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Effect of Protozoa on Bacterial Degradation of an Aromatic Compound

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Prototrophic and growth factor-requiring strains of *Alcaligenes* spp. were used to study the effect of a protozoan, *Tetrahymena pyriformis*, on the degradation of *p*-aminobenzoate. The protozoan inhibited activity of the prototrophic bacterium by reducing its population size. For the growth factor-requiring strain of *Alcaligenes*, *T. pyriformis* provided the required growth factors so that the predator permitted the bacteria to grow and to continue *p*-aminobenzoate degradation. *T. pyriformis* inhibited bacterial activity when the amino acid supply was in excess, but activity of the auxotrophic strain of *Alcaligenes* was stimulated by the protozoan when the amino acid supply was limiting, although the bacterial population size was reduced by the protozoan.

Evidence exists that protozoa play an important role in natural ecosystems. In 1909, Russell and Hutchinson (12) suggested that protozoa might limit bacterial activity by lowering the population size of their prey. Since then, the possible function of protozoa in regulating bacterial populations has been stressed (3, 6). In addition to predation, protozoa have been found to have beneficial effects on bacterial activity. For example, grazing by protozoa has been reported to enhance nutrient cycling (1, 5, 9). It has also been shown that the formation of CO_2 and ammonium in soil is enhanced by protozoa (2, 10). The effect of protozoa on the rates of N_2 fixation (4, 8) and decomposition of complex substances (5) has also been investigated.

The present study was designed to assess the effect of protozoa on the bacterial degradation of an aromatic compound. The study was designed also to explain the possible effects observed should the activity be altered.

MATERIALS AND METHODS

Tetrahymena pyriformis was obtained from D. J. P. Bruns, Cornell University. The protozoa were cultivated under axenic conditions at 30°C in proteose-yeast extract medium without shaking. Two *p*-aminobenzoate (PABA)-utilizing bacteria were isolated from soil. To obtain the organisms, 1.0 g of soil was incubated for 2 weeks in an aqueous solution containing PABA, and the enrichment was then plated on PABA-inorganic salts agar to allow for colony selection. To obtain bacteria nutritionally dependent on protozoa, a sterile glass ring, 20 mm in diameter by 10 mm high, was placed in each of several petri dishes immediately after molten agar was introduced. The medium used was PABA-salts medium amended with 1.5% agar. After the agar had solidified, 0.5 ml of a suspension containing about 3×10^9 *Enterobacter aerogenes* and 0.1 ml of a suspension containing about 10^4 *T. pyriformis* were added to each ring. A 0.1-ml portion of the original enrichment was spread evenly over the plate, which was then incubated at 22°C. Because of the small amount of added carbon source, colonies developed very slowly on the plates. Colonies around the glass rings were selected and purified by isolation of single colonies after streaking on nutrient agar. To identify the organisms, the following tests were conducted: starch and gelatin hydrolysis, nitrate reduction, indole and H_2S production, ammonification, methyl red and Voges-Proskauer reactions, behavior in litmus milk, fermentation of glucose, and activities of catalase, oxidase, and urease. Flagellation was examined by electron microscopy.

The numbers of bacteria were determined by plate counts on nutrient agar. The numbers of protozoa were determined by a modification of the method of Singh (14) as described by Habte and Alexander (7), with *E. aerogenes* as the prey bacterium.

Protozoa and bacteria were washed and suspended in a solution containing 0.85% NaCl, 0.03% KH_2PO_4, and 0.06% Na_2HPO_4. The proteose-yeast extract medium used for cultivating the protozoa contained 2.0% proteose peptone, 0.1% yeast extract, and 0.003% sequestrene (Geigy, Ardsley, N.Y.) in distilled water. The inorganic salts solution used to test bacterial activity contained 2.09 g of K_2HPO_4, 1.0 g of KH_2PO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 10 mg of $CaCl_2 \cdot 2H_2O$, 3 mg of $FeSO_4 \cdot 7H_2O$, 2 mg of $MnCl_2 \cdot 4H_2O$, 1.5 mg of $Co(NO_3)_2 \cdot 6H_2O$, 0.5 mg of $ZnSO_4 \cdot 7H_2O$, and 1,000 ml of distilled water. The PABA-salts medium that was used to test for bacterial degradation of PABA, and also to enrich and isolate the bacteria, was prepared by adding 0.1% NH_4Cl and 0.0012% PABA to the inorganic salts solution. The vitamin-free medium used to assay the presence of growth factors in suspensions of protozoa contained 0.01% vitamin-free acid hydrolysate of casein (Difco Laboratories, Detroit, Mich.) in the inorganic salts solution.

To assess the bacterial degradation of PABA, about 10^4 bacteria were inoculated into 20 to 30 ml of the

PABA-salts medium contained in 125-ml Erlenmeyer flasks, and these cultures were incubated at 30°C without shaking. Samples were taken at regular intervals, and the concentration of PABA was measured spectrophotometrically at 260 nm with a Beckman spectrophotometer, model DU (Beckman Instruments, Inc., Fullerton, Calif.).

To test the effects of protozoa on bacterial activity, protozoa were cultivated in proteose-yeast extract medium for 3 days, at which time they were collected by centrifugation at about 500 × *g* for about 4 min. Protozoa thus collected were washed four times with saline-phosphate buffer. Washed protozoa were suspended in the same solution and then added to the test medium to give about 2×10^4 *T. pyriformis* cells per ml. The degradation of PABA was then measured in the presence of protozoa.

Filtrates of a suspension of *T. pyriformis* containing about 5×10^5 cells per ml were prepared after different periods of incubation at 30°C without shaking, and the ability of these filtrates to support growth of the bacteria in the vitamin-free medium was tested. The growth factor-requiring isolate was suspended in the vitamin-free medium to a final cell number of about 10^5/ml. The filtrate was then added to the medium, which was incubated at 30°C for 3 days without shaking. The optical density resulting from bacterial growth was then measured turbidimetrically at 550 nm with the Beckman model DU spectrophotometer.

RESULTS

Two bacteria able to use PABA as a source of carbon and energy were isolated. Both were identified as strains of *Alcaligenes* spp. One required no growth factors, but the other failed to multiply in a growth factor-free medium. However, it grew readily in a salts solution containing 0.0012% PABA, 0.01% vitamin-free Casamino Acids, and 1.0 μg of D-biotin per ml.

In the presence of *T. pyriformis* alone, the PABA level did not decline as measured at 260 nm, and no detectable growth of the protozoan was evident in a medium with PABA as the sole carbon source.

The prototrophic isolate degraded PABA in the salts solution whether protozoa were present or not (Fig. 1). However, in the presence of *T. pyriformis*, the final bacterial numbers and possibly the rate of bacterial growth were lower than in the absence of protozoa. The period of active degradation of PABA was delayed by about 1 day because of the presence of protozoa. These results show that predation by *T. pyriformis* reduced the bacterial population and affected its activity on the test substrate.

The effect of *T. pyriformis* on the growth factor-requiring isolate was different, however. In this instance, PABA degradation did not occur in the PABA-salts solution unless the protozoan was present (Fig. 2). Moreover, the bacteria did not grow to an appreciable extent in the absence of protozoa. In their presence, however, the bacteria grew and then maintained a nearly constant number of about 5×10^6/ml, a value representing a population of *Alcaligenes* sp. that could be maintained in the face of predation. The loss of PABA was directly related to multiplication of the bacteria. Thus, although the bacterial population was controlled by predation, protozoa enabled the bacteria to proliferate and to destroy the aromatic compound.

An experiment was conducted to determine if the bacterium obtained biotin, the only vitamin it required, from *T. pyriformis*. *T. pyriformis* cells were washed and suspended in 0.02 M phosphate buffer (pH 7.2) to a final density of about 5×10^5 cells per ml, and the suspension was incubated without shaking at 30°C. At days 0, 1, 2, 4, and 6, 5.0 ml of the suspension was passed through a 0.22-μm membrane filter (Millipore Corp., Bedford, Mass.), and 1.0 ml of each filtrate was used as a supplement to 4.0 ml of the vitamin-free medium contained in test tubes. The auxotrophic *Alcaligenes* strain was inoculated into each tube to a final density of about 10^5 cells per ml, and the tubes were incubated for 3 days without shaking at 30°C. No growth was detected by turbidimetric measurement of cultures receiving filtrates of protozoan suspen-

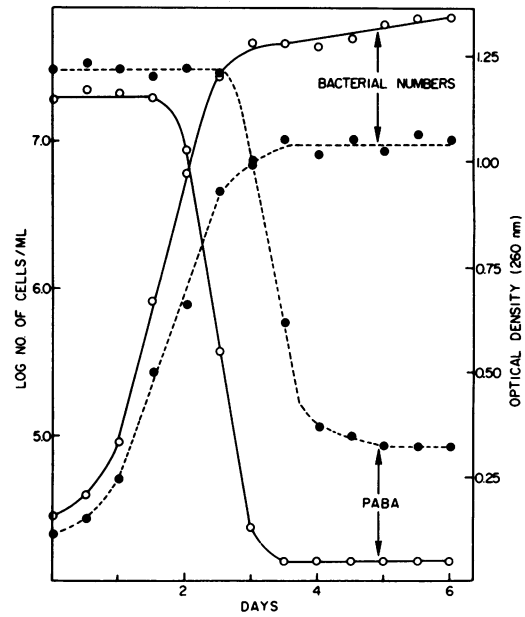

FIG. 1. *Effect of protozoa on numbers of bacteria and PABA degradation (as represented by changes in optical density) by a prototrophic Alcaligenes strain. The initial cell number of T. pyriformis was 2 × 10^4/ml. (Solid lines) Changes in the absence of protozoa; (dotted lines) changes in their presence.*

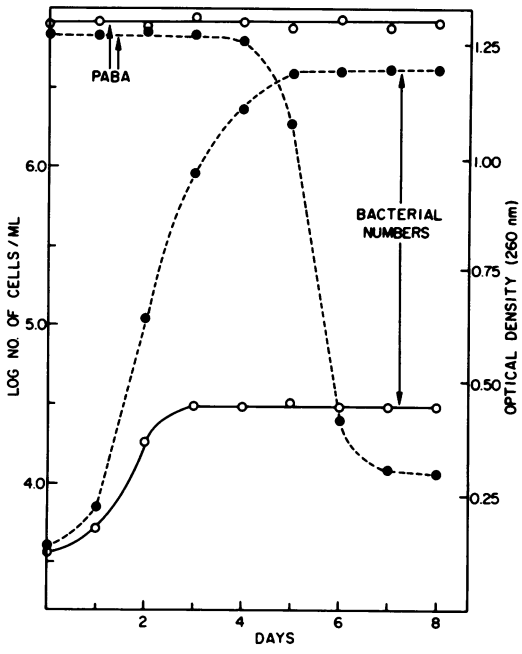

FIG. 2. *Effect of protozoa on numbers of bacteria and PABA degradation (as represented by changes in optical density) by a growth factor-requiring Alcaligenes strain. The initial cell number of T. pyriformis was 2 × 10⁴/ml. (Solid lines) Changes in the absence of protozoa; (dotted lines) changes in their presence.*

sions prepared on day 0, but the turbidity at 550 nm was 0.11, 0.13, 0.13, and 0.16 in bacterial cultures amended with filtrates of *T. pyriformis* suspensions prepared on day 1, 2, 4, and 6, respectively. Thus, the filtrate of the protozoan suspension could replace biotin in supporting bacterial growth.

An experiment was set up to determine if biotin was also present when *T. pyriformis* was feeding on bacteria. For this purpose, washed *E. aerogenes* was suspended in 15 ml of 0.02 M phosphate buffer (pH 7.2) to a final density of about 3×10^8 cells per ml, and half of the 50-ml flasks containing the bacteria received 10^4 *T. pyriformis* cells per ml. After 3 days of incubation at 30°C without shaking, the suspensions were passed through 0.22-μm Millipore filters, and 1.0-ml portions of the filtrates were used as a potential biotin source for growth of the auxotrophic bacterium in 4.0 ml of the vitamin-free medium contained in test tubes. The *Alcaligenes* sp. inoculum contained 5×10^5 cells, and the cultures were incubated for 3 days at 30°C without shaking, after which the increase in turbidity was measured at 550 nm. The optical density was 0.00 if *Alcaligenes* sp. was provided no filtrate, 0.045 if provided the filtrate from *E. aerogenes* alone, and 0.170 if provided the filtrate of *T. pyriformis* that had been grown on *E. aerogenes*. Thus, although some biotin appears to have been released from *E. aerogenes* cells, the concentration was much higher in the protozoan suspension that had fed on these bacteria.

The supply of nutrients in nature is usually low, and a study was thus conducted to assess the effect of protozoa on the activity of bacteria at low nutrient levels. The growth factor-requiring *Alcaligenes* sp. was used as the test organism, and 0.001% casein hydrolysate was added as a limiting nutrient to a medium containing PABA, 1 μg of biotin per ml, and inorganic salts. No ammonium salt was added. PABA was added initially and at day 7 to final concentrations of 0.0012%. At day 10, the protozoan population was 1.5×10^4 cells per ml. Protozoa retarded the initiation of significant PABA degradation, and the final bacterial population was smaller in the presence of protozoa than in their absence (Fig. 3). However, after bacteria reached the stationary phase of growth and fresh PABA was added, *T. pyriformis* apparently promoted the loss of PABA, even though the bacterial population was still smaller in the presence of protozoa than in their absence.

DISCUSSION

Protozoa may have several functions in nature. They may prey on microbes, excrete nutri-

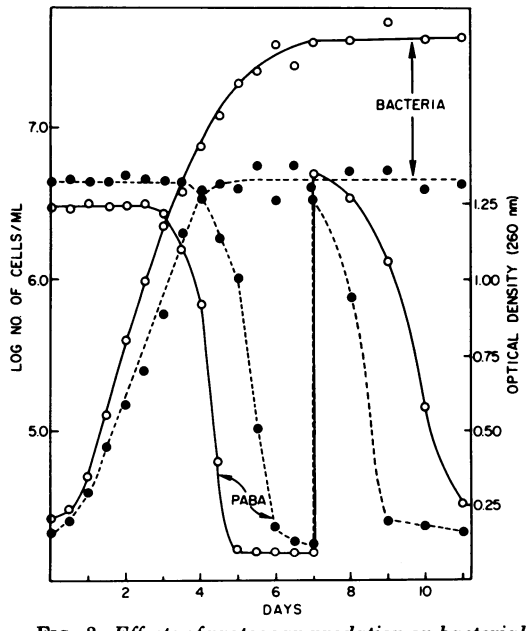

FIG. 3. *Effects of protozoan predation on bacterial degradation of PABA under conditions of nutrient limitation. (Solid lines) Changes in the absence of protozoa; (dotted lines) changes in their presence.*

ents or growth factors, or facilitate nutrient recycling, and their role may depend on the type of microbial prey or the environment in which they are present. However, it is generally believed that predation by protozoa causes a reduction in size of the bacterial population, and thus predators presumably reduce bacterial activity (6, 7).

The excretion of organic compounds by protozoa is well known. The data of Seto and Tazaki (13) indicate that as much as 50% of the carbon consumed by *Tetrahymena* was converted to soluble excretions or particulate substances. The compounds excreted included vitamins, amino acids, and nucleic acids. The present data showing that the auxotrophic strain of *Alcaligenes* sp. depended on *T. pyriformis* for its growth and degradative activity suggest that such excretions may supply needed nutrients for other microorganisms.

A requirement for growth factors by bacteria is common in nature. Thus, more than 50% of the aerobic soil bacteria have been reported to require vitamins (11). Therefore, the provision of growth factors by protozoa may be ecologically important for bacterial activity in nature. The present data show also that the presence of protozoa stimulated bacterial activity under conditions of nutrient limitation, even though the bacterial population was reduced as a consequence of predation. This stimulation might result from nutrient regeneration associated with predation; i.e., the nutrients retained in the bacterial cell are released and recycled, thereby enabling the bacteria to maintain their activity.

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