JOURNAL OF BACTERIOLOGY, Nov. 1970, p. 748-753
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Vol. 104, No. 2
Printed in U.S.A.

Release of Alkaline Phosphatase from Cells of *Pseudomonas aeruginosa* by Manipulation of Cation Concentration and of *p*H[1]

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Received for publication 10 August 1970

Pseudomonas aeruginosa ATCC 9027 contains an inducible alkaline phosphatase. The enzyme is readily removed from 14-hr cells by washes in 0.2 M $MgCl_2$, *p*H 8.4. Similar washes in tris(hydroxymethyl)aminomethane buffer, 20% sucrose, monovalent ions, or water partially release enzyme from the cells. The release of alkaline phosphatase is correlated with an increased release of protein and retention of internal enzymes. The effect of 0.2 M $MgCl_2$ washing upon the cells is minimal since both viability and growth rates remain unchanged as compared to water washing. Although cells are plasmolyzed in both 0.2 M $MgCl_2$ and 20% sucrose, it is evident that plasmolysis alone is unable to account for total enzyme release and that a divalent metal, i.e. Mg^{2+}, augments the release pattern. Growing cells in the presence of increasing concentrations of $MgCl_2$ or at increased *p*H values results in an almost total secretion of the enzyme to the culture filtrate. The findings suggest that *P. aeruginosa* alkaline phosphatase is linked to the exocytoplasmic region through divalent metal ion, presumably Mg^{2+}, bridges.

A very strong case has been made for the localization of the alkaline phosphatase (orthophosphoric monoester phosphohydrolase; EC 3.1.3.1) of *Escherichia coli* in the compartment external to the cytoplasmic membrane (1, 7), and this case has been strengthened by studies on the pattern of its release from this compartment (17). Immunological studies showed that the enzyme is not located at the cell surface (18), but other studies have shown that damage to the outer cell wall layers causes the release of alkaline phosphatase from the cell (5, 12, 14, 17). This body of evidence has served to locate alkaline phosphatase in the periplasmic space of *E. coli.* A number of other degradative enzymes have been localized in the volume external to the cytoplasmic membrane (1, 2, 19, 22) in various gram-negative organisms, and Neu and Chou (15) suggested that the periplasmic compartment of these cells may have a role comparable to that of the lysosome in the eucaryotic cell.

The usual method of releasing the periplasmic enzymes from the cell involves osmotic shock in the presence of ethylenediaminetetraacetic acid (EDTA; reference 15) or spheroplast formation by the action of lysozyme and EDTA (12). Because *Pseudomonas aeruginosa* is lysed by EDTA (6, 23) and because EDTA must be presumed to have a very profound effect on the cytoplasmic membrane, we explored alternative methods for the removal of a presumed periplasmic enzyme from the cells of this organism. We found that simple washing in 0.2 M Mg^{2+} effectively releases 100% of the alkaline phosphatase while the cells remain viable and actively motile.

MATERIALS AND METHODS

Organism and culture conditions. *P. aeruginosa* ATCC 9027 was grown in 2-liter flasks in a medium of the following composition: 0.02 M NH_4Cl, 0.02 M KCl, 0.12 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, 0.5% glucose, 0.5% proteose peptone (Difco), and 0.0016 M $MgSO_4 \cdot 7H_2O$. The medium (inorganic phosphate deficient) was prepared from separately autoclaved solutions as described by Mallette et al. (13) and was adjusted to *p*H 7.4 with 5 N HCl. The above medium, with the addition of 0.6% K_2HPO_4 and 0.6% KH_2PO_4, is known as high-phosphate glucose-ammonium salts-proteose peptone medium. The other high inorganic phosphate medium is that described by Vogel and

[1] Issued as Macdonald College Journal series no. 613.

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Bonner (20). An inoculum of 1 ml from an overnight culture was used per 500 ml of medium with subsequent incubation on a rotary shaker at 37 C. The cells were harvested after 12 to 14 hr of incubation by centrifugation in a Sorvall RC-2B refrigerated centrifuge for 10 min at 13,000 × *g*.

Optical density of cultures. Cells grown in the inorganic phosphate-deficient medium produced (after 8 hr of incubation) large amounts of pigment which absorbed below 500 nm as determined in a Unicam SP800 recording spectrophotometer. The optical density of the culture was measured therefore at 660 nm, since no absorption due to the pigments was evident at this wavelength. Cells grown in the inorganic phosphate-deficient glucose medium were usually clumped in the early log phase but became uniform in the late-log and stationary phases. The optical density of the culture was correlated with the dry weight of the cells by means of a calibration curve following drying to constant weight at 90 C. From this curve it was determined that an optical density of 1.0 is equivalent to 18.5 mg (dry weight of cells) per ml (uncorrected).

Chemicals. *p*-Nitrophenylphosphate, HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonic acid) and Tris were purchased from the Sigma Chemical Co., St. Louis, Mo. Glycylglycine, Bicine (*N,N*-bis-(2-hydroxyethyl)glycine), and Tricine[*N*-Tris(hydroxymethyl)methylglycine] were obtained from Calbiochem, Los Angeles, Calif.

Release of alkaline phosphatase from cells. Standard quantities of cells obtained from cultures grown to 1.2 to 1.4 optical density units were washed with salts such as $MgCl_2 \cdot 7H_2O$, $CaCl_2$, NaCl, KCl, Tris, a combination of Tris and various salts or sucrose. The viability of the cells after washing was determined by serial dilutions in distilled water plated on *Pseudomonas* agar P (Difco). Growth rates were determined after standard inoculations of appropriately washed cells into 500 ml of inorganic phosphate-deficient medium.

Cell extracts. The cells (370 mg dry weight) were ultrasonically disrupted with a Bronson Sonifier at a tip energy of 100 w for three 30-sec intervals. The disrupted cells were centrifuged at 13,000 × *g* for 10 min, and the supernatants were assayed for glucose-6-phosphate dehydrogenase, reduced nicotinamide adenine dinucleotide (NADH) oxidase, and alkaline phosphatase.

Enzyme assays. Alkaline phosphatase was assayed by the methods of Neu and Heppel (16). The reaction was followed at room temperature at 420 nm in a microsample spectrophotometer (either Unicam SP800 or Gilford model 300-N). Known quantities of alkaline phosphatase were added to the assay systems concerned with the various wash treatments to demonstrate the absence of stimulatory or inhibitory effects upon the released enzyme. The assay for glucose-6-phosphate dehydrogenase was similar to that described by Malamy and Horecker (12). NADH oxidase activity was followed in a 1-ml assay system consisting of 4×10^{-4} M NADH, 0.05 M Tris buffer (*p*H 7.6), and extract. The oxidation of NADH was followed at room temperature at 340 nm. One unit of activity in all cases represents the conversion of 1 μmole of substrate to product per min.

Estimation of protein. The method of Lowry et al. (11) was used to estimate protein concentrations with bovine serum albumin as a standard. The optical density of washed cell supernatants was also measured at 260 and 280 nm as described by Warburg and Christian (21).

RESULTS

Before studies on the release of alkaline phosphatase from cells of *P. aeruginosa* were undertaken, it was imperative to determine (i) whether this organism possessed an inducible enzyme system and (ii) the characteristics of such an induced system. The results (Fig. 1) show that alkaline phosphatase is inducible when the organism is grown in minimal medium containing proteose peptone as the sole source of phosphate. In addition, the level of induced enzyme reaches a maximum at approximately 14 hr. Although not reported in Fig. 1, no alkaline phosphatase was detected in cells grown on either inorganic phosphate-deficient medium, amended with 1.2% potassium phosphate, or Vogel's medium.

When induced cells were harvested after growth in minimal medium, as described, and subjected to the osmotic shock procedure of Neu and Heppel (16), it was found that the cells lysed after the addition of EDTA. This observation is in accord with those made by other workers who have attempted EDTA treatment of *P.*

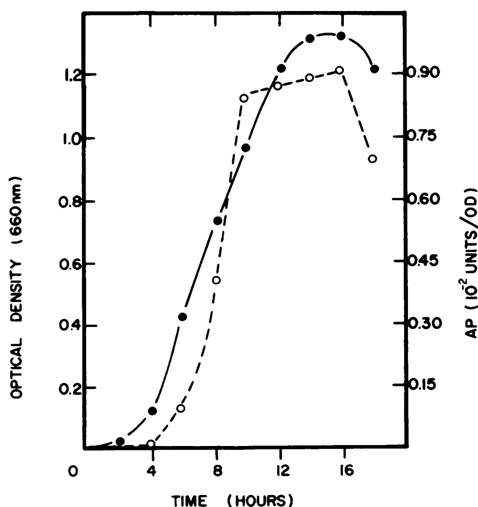

FIG. 1. *Growth of P. aeruginosa and induction of alkaline phosphatase. A 20-ml amount (520 mg dry weight) of cells was centrifuged at each time noted, suspended into 0.01 M Tris (pH 8.4) and disrupted by sonic vibration. Symbols:* ●, *optical density;* ○, *units of alkaline phosphate per optical density unit.*

aeruginosa (3, 6, 10). It was noted that, if EDTA was omitted from the initial shock treatments, and the cells were centrifuged and subsequently resuspended in dilute magnesium, alkaline phosphatase was detected in the magnesium wash supernatant. This effect was further examined, and the results summarized in Table 1 show that 0.2 M Mg^{2+} effectively removes 100% of the enzyme. In addition, Ca^{2+} also releases a substantial portion of alkaline phosphatase. Tris buffer, monovalent ions, 20% sucrose, and water released only part of the total enzyme of the cells. Magnesium alone, on the other hand, adjusted to *p*H 8.4, is as effective as Mg^{2+} and Tris buffer. Substitution of Tris with either HEPES, Bicine, glycylglycine, or Tricine buffers in the Mg^{2+} neither augments nor inhibits the action of Mg^{2+}. It is also noteworthy that washing with 0.01 M Mg^{2+} results in a retention of alkaline phosphatase which is greater than that observed with either Tris buffer or water. This observation will be discussed and extended in a later section.

With respect to the Mg^{2+}, Tris, and water washes, it was noted that, under all conditions employed, the cells remained actively motile. In addition, the viability and growth rates for cells from all three treatments were identical (Table 1). These results may be contrasted with those obtained after osmotic shock (Neu and Heppel) or EDTA treatment (9) of *E. coli*. EDTA-treated cells of *E. coli* remain viable; however, the growth rate of treated cells is decreased at least twofold. Furthermore (Table 1), under conditions in which alkaline phosphatase is released, there is a corresponding increase in protein as determined by the method of Lowry (11) and in the 280/260 ratio which is also indicative of an increased release of protein. The total protein as determined by the method of Lowry (11) released by the effective washes, i.e., 0.2 M Mg^{2+} or 0.2 M Mg^{2+} and Tris buffer, was approximately 5 to 10% that released in the cell-free extract from a corresponding quantity of cells.

Identical treatment of *E. coli* cells grown under similar conditions did not yield alkaline phosphatase (*unpublished observations*).

The results presented in the preceding table strongly suggest that minimal alterations occur within the treated cells. However, upon microscopic examination, it was noted that 0.2 M Mg^{2+} and 20% sucrose-washed cells were plasmolyzed. Photomicrographs of such treated cells are shown in Fig. 2. It is interesting that, although the cells are plasmolyzed in 20% sucrose, it is apparent that plasmolysis alone cannot account for total enzyme release and that a divalent ion, i.e. Mg^{2+}, is a necessary requirement.

Previous findings (Table 1) indicated that washing with 0.01 M Mg^{2+} released less enzyme than when the cells were washed with either Tris buffer or water. This effect was examined further, and the results obtained are illustrated in Fig. 3. It appears from this experiment that a critical level of Mg^{2+}, i.e. 0.01 M, is reached where alkaline phosphatase is bound more strongly than in the case of cells which are washed with either Tris buffer or water. This effect was also observed

TABLE 1. *Effect of various washes upon cells of Pseudomonas aeruginosa*

Treatment	Alkaline phosphatase[a] (units/25 g)		Viability (cells/ml)	Growth rate[b]	280/260	Protein (mg/g)
	Wash	CFE				
0.2 M Mg^{2+} + 0.01 M Tris, *p*H 8.4	85.0	3.3	3.8×10^9	0.134	0.82	2.53
0.2 M Mg^{2+}, *p*H 8.4	88.0	1.1	3.6×10^9	0.134	0.82	1.92
Water	10.4	70.6	3.5×10^9	0.134	0.64	0.86
0.01 M Tris, *p*H 8.4	35.8	44.6	—[c]	0.133	0.68	0.82
0.01 M Mg^{2+} + 0.01 M Tris, *p*H 8.4	4.8	78.0	—	0.134	0.68	0.54
20% Sucrose + 0.01 M Tris, *p*H 8.4	28.8	54.8	—	—	—	—
0.2 M $CaCl_2$ + 0.01 M Tris, *p*H 8.4	57.3	27.4	—	—	0.70	1.42
0.2 M KCl + 0.01 M Tris, *p*H 8.4	52.5	28.2	—	—	0.73	1.78
0.2 M NaCl + 0.01 M Tris, *p*H 8.4	59.2	26.6	—	—	0.75	2.07
Cell-free extract	81.5	—	—	—	0.63	20.50

[a] A 20-ml amount (520 mg, dry weight) of 14-hr cells was centrifuged, suspended into 20 ml of each treatment solution for 15 min at room temperature, and recentrifuged. The resulting washes were assayed for alkaline phosphatase, protein, and 280,260-nm absorbing material. The cell pellet was suspended into 20 ml of Tris buffer, ultrasonically disrupted, and centrifuged, and the cell-free extract (CFE) was assayed for alkaline phosphatase.

[b] Growth rate is expressed as the change in optical density (OD) per hour between the 6th and 10th hr.

[c] Test not performed.

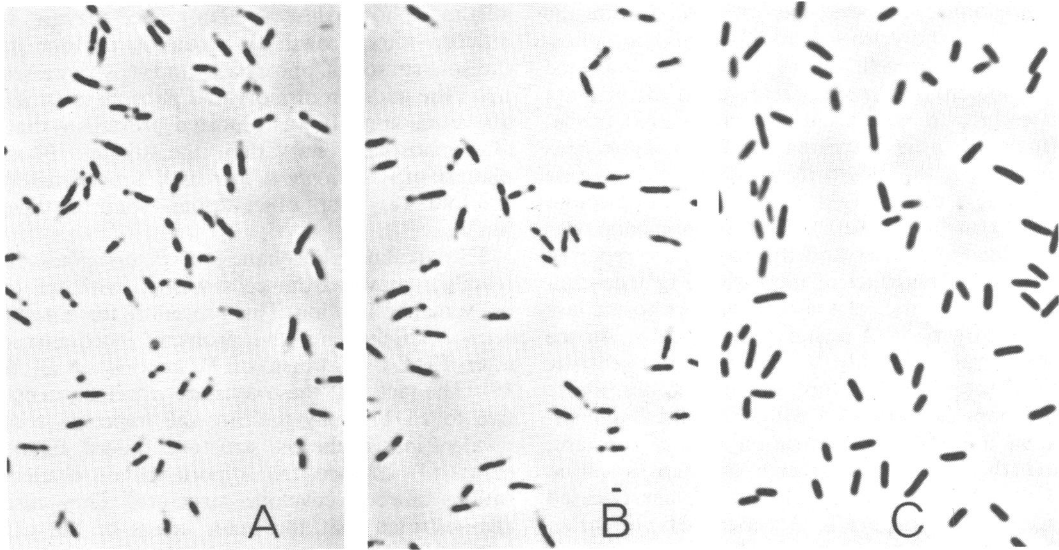

FIG. 2. *Plasmolysis effects upon cells of P. aeruginosa treated with (A) 0.2 M Mg^{2+}, (B) 20% sucrose, and (C) water.*

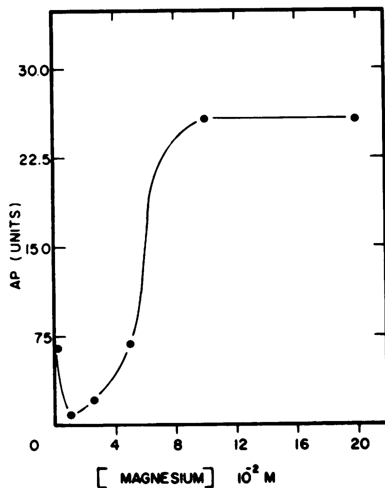

FIG. 3. *Release of alkaline phosphatase from cells of P. aeruginosa after washes in various concentrations of $MgCl_2$. A 20-ml amount (445 mg dry weight) of 12-hr cells was centrifuged, suspended into 20 ml of the various $MgCl_2$ solutions for 15 min, and recentrifuged. The $MgCl_2$ supernatants were then assayed for alkaline phosphatase. The units were calculated per 500 ml of culture.*

with 0.1 M $CaCl_2$. Increasing the concentration of Mg^{2+} beyond this level results in a proportionate increase in the amount of enzyme stripped from the cells. The maximum release of alkaline phosphatase occurs at a concentration of 0.01 M at *p*H 8.4.

Although it was shown previously (Table 1) that Mg^{2+} washing affects neither the cell viability nor the growth rate of *P. aeruginosa*, it was necessary to establish the fact that cell lysis or removal of internal enzymes (16) did not occur. The results of Table 2 show definitively that cytoplasmic or cytoplasmic-membrane marker enzymes (glucose-6-phosphate dehydrogenase and NADH oxidase, respectively) are not released after Mg^{2+}, Tris buffer, or water washes or during growth, and that the removal of alkaline phosphatase is enhanced by Mg^{2+}.

TABLE 2. *Release patterns of internal and external enzymes from Pseudomonas aeruginosa*

Treatment[a]	Enzyme released[b]		
	G-6-P dehydrogenase	NADH oxidase	Alkaline phosphatase
	units/25 g dry wt	*units/25 g dry wt*	*units/25 g dry wt*
0.2 M Mg^{2+}, *p*H 8.4	0	0	85.0
0.01 M Tris, *p*H 8.4	0	0	35.6
Water	0	0	16.6
Cell-free extract	138.5	14.5	81.8
Culture filtrate	0	0	68.8

[a] A 20-ml amount (520 mg dry weight) of 14-hr cells was centrifuged, suspended into 20 ml of each treatment solution for 15 min at room temperature, and recentrifuged. The resulting washes were assayed for each enzyme as described.

[b] Results are expressed as units per gram of dry weight.

Preliminary experiments concerned with the induction and release patterns of alkaline phosphatase from cells of *P. aeruginosa* indicated that the enzyme was not released to the medium after growth of the culture on minimal media. However, after repeated transfers upon this medium, it was noted that alkaline phosphatase was, in fact, released to the culture medium after growth (Table 2). This phenomenon was investigated further and the results are reported in Fig. 4. The effect of increasing Mg^{2+} concentration upon the release of alkaline phosphatase from growing cells is shown in Fig. 4A. As the concentration of Mg^{2+} is increased, essentially all of the enzyme is found in the culture filtrate and none is associated with the cells. Similarly, when the Mg^{2+} concentration is kept constant, and the *p*H of the growth medium is varied (Fig. 4B), the amount of phosphatase released increases as the *p*H is increased. At *p*H values tested below 6.8, essentially all of the enzyme is associated with the cells. In all cases cited above, the total amount of enzyme obtained in both the culture filtrate and the cells is approximately 144 units/25 g (dry weight).

DISCUSSION

The studies reported in the present communication show that *P. aeruginosa* contains an inducible alkaline phosphatase system. The enzyme is induced after growth on proteose peptone as the sole source of phosphate, and it is repressed upon the addition of inorganic phosphate to the above medium. It was reported previously that, after phosphate starvation, the alkaline phosphatase of *P. aeruginosa* increased approximately 500-fold (8); our observations confirm these findings.

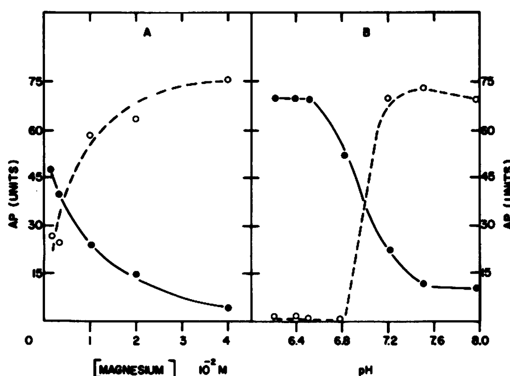

FIG. 4. *Release patterns of alkaline phosphatase from growing cells of P. aeruginosa. Cultures were grown for 14 hr under conditions of (A) varying concentrations of $MgCl_2$ and (B) varying pH. The pH of the medium was measured after autoclaving. Twenty-milliliter samples (520 mg dry weight) from a culture of standardized optical density were removed, centrifuged, and suspended into 20 ml of 0.2 M $MgCl_2$ to determine total cellular phosphatase (●). Samples of the culture filtrate (○) were examined directly for alkaline phosphatase activity. The concentration of magnesium in A is that amount added to the inorganic phosphate-deficient medium in addition to the 1.35 × 10^{-4} M magnesium which is supplied in the proteose peptone.*

The alkaline phosphatase of *P. aeruginosa* is readily removed from cells washed with 0.1 or 0.2 M magnesium ion. This procedure for enzyme release circumvents the problems encountered after EDTA treatment of *P. aeruginosa* (3, 6, 10). The fact that these cells are extremely sensitive to EDTA may indicate the importance of divalent ions in the cell structure. Indeed, Eagon et al. (4) stressed the importance of divalent cations in cell envelope structure. They also demonstrated that the outer layers of the cell wall of *P. aeruginosa* contain significantly high concentrations of both Mg^{2+} and Ca^{2+} and that EDTA causes the solubilization and release of a large proportion of the cellular lipopolysaccharide. The present finding, therefore, that alkaline phosphatase is released by washing in high Mg^{2+}, strongly suggests that the *P. aeruginosa* enzyme is bound by electrostatic forces which probably involve linkages, mediated by Mg^{2+}, between anionic centers in the enzyme and components of the cell external to the cytoplasm. This latter conclusion is strengthened by the observation that cytoplasmic or cytoplasmic membrane enzymes are not released by this treatment. Similarly, although the cells are plasmolyzed after Mg^{2+} or sucrose washes, it is evident that plasmolysis alone is unable to account for total enzyme release. It is suggested, therefore, that the reaction of high Mg^{2+} proceeds by way of competition with the anionic centers, resulting in the removal of the enzyme.

The fact that a certain amount of enzyme is released by other treatments, i.e., Tris, water, sucrose, or monovalent ions, may be due to the ability of the cell to secrete enzyme to the external medium during growth (Fig. 4). This secretion could result from bond weakening due to changes in *p*H or Mg^{2+} concentration. This conclusion is substantiated by the observation that 0.01 M Mg^{2+} wash results in less enzyme release than the above treatments. It seems reasonable that under this condition the number of electrostatic bonds is increased, which would thence yield stronger enzyme associations.

It is evident from the results presented here that conditions necessary for the removal of alkaline phosphatase exert little or no effect upon the cell, since neither viability nor growth rates are

affected by this treatment. The present study indicates, therefore, that such treatments will be of great value in elucidating the localization of the exocytoplasmic alkaline phosphatase of *P. aeruginosa*, and that any effects upon metal-lipoprotein-lipopolysaccharide complexes which are exerted by EDTA may be kept to a minimum. Such studies on the localization of *P. aeruginosa* alkaline phosphatase are currently under investigation.

ACKNOWLEDGMENT

The generous support of the National Research Council of Canada is gratefully acknowledged.

LITERATURE CITED

1. Brockman, R. W., and L. A. Heppel. 1968. On the localization of alkaline phosphatase and cyclic phosphodiesterase in *Escherichia coli*. Biochemistry 7:2554–2562.
2. Cedar, H., and J. H. Schwartz. 1968. Production of L-asparaginase II by *Escherichia coli*. J. Bacteriol. 96:2043–2048.
3. Eagon, R. G., and K. J. Karson. 1965. Lysis of cell walls and intact cells of *Pseudomonas aeruginosa* by ethylenediaminetetraacetic acid and by lysozyme. Can. J. Microbiol. 11:193–201.
4. Eagon, R. G., G. R. Simmons, and K. J. Karson. 1965. Evidence for the presence of ash and divalent metals in the cell wall of *Pseudomonas aeruginosa*. Can. J. Microbiol. 11:1041–1042.
5. Feingold, D. S., J. N. Goldman, and H. M. Kuritz. 1968. Locus of the lethal event in the serum bactericidal reaction. J. Bacteriol. 96:2127–2131.
6. Gray, G. W., and S. G. Wilkinson. 1965. The action of EDTA on *Pseudomonas aeruginosa*. J. Appl. Bacteriol. 28:153–164.
7. Heppel, L. A. 1967. Selective release of enzymes from bacteria. Science 156:1451–1455.
8. Hou, C. I., A. F. Gronlund, and J. J. R. Campbell. 1966. Influence of phosphate starvation on cultures of *Pseudomonas aeruginosa*. J. Bacteriol. 92:851–855.
9. Leive, L. 1965. Actinomycin sensitivity in *Escherichia coli* produced by EDTA. Biochem. Biophys. Res. Commun. 18:13–17.
10. Leive, L. 1968. Studies on the permeability change produced in coliform bacteria by ethylenediaminetetraacetate. J. Biol. Chem. 243:2373–2380.
11. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin reagent. J. Biol. Chem. 193:265–275.
12. Malamy, H. M., and B. L. Horecker, 1964. Release of alkaline phosphatase from cells of *E. coli* upon lysozyme spheroplast formation. Biochemistry 3:1889–1893.
13. Mallette, M. F., C. I. Cowan, and J. J. R. Campbell. 1964. Growth and survival of *Escherichia coli* in medium limited in phosphate. J. Bacteriol. 87:779–785.
14. Mangariotti, G., D. Apirion, and D. Schlessinger. 1966. Selection of sucrose dependent *Escherichia coli* to obtain envelop mutants and fragile cultures. Science 153:892–896.
15. Neu, H. C., and J. Chou. 1967. Release of surface enzymes in Enterobacteriaceae by osmotic shock. J. Bacteriol. 94:1934–1945.
16. Neu, H. C., and L. A. Heppel. 1965. The release of enzymes from *Escherichia coli* by osmotic shock and during the formation of spheroplasts. J. Biol. Chem. 240:3685–3692.
17. Rogers, D. 1968. Osmotic pools in *Escherichia coli*. Science 159:531–532.
18. Schlesinger, M. J., and R. Olsen. 1968. Expression and localization of *Escherichia coli* alkaline phosphatase synthetized in *Salmonella typhimurium* cytoplasm. J. Bacteriol. 96:1601–1605.
19. Voelz, H., and R. O. Ortigoza. 1968. Cytochemistry of phosphatase in *Myxococcus xanthus*. J. Bacteriol. 96:1357–1365.
20. Vogel, H. J., and D. M. Bonner. 1957. Acetylornithinase of *Escherichia coli:* partial purification and some properties. J. Biol. Chem. 218:97–106.
21. Warburg, O., and W. Christian. 1942. Isolierung und Krystallization des Gerungsferments. Enolase. Biochem. Z. 310:421–483.
22. Ward, J. B., and L. Glaser. 1968. An *E. coli* mutant with cryptic UDP-sugar hydrolase and altered metabolite regulation. Biochem. Biophys. Res. Commun. 31:671–677.
23. Wilkinson, S. G. 1967. The sensitivity of pseudomonads to ethylenediaminetetra-acetic acid. J. Gen. Microbiol. 47:67–76.