electron-transport system activity of the organism, including the enzymes hydrogenase, succinic dehydrogenase, nitrate reductase, and cytochromes *b* and *c*. The situation may be similar to the disappearance of a coupling to oxygen in the succinoxidase system of *Micrococcus lysodeikticus* when protoplasts are prepared (3).

We recently showed that all of the cell hydrogenase is bound to insoluble residue remaining after alkaline EDTA lysis and can be released from the residue at *p*H 11.0 (Aspen and Wolin, Bacteriol. Proc., p. 96, 1964). The hydrogenase can be reconstituted with insoluble material left after the *p*H 11.0 treatment by combining both fractions at *p*H 7.0. The lytic procedures described in this paper should, therefore, provide a tool for the examination of the relationship of the various surface structures of *V. succinogenes* and the binding between enzymes and the particulate structures of the organism.

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