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Lysis of *Trypanosoma cruzi* by *Pseudomonas fluorescens*

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Trypomastigotes of *Trypanosoma cruzi* isolated from the blood of infected mice were lysed within 24 h by an extracellular substance produced by *Pseudomonas fluorescens*. Isolation of the anti-trypanosomal factor (ATF) was accomplished by growth of the organisms in a defined medium, extracellular secretion by the sedimented cells, sterilization by filtration, lyophilization, dialysis, and gel filtration. Chromatographic separation with Sephadex G-25 and G-200 disclosed the occurrence of three active fractions. ATF-I(1) exhibited a molecular weight higher than 440,000. ATF-II and ATF-III were considerably smaller (molecular weights approximately 1,355 and 1,060, respectively). The lytic substance contained protein and lipopolysaccharide, was resistant to heat and freezing, was not proteolytic or hemolytic, and was not inhibited by trypsin but was suppressed by pronase.

During studies of *Trypanosoma cruzi*, we observed that the number of trypomastigotes isolated from the blood of infected mice markedly diminished when maintained in vitro at 4°C for 3 days. Microscopic examination revealed that the parasite samples had become contaminated by a small, motile, rod-shaped microorganism that was attracted to the parasites, particularly to the posterior end. As a result of the interaction, the trypomastigotes became immobilized, and most were lysed within 24 h. A gram-negative bacterium isolated in blood agar and nutrient broth cultures was identified as *Pseudomonas fluorescens* by the Clinical Pathology Department, National Institutes of Health. We considered these observations to be significant not only in terms of cellular biology and physiology, but also because of their potential significance in the chemotherapy of Chagas' disease; further studies were therefore conducted to analyze the parasite-bacterium interaction.

MATERIALS AND METHODS

Parasites. The Tulahuen strain of *T. cruzi* is routinely maintained in this laboratory by blood passaging every 6 days in NIH-Swiss 16- to 20-g male mice; 4×10^6 to 5×10^6 trypomastigotes are usually inoculated intraperitoneally (14). The infection becomes acute after 6 days, producing marked pathology of liver and spleen, and blood counts of 10^8 parasites per ml are often obtained. The animals are not immunosuppressed. Blood samples are obtained under ether anesthesia directly from the heart after exposure of the cardiac surface. Parasites were isolated from blood by a two-step procedure (15) involving separation of the erythrocytes with Lymphoprep (9.6% sodium-*N*-methyl-3,5-diacetamido-2,4,6-triiodobenzoate and 5.5% Ficoll; specific gravity, 1.077; Nyegaard and Co., Oslo, Norway) and elution through a DEAE-cellulose column to remove lymphocytes and platelets. The eluted flagellates exhibited the usual lanceolate shape (6), were actively motile and elicited typical infections. Two myotropic strains of *T. cruzi*, the House 510 and the House 11, of Costa Rican and Nicaraguan origin, respectively, trypomastigotes of *T. rhodesiense* 1886, *T. equiperdum*, and promastigotes of *Leishmania brasiliensis* were used also.

Bacteria. *P. fluorescens* was maintained in nutrient agar slants (Difco Laboratories, Detroit, Mich.) at 4°C. *Escherichia coli* B63X152 and *Bacillus subtilis* 64X395 were obtained from the Laboratory of Streptococcal Diseases, National Institute of Allergy and Infectious Diseases; and *Pseudomonas aeruginosa*, isolated from a patient, was furnished by the Clinical Pathology Department, National Institutes of Health. These species were cultured as described below for *P. fluorescens* but were incubated at 37°C instead of 26°C since they grow best at this temperature.

Preparation of the anti-trypanosome factor. The cultural conditions for preparation of the anti-trypanosome factor (ATF) were essentially those described by Stinson and Hayden (24). Each of eight agar slants was inoculated with a loopful from the stock culture and incubated for 24 h at 26°C. Each of eight flasks containing 100 ml of a defined medium (120 mM Tris-chloride [pH 7.2]–10 mM KCl–20 mM $(NH_4)_2SO_4$–1.6 mM $CaCl_2$–0.1% tryptose [Difco]–50 mM glucose) was inoculated with a 2-ml nutrient broth wash of the agar slants (optical density at 540 nm adjusted to 100 Klett units) and incubated at 26°C for 16 h with constant shaking. The cultures were centrifuged at $2,000 \times g$ for 10 min, and the sedimented cells were washed three times in a medium containing 120 mM Tris-chloride (pH 7.2), 20 mM $(NH_4)_2SO_4$, 10 mM KCl, 1.6 mM $MgSO_4$, and 6 mM glucose. They were resuspended in 200 ml of the same medium and incubated for 4.5 h at 26°C with constant shaking. The suspension was cen-

trifuged as described above, and the supernatant fluid was filtered through a Millipore filter (0.45 μm), lyophilized, and dialyzed against distilled water (three 500-ml volumes) through Spectrapor tubing of 6,000 to 8,000 molecular-weight cutoff. The dialyzed fraction was lyophilized, reconstituted in 50 mM NH_4HCO_3 (pH 8.0), and chromatographed in a Sephadex G-200 column equilibrated with the same buffer. The dialysates were lyophilized and dialyzed as described above, except that tubing of 1,000 molecular-weight cutoff was used and the recovered fractions were eluted through a Sephadex G-25 column. The dimensions of the columns were 86 by 1.5 cm, and elutions were performed at 12 ml/h. An alternative procedure was to elute the crude samples without first desalting them. Although a higher yield of the fractions was obtained this way, the method led to shrinking of the columns; therefore, to avoid this complication when using this procedure, we did not elute more than 5 ml of the crude sample, and we washed the columns after each elution with 160-ml volumes of buffer. Sterilized water was used throughout, and gentamicin (Schering Corp., Kenilworth, N.J.) was added to all buffers at a concentration of 50 μg/ml. All filtrates were checked for sterility by incubation in blood agar and nutrient broth. Protein was determined by the procedure of Lowry et al. (10).

Trypanosome-bacterium interaction. Two methods were used for determination of trypanosome-bacterium interaction: (i) microscopic examination and (ii) readings of turbidity changes (Klett units at 540 nm). For the microscopic examination, 0.15 or 0.30 ml of a buffer suspension containing at least 2.5×10^7 parasites per ml was mixed with 0.30 ml of a 1% suspension of *Pseudomonas* cells (approximately 3.6×10^6 cells from a 24-h culture) or 0.30 ml of the ATF equivalent to 1 mg of protein per ml. They were examined immediately after mixing, after 10 min, and after 12 and 24 h. The mixtures were maintained at 4°C. Our observations were based on the examination of the trypanosome-bacterium mixture for 1 min because of the possibility that drying of the covered preparations would interfere with an accurate appraisal of the parasites. To check for the lytic effect spectrophotometrically, turbidity readings were made every 5 min after the addition of ATF to the parasite-buffer suspension. Phosphate-buffered saline (pH 8.0), routinely used to isolate the trypanosomes from blood, was used in the assays. It consisted of 95 mM Na_2HPO_4, 5 mM NaH_2PO_4, and 73 mM NaCl.

Extracellular nature of the ATF. To assess the extracellular nature of the ATF, i.e., its production during the logarithmic period of growth (19), cultures were grown in nutrient broth; at various periods, 5-ml samples were withdrawn and their turbidity was assessed. Samples from the 0-, 3-, 6-, 8-, 12-, 24-, and 48-h intervals were diluted 10-fold with isotonic saline, and 0.1 ml was plated in nutrient agar to test for bacterial viability. In addition, 5- to 10-ml samples were centrifuged and filtered as described above, concentrated 10-fold (Minicon B-15; Amicon Corp., Lexington, Mass.), and tested against the trypanosomes. Similar growth curves were completed for *E. coli*, *B. subtilis*, and *P. aeruginosa*. ATF activity in sedimented cells was tested after washing (three times) with phosphate-buffered saline (pH 7.4) and sonication for 3 min at 4°C (on a Biosonik IV set at 50; Bronwill Scientific Inc., Rochester, N.Y.). The suspensions were centrifuged at 2,000 × *g* for 20 min, and the supernatant fluid was filtered, concentrated as described above, and tested against the flagellates.

Test for the hemolytic effect of the ATF. Samples of human, horse, rabbit, sheep, and mouse fresh blood (5 ml each) were washed repeatedly with 10 ml of phosphate-buffered saline until the supernatant fraction was completely clear. The cells were centrifuged at 2,000 × *g* for 10 min and resuspended in the same buffer to a concentration of 1%. Each blood sample was divided into two fractions of 5 ml each; to one fraction 0.1 ml of buffer was added, and to the other was added 0.1 ml of the ATF equivalent to 100 μg of protein. The tubes were incubated at 37°C for 3 h, centrifuged, and checked for hemolysis.

Proteolytic activity was examined as described by Mayerhofer et al. (11). Solutions of the active fractions equivalent to 100 μg of protein were absorbed in 12-mm filter paper disks and placed on the surface of agar plates prepared with 15% skim milk. They were observed for zones of proteolysis at 22°C for 48 h.

Properties of the ATF. To assess the activity of trypsin and pronase on the ATF, 5 μg of trypsin (Fischer Scientific Co., Fairlawn, N.J.) or 20 μg of pronase (protease from *Streptomyces griseus*; Sigma Chemical Co., St. Louis, Mo.) per 200 μg of ATF protein was used in trypanosome isolation buffer (described above). The solutions were incubated at 25°C (trypsin) and 40°C (pronase) for 1 h. At the end of incubation, the solutions were boiled for 1 min to terminate enzyme action. Buffer suspensions (0.15 ml) containing 2.5×10^7 trypanosomes and 0.30 ml of the enzyme-treated ATF were mixed and observed at 22°C after 10 min and after 1 and 3 h. Parasite suspensions exposed to untreated ATF and control suspensions of untreated parasites were observed also.

The *Limulus* amoebocyte lysate test for endotoxin (7, 20) was used to detect the presence of lipopolysaccharide (LPS). The assayed fractions contained 1 mg of ATF protein per ml.

RESULTS

Light microscopy. In the presence of *P. fluorescens*, trypomastigotes of *T. cruzi* were quickly immobilized. Usually, one bacterium approached a single flagellate and, with persistent to-and-fro movements directed to the posterior end (site of the kinetoplast and flagellar origin), stopped the movement of the flagellate; soon after, other rods appeared in other areas of the surface of the parasite. Giemsa-stained smears disclosed a rounding up of the parasite with the flagellum coiled around the cell body; as the incubation time increased, disintegration of the kinetoplast and of the nucleus occurred. The cell bodies became considerably reduced in size and assumed bizarre shapes (Fig. 1A to D). Similar results were obtained when the culture supernatant fractions were tested (Fig. 1E). These produced immobilization and cellular disintegration within 12 h or less. Cell membranes detached from the parasites were observed, and some parasites, although still intact, were no-

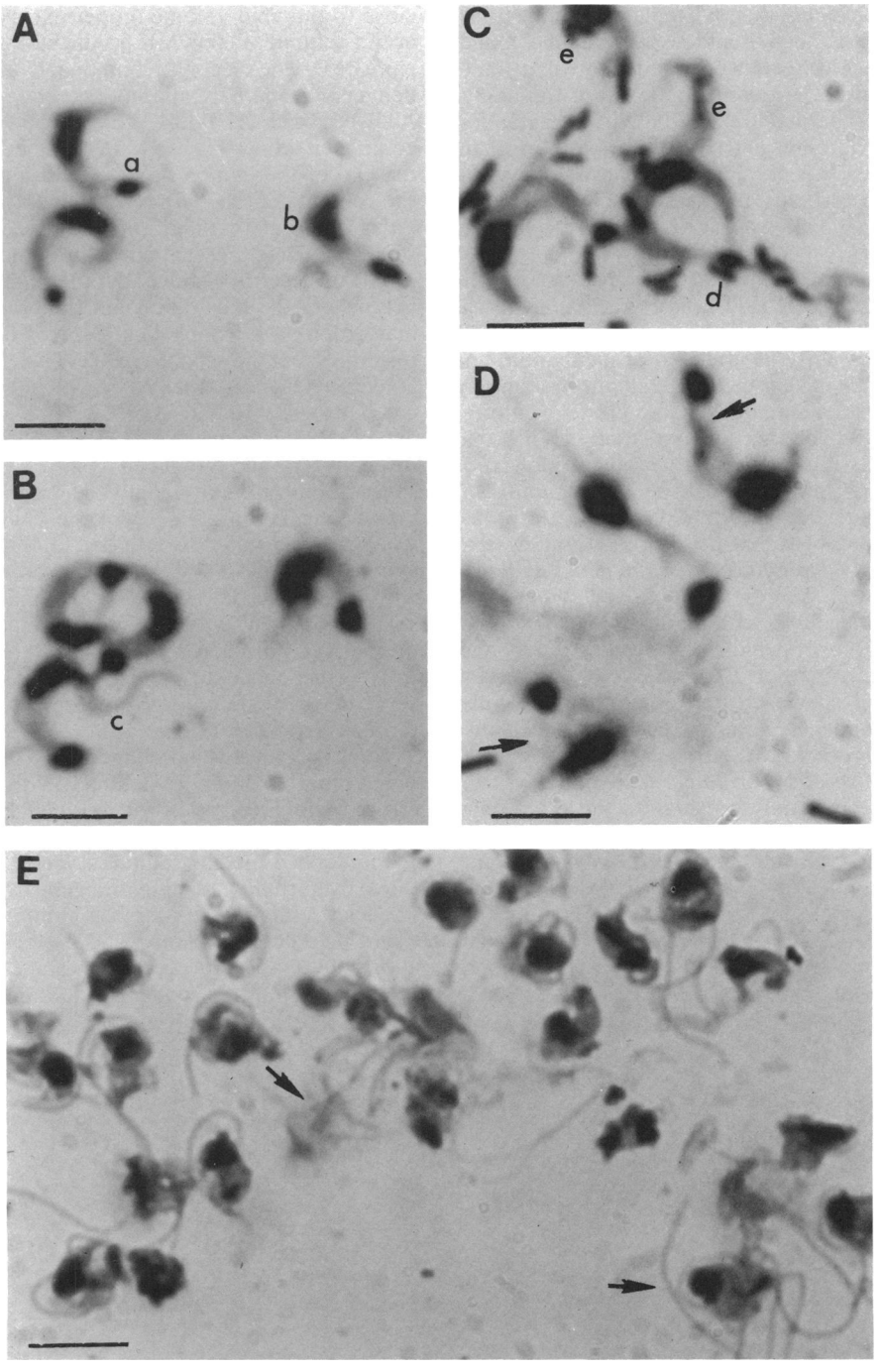

FIG. 1. Normal trypomastigotes, after incubation with *P. fluorescens*, and after treatment with ATF-II. Giemsa-stained smears. Bar = 10 μm. (A) Normal trypomastigotes immediately after column elution at 22°C and (B) after 24 h at 4°C. Kinetoplast (a), nucleus (b), and flagellum (c) are clearly visible. (C) Trypomastigotes and *P. fluorescens* after incubation for 10 min at 22°C. Note bacteria (d) close to kinetoplast and (e) on other areas of the surface of the parasite. (D) Trypomastigotes and *P. fluorescens* after incubation for 24 h at 4°C. Structural integrity damaged; organisms have lost the flagellum and appear as structures consisting of kinetoplast, nucleus, and a disintegrating plasma membrane. (E) Trypomastigotes after treatment with ATF-II (1.3 mg of protein per 5 ml of buffer against 1.5×10^8 parasites) after 4 h at 4°C. Note structural disintegration and many detached flagella (arrows).

ticeably swollen; their protoplasm became transparent with an apparent loss of organelle boundaries. These effects were produced when 100 to 300 μg of ATF protein was used in the assays and was observed in 95 to 100% of the parasites. Spectrophotometric analysis of the ATF-trypanosome suspensions disclosed a progressive decrease in turbidity as the lytic effect increased (Fig. 2). ATF-II was distinctly more lytic than ATF-I(1).

Extracellular nature of the ATF. Our results indicated that the ATF was extracellular. It was produced during the logarithmic period of growth, as early as after 8 h of incubation, and was absent from the supernatant fraction of washed sedimented cells from growing cultures which had been thoroughly disrupted with sonication. Assays performed with 300 μg of crude protein from the bacterial species *B. subtilis*, *E. coli*, and *P. aeruginosa*, obtained as described above, and with 300 μg of LPS from *E. coli*, *Salmonella typhimurium*, *Salmonella typhosa*, and *Serratia marcescens*, obtained commercially, were negative, with the notable exception of *P. aeruginosa*, which produced a lytic effect resembling that of *P. fluorescens*. All of the flagellated species tested other than the Tulahuen strain, i.e., strains House 510 and House 11 of *T. cruzi*, the African trypanosomes *T. rhodesiense* and *T. equiperdum*, and *L. brasiliensis* were equally sensitive to the ATF.

Column chromatography. Gel filtration of the ATF-I (fraction obtained with dialysis through tubing of 6,000 to 8,000 molecular-weight cutoff) with Sephadex G-200 disclosed four components (Fig. 3). AFT-I(1) exhibited a molecular weight higher than 440,000 (440K); it was difficult to assess its molecular weight because it was eluted close to the void volume (upper exclusion limit of the column of 600K). It produced lysis of the parasites. The second component, ATF-I(2), between 43 and 67K, did not lyse the parasites. ATF-I(3) and AFT-I(4), considerably smaller components than 13.7K, were obtained close to the end of the elution (lower exclusion limit of the column of 5,000). They exhibited lytic properties. Chromatography of undialyzed crude samples yielded essentially the same elution profile as described above, but the small components were the most abundant fractions. Chromatography with Sephadex G-25 of dialysates resulting from the separation of ATF-I described above yielded essentially two fractions, designated as ATF-II and AFT-III (Fig. 4). They exhibited molecular weights of 1,355 and 1,060, respectively, and produced lysis of the parasites. Gel filtration of undialyzed crude samples yielded a fraction eluted with the void volume which corresponded to ATF-I(1 to 4) described above and ATF-II and -III. As was observed with the Sephadex G-200 fractionations, the small components were the most abundant. Further analysis disclosed that ATF-I(3) and ATF-I(4) corresponded to ATF-II and -III, as observed when they were rechromatographed through Sephadex G-25.

Total protein levels obtained from the crude supernatant fractions (contained in the 200-ml volume after secretion of sedimented cells) ranged from 20 to 55 mg. Similarly, the growth of the defined medium cultures varied, as indicated by turbidity readings ranging from 54 to 123 Klett units in a total of 44 cultures. There was a marked reduction of the protein content during the dialysis procedure in some samples as high as 50%; consequently, the amounts of

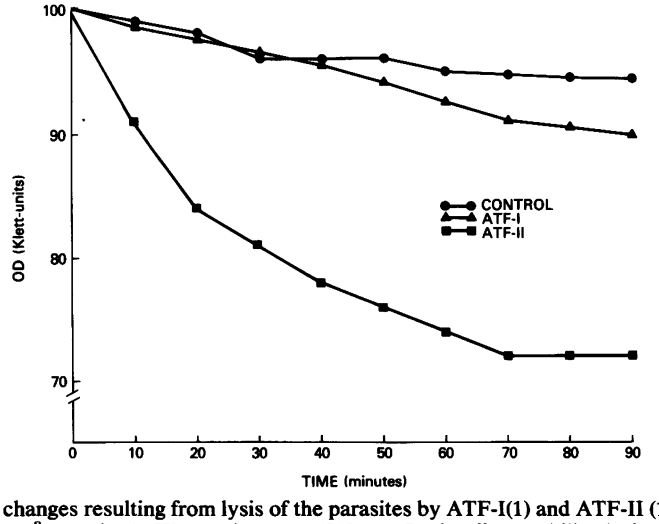

FIG. 2. Turbidity changes resulting from lysis of the parasites by ATF-I(1) and ATF-II (1.3 mg of protein per 5 ml of buffer against 10^8 parasites). Klett units are at 540 nm. Lytic effect stabilized after 70 min.

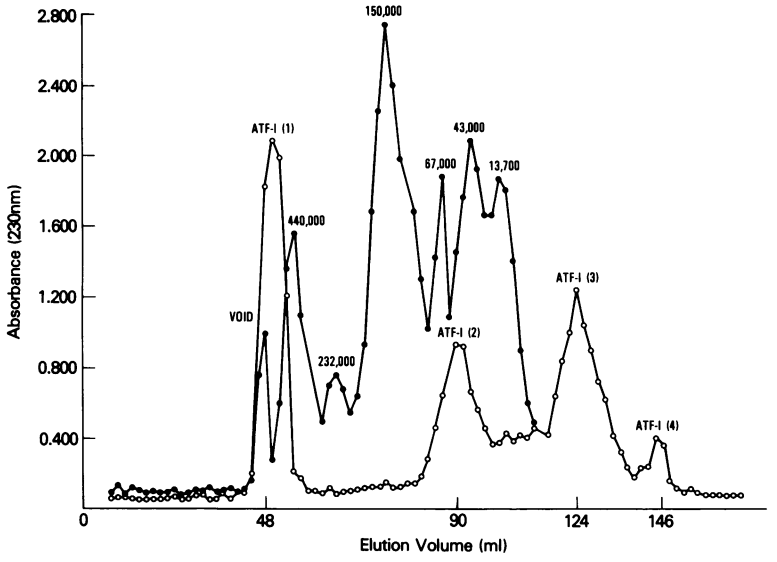

FIG. 3. Chromatography of ATF-I obtained after dialysis of crude sample in Sephadex G-200. Four fractions were recovered: ATF-I(1), (2), (3), and (4). Molecular-weight markers: ferritin (440K), catalase (232K), aldolase (150K), bovine serum albumin (67K), ovalbumin (43K), and ribonuclease (13.7K). Void volume (46 ml) was obtained with dextran 2,000.

the active fractions recovered were variable. However, when chromatography of the crude samples was performed without previous desalting, and ammonium bicarbonate, a volatile buffer, was used for elution, we obtained more uniform and considerably higher (as high as 97%) total yields of the active fractions.

Properties. ATF-I(1) lyophilized as a fine white powder. ATF-II and ATF-III were beige in color and coarser in appearance. They resembled needles of crystals. All of the fractions were resistant to heat (boiling for 5 min) and freezing (−80°C); they were not inhibited by trypsin but were suppressed by pronase. They did not produce hemolysis and were not proteolytic. ATF-I(1) contained the highest amount of LPS (1.25×10^7 ng/ml). ATF-II and -III contained considerably less, 1.25×10^3 ng/ml. The samples assayed contained 1 mg of ATF protein per ml.

DISCUSSION

P. fluorescens is a saprophytic bacterium (27) occurring in soil and commonly found as a contaminant of water, milk, and other refrigerated dairy foods (16–18, 23). It is psychrophilic and grows best at room temperature. Early in our study, we established that the original contamination occurred through the distilled water. Our adopted procedures of autoclaving the water used for dialysis and buffers and adding gentamicin to these media were excellent aids to avoiding contamination of the assayed fractions.

The production of bacterial extracellular substances, including secondary metabolites from the fluorescent pseudomonads which exhibit antibiotic activity, has been a subject of extensive study (1, 3, 4, 5, 7, 8, 13, 17, 19–21, 26). The presence of such substances in a bacterial medium, however, does not necessarily indicate that they are produced physiologically rather than as a result of cell autolysis. To establish the extracellular nature of a substance, it should be demonstrated that it is produced during the logarithmic period of growth and that substantial amounts are not found inside the cells. The assays performed in our study with culture filtrates obtained after different periods of growth clearly indicated that the ATF was extracellular. It was detected as early as 8 h in the log period of growth and, when cells of the growing cultures were subjected to sonication, no additional lytic activity was released.

Lytic substances produced by species of the genus *Pseudomonas* have been described by many investigators. For example, lysis of *Staphylococcus aureus* was produced by both *P. aeruginosa* and *P. fluorescens* (28). A protease was characterized from strain 26 of *P. fluorescens*, and lipase activity was exhibited by both *P. fluorescens* and *P. fragi* (16–18, 22). However, these substances appear to be different from those reported in this study, e.g., in regard to molecular weight and resistance to heat and freezing. By contrast, three other reports are particularly relevant because they are comparable to our observations on the properties of the

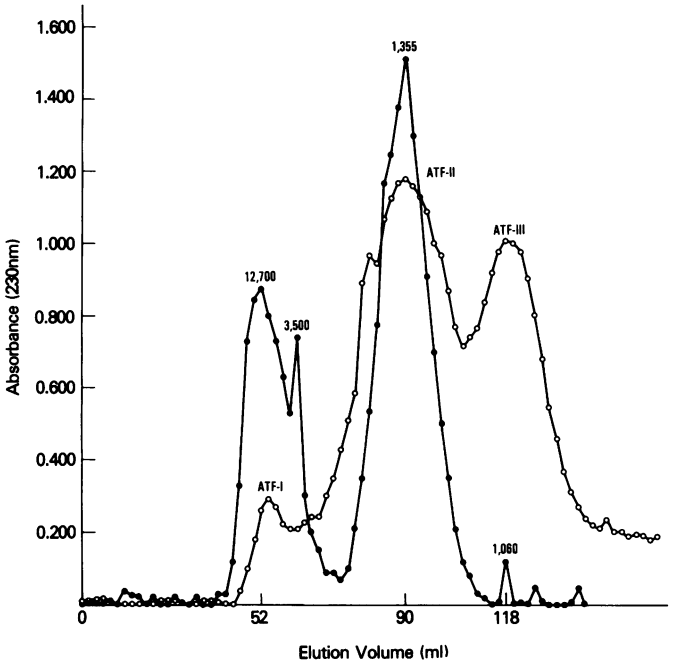

FIG. 4. Chromatography of dialysates from crude sample in Sephadex G-25. Shown are two main fractions, ATF-II and ATF-III, and a minimal ATF-I. Molecular-weight markers: cytochrome oxidase (12.7K), insulin B(3.5K), vitamin B_{12} (1,355), and bradykinin (1,060). Void volume (52 ml) was obtained with dextran 2,000.

ATF. Meinke and Berk (12) described a factor from a strain of *P. aeruginosa*, isolated from a patient, that was non-proteolytic and nonhemolytic, withstood boiling for 30 min, and produced toxic effects in mice similar to those we observed with ATF-I (unpublished data). It was particularly interesting that of all the bacterial species tested in this study, only *P. aeruginosa* produced a lytic effect comparable to that of *P. fluorescens*. Caceres et al. (2) described a lytic effect on epimastigote forms of *T. cruzi* by a species of *Aspergillus* occurring as a contaminant of the cultured flagellates. A loss of infectivity to tissue culture cells and to mice was observed also. A more recent review by Kiprianova and Smirnov (8) was especially significant; it referred to a study reported by Gräf and Bickel (5) regarding a polypeptide antibiotic from *P. fluorescens* which produced lysis of the parasitic flagellates *Trichomonas vaginalis*, *L. donovani* promastigotes, and *T. brucei*. The properties of this compound are, however, different from those of the ATF. It exhibited a molecular weight of 7,000, was insoluble in water, and produced a strong hemolytic effect. Additionally, it did not inhibit the growth of *S. aureus*; ATF-II exerted a strong bacteriostatic effect on this bacterium (unpublished data).

It was of interest that gel filtration of the ATF-I (obtained after the first dialysis of the crude) yielded the same elution profile as that of the undialyzed samples. We questioned, however, the presence of ATF-I(3) and ATF-I(4) since they are considerably smaller than the molecular-weight cutoff (6,000 to 8,000) of the membrane used. We attributed this occurrence to the limitations of the procedure when membrane tubing was used, since they were retained even after long periods of dialysis. It was curious also that gel filtration of the crude supernatant samples without previous desalting yielded more abundant amounts of the small components. This finding suggested that the ATF-I acted as a precursor of these fractions; however, the extent to which these observations were related to other conditions, such as the occurrence of a cycle in the production of the active fractions by the *Pseudomonas*, remains to be elucidated.

Other aspects of the ATF are being examined currently. For example, ultrastructural studies with ATF-II revealed changes in the kinetoplast and nucleus that were more extensive than those reported previously in studies with Lampit (25), the most effective drug used today in controlling *T. cruzi* infections. The observed effects on these structures after the immobilization of the parasites indicated a logical consequence of the observed tropism of the *Pseudomonas* for the posterior end of the parasite. The flagellum originates at this site, which is also the location

of the kinetoplast, the DNA-containing organelle closely associated with the mitochondrion.

The importance of the pseudomonads as producers of antibiotics dates back to 1899, when it was observed that a concentrated cell-free culture fluid of *P. aeruginosa* exerted a bactericidal effect on several kinds of bacteria. The active factor, pyocyanase, was used extensively in the therapy of diphtheria, influenza, and meningitis (9). The present findings with *T. cruzi* are encouraging because of their potential usefulness in the chemotherapy of Chagas' disease; however, it is hoped that they will also renew interest in the industrial application of the *Pseudomonas* secondary metabolites, which has declined considerably for many years.

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LITERATURE CITED

1. **Alford, J. A., and D. A. Pierce.** 1963. Production of lipase by *Pseudomonas fragi* in a synthetic medium. J. Bacteriol. **86**:24–29.
2. **Caceres, O., M. C. S. Salgueiro, E. Kimura, G. Moraes, and T. A. R. Machado.** 1979. Açao do *Aspergillus* sp. sobre o *Trypanosoma cruzi "in vitro."* Rev. Inst. Med. Trop. Sao Paulo **21**:5–12.
3. **Callahan, L. T., III.** 1976. *Pseudomonas aeruginosa* exotoxin: purification by preparative polyacrylamide gel electrophoresis and the development of a highly specific antitoxin serum. Infect. Immun. **14**:55–61.
4. **Doi, O., and S. Nojiona.** 1971. Phospholipase C from *Pseudomonas fluorescens*. Biochim. Biophys. Acta **248**:234–244.
5. **Gräf, W., and H. Bickel.** 1961. Antibiotische Eigenschaften und Toxizität des Wirkstoffes aus *Pseudomonas fluorescens*. Arch. Hyg. Bakteriol. **145**:21–25.
6. **Hoare, C.** 1972. The trypanosomes of mammals. Blackwell Scientific Publications, Oxford.
7. **Hochstein, H. D., R. J. Elin, J. F. Cooper, E. B. Seligman, Jr., and S. M. Wolff.** 1973. Further developments of Limulus amoebocyte lysate test. Bull. Parenter. Drug Assoc. **27**:139–148.
8. **Kiprianova, E. A., and V. V. Smirnov.** 1981. *Pseudomonas fluorescens*, a producer of antibiotic substances. Antibiotiki (Moscow) **26**:135–143.
9. **Leisinger, T., and R. Margraff.** 1979. Secondary metabolites of the fluorescent pseudomonads. Microbiol. Rev. **43**:422–442.
10. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
11. **Mayerhofer, H. J., R. T. Marshall, C. H. White, and M. Lu.** 1973. Characterization of a heat-stable protease of *Pseudomonas fluorescens* P26. Appl. Microbiol. **25**:44–48.
12. **Meinke, G., and R. S. Berk.** 1970. *In vivo* studies with a toxic fraction of *Pseudomonas aeruginosa*. Proc. Soc. Exp. Biol. Med. **135**:360–363.
13. **Mencher, J. R., H. Ng, and J. A. Alford.** 1965. The extracellular nature of the lipases of *Pseudomonas fragi*. Biochim. Biophys. Acta **106**:628–630.
14. **Mercado, T. I.** 1976. *Trypanosoma cruzi*: lactate dehydrogenase isoenzymes and infections in mice. Exp. Parasitol. **40**:411–420.
15. **Mercado, T. I., and K. Katusha.** 1979. Isolation of *Trypanosoma cruzi* from the blood of infected mice by column chromatography. Prep. Biochem. **9**:97–106.
16. **Nashif, S. A., and F. E. Nelson.** 1953. The lipase of *Pseudomonas fragi*. II. Factors affecting lipase production. J. Dairy Sci. **36**:471–480.
17. **Nashif, S. A., and F. E. Nelson.** 1953. The lipase of *Pseudomonas fragi*. III. Enzyme action in cream and butter. J. Diary Sci. **36**:481–488.
18. **Pinheiro, A. J. R., B. J. Liska, and C. E. Parmelee.** 1965. Heat stability of lipases of selected psychrophilic bacteria in milk and Purdue Swiss-type cheese. J. Dairy Sci. **48**:983–984.
19. **Pollock, M. R.** 1962. Exoenzymes, p. 121–278. *In* I. C. Gunsalus and R. Y. Stanier (ed.), The bacteria, vol. IV. Academic Press, Inc., New York.
20. **Rastogi, S. C., E. B. Seligmann, Jr., H. D. Hochstein, J. H. Dawson, L. G. Farag, and R. E. Marquina.** 1979. Statistical procedure for evaluating the sensitivity of *Limulus* amoebocyte lysate by using a reference lysate. Appl. Environ. Microbiol. **38**:911–915.
21. **Reynolds, M. T., F. R. Falkiner, R. Hardy, and C. T. Keane.** 1979. Differentiation of fluorescent *Pseudomonas* by their effect on milk agar. J. Med. Microbiol. **12**:379–382.
22. **Severina, L. O., and N. A. Bashkatova.** 1979. Isolation and properties of lipase from *Pseudomonas fluorescens* 533-5b. Biokhimiya **44**:116–124.
23. **Speck, M. L., and D. M. Adams.** 1976. Heat resistant proteolytic enzymes from bacterial sources. J. Dairy Sci. **59**:786–789.
24. **Stinson, M. W., and C. Hayden.** 1979. Secretion of phospholipase C by *Pseudomonas aeruginosa*. Infect. Immun. **25**:558–564.
25. **Villalta, S., W. de Souza, and W. Leon.** 1979. The effect of Lampit on *T. cruzi* in mice organs and in the blood stream. Z. Parasitenkd. **61**:1–27.
26. **Whiteside, T. L., and W. A. Corpe.** 1969. Extracellular enzymes produced by *Pseudomonas* sp. and their effect on cell envelopes of *Chromobacterium violaceum*. Can. J. Microbiol. **15**:81–92.
27. **Zwadyk, P.** 1976. *Pseudomonas*, p. 597–598. *In* W. K. Joklik and H. P. Willett (ed.), Zinsser microbiology, 16th ed. Appleton-Century-Crofts, New York.
28. **Zyskind, J. W., P. A. Pattee, and M. Lacke.** 1965. Staphylolytic substance from a species of *Pseudomonas*. Science **147**:1458–1459.