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Secretion of Phospholipase C by *Pseudomonas aeruginosa*

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The conditions necessary for the secretion of phospholipase C (phosphatidylcholine cholinephosphohydrolase) by *Pseudomonas aeruginosa* were studied. Enzyme secretion by washed cell suspensions required a carbon source and ammonium, potassium, and calcium ions. The calcium requirement could be substituted by magnesium and strontium but not by copper, manganese, cobalt, or zinc. During growth in liquid medium, cells secreted phospholipase C during late logarithmic and early stationary phases. Secretion was repressed by the addition of inorganic phosphate but not by organic phosphates, glucose, or sodium succinate. Studies with tetracycline indicated that de novo protein synthesis was necessary for the secretion of phospholipase C and that the exoenzyme was not released from a preformed periplasmic pool. Similarly, extraction of actively secreting cells with 0.2 M $MgCl_2$ at pH 8.4 solubilized large quantities of the periplasmic enzyme alkaline phosphatase but insignificant amounts of phospholipase C. Bacteria continued to secrete enzyme for nearly 45 min after the addition of inorganic phosphate or rifampin.

The extracellular enzymes secreted by *Pseudomonas aeruginosa* have been subjected to intensive study in recent years in an attempt to define the virulence factors of this opportunistic pathogen. Several of these substances have been shown to exhibit cytotoxicity in vivo or in vitro or both including two adenosine diphosphate ribosyltransferases (11, 12, 16, 23), several proteases (10, 13, 17), and at least one phospholipase C (15, 17).

Of these substances, the role of phospholipase C in the pathogenesis of *Pseudomonas* infections is the least characterized. Liu (15) reported that the injection of a partially purified preparation of phospholipase C from *P. aeruginosa* into experimental animals resulted in liver necrosis and skin abscesses. More recently, Taguchi and Ikezawa (29, 31) found that purified phospholipase C from nonvirulent *Pseudomonas aureofaciens* hydrolyzed phospholipid components of horse and sheep erythrocyte membranes and membranes isolated from *Escherichia coli*.

The preparation of large quantities of purified phospholipase C is necessary for further characterization of this enzyme. In this report, we define the conditions which regulate the synthesis and secretion of phospholipase C by *P. aeruginosa* and describe some characteristics of the crude enzyme preparation.

MATERIALS AND METHODS

Chemicals. L-α-Phosphatidylcholine (type III-E), phosphorylcholine chloride, L-α-phosphatidic acid, alkaline phosphatase (type III, *E. coli*), phosphatidylcholine cholinephosphohydrolase (type I, *Clostridium perfringens* phospholipase C), and tetracycline were obtained from Sigma Chemical Co., St. Louis, Mo. Rifampin was purchased from Calbiochem, San Diego, Calif. All other compounds and reagents were from local distributors and were analytical grade.

Bacterium and cultural conditions. *P. aeruginosa* ATCC 15692 was grown in tryptose minimal medium (modified from Cheng et al. [4]) containing 120 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, 0.1% tryptose (Difco), 20 mM $(NH_4)_2SO_4$, 1.6 mM $CaCl_2$, 10 mM KCl, and 50 mM glucose or 24 mM sodium succinate. Cultures were incubated at 30°C for 14 to 18 h on a New Brunswick gyratory shaker. Growth was followed by measuring turbidity with a Klett-Summerson photoelectric colorimeter (no. 66 filter).

For studies of secretion by growing cells, logarithmic-growth-phase cultures (100 to 200 Klett units) were diluted with sterile medium to a turbidity of 25 Klett units (2×10^8 cells per ml) and returned to the incubator shaker. At various intervals 2-ml samples of the cell suspension were withdrawn and centrifuged for 10 min at $8,000 \times g$. The clear supernatant fluids were removed and assayed for phospholipase C activity.

For studies of secretion by nongrowing cells, late-logarithmic-growth-phase cultures (200 to 250 Klett units) were harvested by centrifugation, and the cell pellets were washed twice with 0.12 M Tris-hydrochloride buffer, pH 7.2. The final washed cell pellet was suspended to a turbidity of 600 Klett units (4×10^9 cells per ml) in secretion medium containing 120 mM Tris-hydrochloride buffer at pH 7.2, 10 mM KCl, 20 mM $(NH_4)_2SO_4$, 1.6 mM $MgSO_4$, and 6 mM glucose.

The resulting suspensions (50-ml cultures in 250-ml Erlenmyer flasks) were incubated at 30°C on a gyratory shaker. Samples were withdrawn and treated in the manner described above for growing cultures.

Enzyme assay. Egg yolk phosphatidylcholine (100 mg) was dissolved in 1 ml of absolute ethanol and added to 5 ml of 0.25 M Tris-hydrochloride buffer, pH 7.2. A homogeneous suspension was achieved by ultrasonic radiation (Biosonic III; Bronwill Scientific Co.) for 5 min at 4°C. Sodium deoxycholate was added to a concentration of 0.25% in a final volume of 5.5 ml. This mixture was prepared each day and tested with standard amounts of *C. perfringens* phospholipase C.

The clear supernatant fluids from the bacterial suspensions were assayed for phospholipase C activity by using a modified acid-soluble phosphorus method (14, 21). The reaction mixture contained 125 mM Tris-hydrochloride buffer at pH 7.2, 5 mM $CaCl_2$, 0.2% bovine serum albumin, 0.02 U of *E. coli* alkaline phosphatase, and 200 μl of phosphatidylcholine solution in a final volume of 1 ml. After 30 min at 37°C, the reaction was terminated by the addition of 1 ml of 10% trichloroacetic acid, and the resulting precipitate was removed by centrifugation. Phosphate concentration of the supernatant fluids was determined by the method of Bartlett (1). One unit of phospholipase C activity is defined as the amount of enzyme that hydrolyzes 1 nmol of substrate per min at 37°C.

Alkaline phosphatase activity was determined at room temperature by monitoring the hydrolysis of *p*-nitrophenyl phosphate (6) with a Zeiss PMQ II spectrophotometer.

Chromatography. Thin-layer chromatography was performed on Silica Gel G (Whatman) by using a solvent system of *n*-butanol–ethanol–acetic acid–water (8:2:1:3, vol/vol) (22). The positions of chromatographed substances were detected with iodine vapor.

RNA synthesis. Ribonucleic acid (RNA) synthesis was determined by measuring [5,6-^{3}H]uridine (40 Ci/mmol; New England Nuclear Corp., Boston, Mass.) incorporation into total cellular RNA. Washed cells were incubated in tryptose minimal medium containing, per ml, 0.8 μCi of labeled uridine and 2 μg of unlabeled uridine. At various time intervals, 1-ml samples were added to 2 ml of ice-cold 5% trichloroacetic acid. After 30 min, the cell suspensions were centrifuged at 10,000 × *g* for 10 min. The pellets were washed twice with 5 ml of cold 5% trichloroacetic acid and then with 5 ml of 1% acetic acid. The preparations were dried at 50°C, treated with tissue solubilizer (Scinti-Gest; Fisher Scientific Co.), and quantitatively transferred to scintillation vials. Radioactivity was determined by liquid scintillation counting in a Nuclear Chicago Mark II spectrometer.

RESULTS

Enzyme secretion during growth. *P. aeruginosa* cells growing in tryptose minimal medium supplemented with sodium succinate secreted phospholipase C during late logarithmic and early stationary growth (Fig. 1). The delay in the appearance of extracellular enzyme suggested that its synthesis was repressed by some component in the culture medium. Most

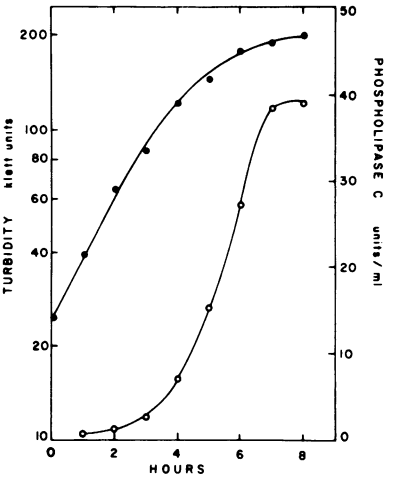

FIG. 1. *Secretion of phospholipase C by growing P. aeruginosa cells. Symbols:* ○, *extracellular enzyme;* ●, *turbidity.*

enzyme secretion systems in bacteria have been shown to be regulated by either catabolite or end product repression (7, 24). The pH of the culture medium remained constant (pH 7.2) throughout the 8-h growth period.

The products formed by the action of the unconcentrated spent culture medium on phosphatides were examined by thin-layer chromatography (Fig. 2). Phosphatidylcholine was hydrolyzed to diglyceride and phosphorylcholine. The small quantity of free choline present may have been due to the action of secreted alkaline phosphatase on phosphorylcholine.

Requirements for enzyme secretion. The nutritional conditions necessary for maximal enzyme secretion were studied by using washed late-logarithmic-phase cells suspended in medium at four to five times the original cell density (600 Klett units). These conditions permitted synthesis and release of measurable quantities of enzyme over relatively short periods of time without the necessity of cell growth. At this cell density, growth (as indicated by changes in turbidity) did not vary by more than 10% during these experiments. A high rate of secretion was obtained in a medium consisting of Tris-hydrochloride buffer (pH 7.2) containing glucose, K^+, NH_4^+, and Mg^{2+} (Fig. 3). Deletion of any of these components from the medium resulted in a pronounced reduction in the rate of secretion. The requirement for K^+ could not be substituted by Na^+ or Li^+ (data not shown).

The effects of selected divalent cations on the secretion of phospholipase C by washed suspensions of *P. aeruginosa* are summarized in Table 1. Calcium was the most effective in supporting enzyme secretion, followed by magnesium and

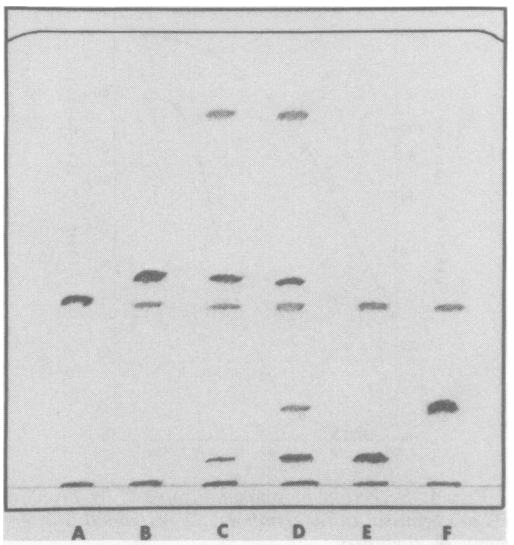

FIG. 2. *Tracing of a thin-layer chromatogram of phosphotides and reaction products of phospholipase C. Lane A, lysopalmitoylphosphatidylcholine; lane B, phosphatidylcholine; lane C, phosphatidylcholine after 24 h of incubation with C. perfringens phospholipase C; lane D, phosphatidylcholine after 24 h of incubation with P. aeruginosa extracellular products; lane E, phosphorylcholine; lane F, choline.*

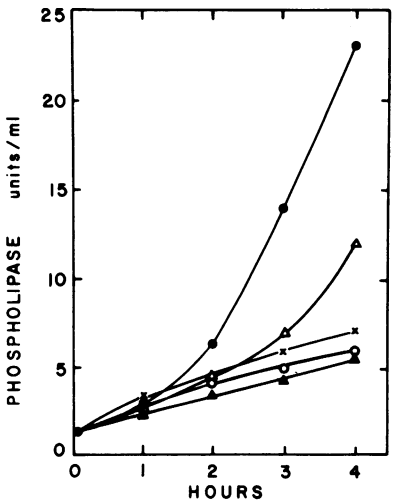

FIG. 3. *Secretion of phospholipase C by washed P. aeruginosa cells in various media. Symbols: ●, complete medium; △, Mg^{2+} omitted; ×, glucose omitted; ○, NH_4^+ omitted; ▲, K^+ omitted.*

strontium. Copper and manganese were completely ineffective, and zinc and cobalt were inhibitory.

The addition of sodium succinate as the sole carbon and energy source resulted in maximal extracellular enzyme production (Table 2). Glucose supported a moderately high rate of enzyme secretion, whereas Casamino Acids were ineffective. A combination of Casamino Acids and glucose supported the same level of secretion by *P. aeruginosa* as did medium containing only glucose. Moreover, the concentration of carbon sources did not affect the initial rates of secretion since no lag period was observed when the initial quantity of carbon source was increased. Instead, high concentrations of carbon source re-

TABLE 1. *Effect of selected cations on enzyme secretion*[a]

Addition	Relative rate of enzyme secretion
Ca^{2+}	100
Mg^{2+}	84
Sr^{2+}	84
Mn^{2+}	39
Cu^{2+}	37
Zn^{2+}	25
Co^{2+}	13
None	37

[a] Bacteria were suspended in secretion medium containing various divalent cations at final concentrations of 1 mM. Relative rates of phospholipase C secretion were determined during the linear period between 2 and 4 h. Results are normalized to the rate of enzyme secretion observed for cells incubated in the presence of calcium.

TABLE 2. *Effect of carbon source on enzyme secretion*[a]

Addition(s)	Enzyme activity (U/ml)
Glucose	
2 mM	11
6 mM	16
10 mM	16
Succinate	
6 mM	11
12 mM	22
24 mM	29
Casamino Acids	
0.01%	6
0.05%	7
0.10%	7
Glucose (6 mM) + 0.1% Casamino Acids[b]	15
None	5

[a] Bacteria were suspended in secretion medium containing various carbon sources at the indicated concentrations. After 4 h at 30°C, the culture fluid was assayed for phospholipase C activity.

[b] A cell suspension containing 6 mM glucose was preincubated for 2 h before the addition of Casamino Acids. The concentration of extracellular enzyme was determined after an additional 2 h at 30°C.

sulted in more extended periods of maximal secretion.

Studies of the effect of pH on phospholipase C secretion indicated an optimum range of pH 7.0 to 8.0.

Repression of secretion. Early studies by Liu (15, 17) suggested that the presence of inorganic phosphate prevented the secretion of phospholipase C by *P. aeruginosa* growing on an agar medium. In the present study this mechanism was studied by using actively growing cultures in tryptose minimal medium. Synthesis and secretion of phospholipase C were repressed during the early logarithmic phase of growth (Fig. 1), when the concentration of inorganic phosphate was greatest (0.15 mM). Presumably, after its depletion at midlogarithmic growth, the repression was relieved and the synthesis and secretion of phospholipase C were initiated. The nutritive requirement for phosphate at this time was satisfied by the hydrolysis of organic phosphates present in tryptose (Difco) by the periplasmic enzyme alkaline phosphatase (4).

If phospholipase C synthesis and secretion are regulated by the presence of inorganic phosphates, as suggested by these observations, then it should be possible to prevent or interrupt the production of phospholipase C by the addition of this substance to cell suspensions. Figure 4 shows that the addition of inorganic phosphate before the onset of secretion delayed the appearance of extracellular phospholipase C for 6 h, whereas its addition during the period of active secretion stopped enzyme production within 60 min. Tryptose addition, at a concentration 10-fold higher than that present at zero time, did not affect actively secreting cells. The bacterial growth rate was not affected appreciably by the addition of either substance.

Effects of tetracycline and rifampin on phospholipase C secretion. The lag period between the addition of inorganic phosphate and the cessation of phospholipase C secretion may have been due to the release of a preformed enzyme pool localized in the periplasmic space. Accumulation of a cell-bound enzyme has been shown to occur in enzyme secretion by other microorganisms (7, 24, 25). The release of these enzyme pools occurred in the absence of further protein synthesis, since the addition of protein synthesis inhibitors, such as chloramphenicol, did not immediately stop secretion. A similar experiment was conducted with phospholipase C-secreting *P. aeruginosa* cells (Fig. 5). Enzyme secretion was immediately inhibited by tetracycline, regardless of the time of addition, suggesting that de novo protein synthesis was required for continued enzyme secretion and that accumulation of a preformed phospholipase C pool was unlikely.

Periplasmically localized proteins, such as alkaline phosphatase and binding proteins, have been solubilized from *P. aeruginosa* by extraction with 0.2 M $MgCl_2$ at pH 8.4 (4, 27). This procedure solubilizes lipopolysaccharide and periplasmic components without cell lysis or loss of cell viability. Extraction of secreting *P. aeruginosa* by this procedure released considerable alkaline phosphatase activity but no de-

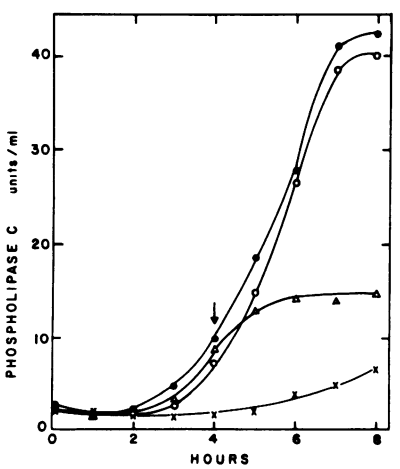

FIG. 4. *Effect of inorganic phosphate and tryptose on phospholipase C secretion by growing cells. Symbols (additions): ○, none; ×, 10 mM phosphate at zero time; △, 10 mM phosphate at 4 h; ●, 1% tryptose at 4 h.*

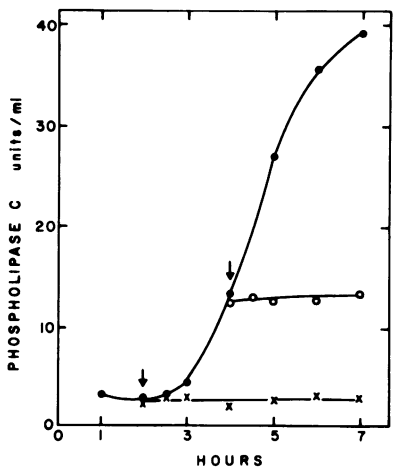

FIG. 5. *Effect of tetracycline on phospholipase C secretion by growing cells. Tetracycline was added at various times to give a final concentration of 10 μg/ml. Symbols (tetracycline additions): ●, none; ×, at 2 h; ○, at 4 h.*

tectable phospholipase C activity, suggesting that the latter enzyme does not accumulate in the periplasmic space.

The continued secretion of extracellular enzymes by bacteria after adjustment from nonrepressive to repressive conditions has also been observed by other investigators. Both et al. (3) and Glenn et al. (8) found that *Bacillus amyloliquefaciens* could synthesize and secrete protease for over 60 min in the presence of repressive concentrations of amino acids and in the presence of the RNA synthesis inhibitors rifampin and actinomycin D. Evidence was presented which indicated that protease synthesis during this interval was due to a large pool of preformed messenger RNA (mRNA). They proposed that the exoenzyme mRNA molecules migrate from the gene to translational sites on the membrane and that the pool consisted of mRNA in transit. If hyperproduction of mRNA is a consequence of the mechanism of enzyme secretion in bacteria, then *P. aeruginosa* should also exhibit a rifampin-insensitive phase of phospholipase C production. Such a mechanism could also explain the phosphate-insensitive phase of secretion.

Addition of rifampin to actively secreting cells did not inhibit enzyme secretion for approximately 45 min, whereas the incorporation of [^{3}H]uridine into trichloroacetic acid-precipitable material was immediately inhibited (Fig. 6). Synthesis and secretion of phospholipase C appear to have taken place in the absence of further mRNA synthesis.

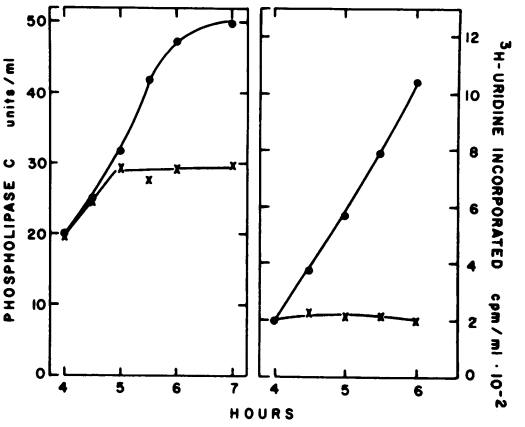

FIG. 6. *Effect of rifampin on phospholipase C secretion and RNA synthesis by growing cells. At 4 h [^{3}H]uridine was added and the culture was divided into two equal suspensions, one of which received 30 μg of rifampin per ml. Symbols: ●, no addition; ×, rifampin added at 4 h.*

DISCUSSION

Extracellular phospholipase C was secreted by *P. aeruginosa* cells during logarithmic and stationary phases of growth in tryptose minimal medium. The production of exoenzymes at the end of exponential growth is typical of most enzyme secretion systems in bacteria (7, 24). This is often due to catabolite repression where high concentrations of a readily metabolizable carbon source early in the growth cycle prevent the expression of genes responsible for synthesis of exoenzymes. The secretion of phospholipase C, however, appears to be regulated by end product repression rather than by catabolite repression, since the addition of inorganic phosphate to actively secreting cells interrupted the synthesis of enzyme and the addition of glucose and succinic acid did not. This also suggests that phospholipase C may be a component of a phosphate-scavenging mechanism in *P. aeruginosa*. Under cultural conditions where inorganic phosphate has been depleted, the phospholipase C genes become derepressed and synthesis and secretion are initiated. The nutrient requirement for phosphate is subsequently satisfied by the release of phosphomonoesters from phosphatides by the action of phospholipase C, followed by the liberation of free phosphate by the periplasmic enzyme alkaline phosphatase (4).

End product repression has also been shown to regulate the production of a number of other exoenzymes in bacteria. Amino acids repress the synthesis of extracellular proteases in members of the genera *Bacillus*, *Sarcina*, and *Arthrobacter* (7, 24). Inorganic phosphate has also been reported to repress the synthesis of extracellular ribonuclease of *Bacillus subtilis* (19) and alkaline phosphatase of *Micrococcus sodonensis* (9) and *P. aeruginosa* (4).

Divalent cations (Ca^{2+} or Mg^{2+}) were required for phospholipase secretion by washed cell suspensions and growing cultures of *P. aeruginosa*. This observation was not unexpected since these cations are essential for growth and have been shown to be important in maintaining the structure and function of the cytoplasmic and outer membranes of this bacterium (4, 5, 18). Magnesium ion has also been shown to be necessary for enzyme secretion by *Pseudomonas lemoignei* (28).

The effect of cations on the enzymatic activity of phospholipase C is more difficult to evaluate. Conventionally, this relationship is studied by first removing cations from an enzyme preparation with ethylenediaminetetraacetic acid and then attempting to restore activity by the readdition of selected cations (26, 30). In preliminary experiments in our laboratory, extended dialysis

of phospholipase C against 10^{-3} M ethylenediaminetetraacetic acid did not completely inactivate the enzyme (65%) and the activity could only be partially restored by the addition of cations such as Mn^{2+} (90%), Ca^{2+} (78%), and Mg^{2+} (70%). Other cations tested, including Zn^{2+} and Cu^{2+}, were either ineffective or slightly inhibitory. Studies of this type, however, must be interpreted with caution since it is not clear whether the cation is important in maintaining the proper configuration of the enzyme or of the substrate. Taguchi and Ikezawa (30) found that the effects of divalent cations on the activity of phospholipase C of *Clostridium novyi* type A were directly related to the physical state of the phospholipid micelles used as substrates. The cation requirement varied with the substrate being studied. These workers also found that Zn^{2+} could reactivate ethylenediaminetetraacetic acid-treated phospholipase C but that excess concentrations modified substrate micelles in a way unfavorable for enzymatic attack. From these observations, it appears that atomic absorption analysis of purified extracellular enzyme is the most reliable means of determining the presence of metal ions in this protein.

Several investigators (7, 24) have proposed that syntheses of exoenzymes occur on membrane-bound ribosomes in bacteria. At these specific membrane sites, protein synthesis is accompanied by simultaneous extrusion of the growing peptide chain through the cytoplasmic membrane. The close coupling of synthesis and secretion is reflected in the ability of translation inhibitors to simultaneously interrupt both processes in many bacteria (7, 24). Our studies of tetracycline inhibition of phospholipase C secretion indicate that this mechanism may also function in *P. aeruginosa*. Moreover, these data indicated that the enzyme diffuses rapidly through the peptidoglycan and outer membrane structures to the surrounding milieu, since an accumulated periplasmic pool could not be detected by secretion studies or by direct extraction procedures. Extraction of actively secreting cells with 0.2 M $MgCl_2$ released large quantities of the periplasmic enzyme alkaline phosphatase. This procedure has been shown to dissociate lipopolysaccharides from the outer membrane of *P. aeruginosa* and to solubilize periplasmic proteins without loss of cell viability (4, 27).

The inability of inorganic phosphate and the transcription inhibitor rifampin to promptly interrupt phospholipase C secretion is also consistent with the membrane translation-secretion model. Numerous studies (2, 3, 7, 8, 24, 28) with a variety of bacteria have shown that mRNA accumulates during the period of active secretion and that this pool can support exoenzyme synthesis for extended periods under repressive conditions or in the presence of transcription-inhibiting drugs. This accumulation of mRNA occurs as a result of an imbalance in the rates of transcription and mRNA degradation. The physiological role of the mRNA pool is obscure since it is not observed in all cases of enzyme secretion (7, 20) and has been shown to undergo drastic quantitative fluctuations in some bacteria without affecting the rate of exoenzyme synthesis and secretion (20).

LITERATURE CITED

1. **Bartlett, G. R.** 1958. Phosphorus assay in column chromatography. J. Biol. Chem. **234**:466–468.
2. **Boethling, R. S.** 1975. Regulation of extracellular protease secretion in *Pseudomonas maltophilia*. J. Bacteriol. **123**:954–961.
3. **Both, G. W., J. L. McInnes, J. E. Hanlon, B. K. May, and W. H. Elliott.** 1972. Evidence for an accumulation of messenger RNA specific for extracellular protease and its relevance to the mechanism of enzyme secretion in bacteria. J. Mol. Biol. **67**:199–207.
4. **Cheng, K.-J., J. M. Ingram, and J. W. Costerton.** 1970. Release of alkaline phosphatase from cells of *Pseudomonas aeruginosa* by manipulation of cation concentration and of pH. J. Bacteriol. **104**:748–753.
5. **Gilleland, H. E., Jr., J. D. Stinnett, and R. G. Eagon.** 1974. Ultrastructural and chemical alteration of the cell envelope of *Pseudomonas aeruginosa*, associated with resistance to ethylenediaminetetraacetate resulting from growth in Mg^{2+}-deficient medium. J. Bacteriol. **117**:302–311.
6. **Garen, A., and C. Levinthal.** 1960. A fine-structure genetic and chemical study of the enzyme alkaline phosphatase of *E. coli*. I. Purification and characterization of alkaline phosphatase. Biochim. Biophys. Acta **38**:470–483.
7. **Glenn, A. R.** 1976. Production of extracellular proteins by bacteria. Annu. Rev. Microbiol. **30**:41–62.
8. **Glenn, A. R., G. W. Both, J. L. McInnes, B. K. May, and W. H. Elliott.** 1973. Dynamic state of the messenger RNA pool specific for extracellular protease in *Bacillus amyloliquefaciens*: its relevance to the mechanism of enzyme secretion. J. Mol. Biol. **73**:221–230.
9. **Glew, R. H., and E. C. Heath.** 1971. Studies on the extracellular alkaline phosphatase of *Micrococcus sodonensis*. J. Biol. Chem. **246**:1566–1574.
10. **Heckly, R. J.** 1971. Toxins of *Pseudomonas*, p. 473–491. *In* S. Ajl (ed.), Microbial toxins, vol. 3. Academic Press Inc., New York.
11. **Iglewski, B. H., and D. Kabat.** 1975. NAD-dependent inhibition of protein synthesis by *Pseudomonas aeruginosa* toxin. Proc. Natl. Acad. Sci. U.S.A. **72**:2284–2288.
12. **Iglewski, B. H., J. Sadoff, M. J. Bjorn, and E. S. Maxwell.** 1978. *Pseudomonas aeruginosa* exoenzyme S: an adenosine diphosphate ribosyltransferase distinct from toxin A. Proc. Natl. Acad. Sci. U.S.A. **75**:3211–3215.
13. **Kreger, A. S., and L. D. Gray.** 1978. Purification of *Pseudomonas aeruginosa* proteases and microscopic characterization of pseudomonal protease-induced rabbit corneal damage. Infect. Immun. **19**:630–648.
14. **Kurioka, S., and P. V. Liu.** 1967. Improved assay method for phospholipase C. Appl. Microbiol. **15**:551–555.
15. **Liu, P. V.** 1966. The roles of various fractions of *Pseudomonas aeruginosa* in its pathogenesis. II. Effects of lecithinase and protease. J. Infect. Dis. **116**:112–116.

16. **Liu, P. V.** 1966. The roles of various fractions of *Pseudomonas aeruginosa* in its pathogenesis. III. Identity of the lethal toxins produced *in vitro* and *in vivo*. J. Infect. Dis. **116**:481-489.
17. **Liu, P. V.** 1974. Extracellular toxins of *Pseudomonas aeruginosa*. J. Infect. Dis. **130**(Suppl.):594-599.
18. **Matsushita, K., O. Adachi, E. Shinagawa, and M. Ameyama.** 1978. Isolation and characterization of outer and inner membranes from *Pseudomonas aeruginosa* and effect of EDTA on the membranes. J. Biochem. **83**:171-181.
19. **May, B. K., R. L. Walsh, W. H. Elliott, and J. R. Smeaton.** 1968. Mechanism of the paradoxical stimulation of ribonuclase synthesis in *Bacillus subtilis* by actinomycin D. Biochim. Acta **169**:260-262.
20. **O'Connor, R., W. H. Elliott, and B. K. May.** 1978. Modulation of an apparent mRNA pool for extracellular protease in *Bacillus amyloliquefaciens*. J. Bacteriol. **136**:24-34.
21. **Ohsaka, A., and T. Sugahara.** 1968. Quantitative determination of *Clostridium welchii* phospholipase C activity in aqueous reaction medium. J. Biochem. **64**:335-345.
22. **Okawa, J., and T. Yamaguchi.** 1975. Studies on phospholipases from *Streptomyces*. III. Purification and properties of *Streptomyces hachijoensis* phospholipase C. J. Biochem. **78**:537-545.
23. **Pavlovskis, O. R., and A. H. Shackelford.** 1974. *Pseudomonas aeruginosa* exotoxin in mice: localization and effect on protein synthesis. Infect. Immun. **9**:540-546.
24. **Priest, F. G.** 1977. Extracellular enzyme synthesis in the genus *Bacillus*. Bacteriol. Rev. **41**:711-753.
25. **Shatzman, A. R., and A. J. Kosman.** 1977. Regulation of galactose oxidase synthesis and secretion in *Dactylium dendroides*: effects of pH and culture density. J. Bacteriol. **130**:455-463.
26. **Sonoki, S., and H. Ikezawa.** 1976. Studies on phospholipase C from *Pseudomonas aureofaciens*. II. Further studies on the properties of the enzyme. J. Biochem. **80**:361-366.
27. **Stinson, M. W., M. A. Cohen, and J. M. Merrick.** 1976. Isolation of dicarboxylic acid- and glucose-binding proteins from *Pseudomonas aeruginosa*. J. Bacteriol. **128**: 573-579.
28. **Stinson, M. W., and J. M. Merrick.** 1974. Extracellular enzyme secretion by *Pseudomonas lemoignei*. J. Bacteriol. **119**:152-161.
29. **Taguchi, R., and H. Ikezawa.** 1976. Studies on the hemolytic and hydrolytic actions of phospholipases against mammalian erythrocyte membranes. Arch. Biochem. Biophys. **173**:538-545.
30. **Taguchi, R., and H. Ikezawa.** 1977. Phospholipase C from *Clostridium novyi* type A. II. Factors influencing the enzyme activity. J. Biochem. **82**:1217-1223.
31. **Taguchi, R., and H. Ikezawa.** 1977. Hydrolytic action of phospholipases on bacterial membranes. J. Biochem. **82**:1225-1230.