

## Regulation of Predation by Prey Density: the Protozoan-*Rhizobium* Relationship

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*Tetramitus rostratus* and strains of *Hartmanella*, *Naegleria*, and *Vahlkampfia* consumed large numbers of *Rhizobium meliloti* cells in a salt solution, but protozoan multiplication and the bacterial decline stopped when the prey density fell to about  $10^6$  to  $10^7$  cells/ml. At higher prey densities, the maximum numbers of *Hartmanella* sp. and *Naegleria* sp. were proportional to the quantity of *R. meliloti* initially provided to the amoebas. When supplemental rhizobia were supplied to *Hartmanella* sp. or *Naegleria* sp. after their active feeding had terminated, presumably because the remaining  $10^6$  or  $10^7$  bacteria/ml could not be captured, replication of the protozoa was initiated. The rate of elimination of rhizobia present in large populations was proportional to the initial abundance of *Naegleria* sp., but the final numbers of amoebas and surviving *R. meliloti* cells were independent of initial numbers of predators. The surviving bacteria were not intrinsically resistant to attack because 98% of the survivors, when concentrated, were consumed. It is suggested that large populations of bacteria in nature may be reduced in size by predatory protozoa, but many of the prey cells will not be eliminated.

The results of a recent study show that biological factors are involved in the decline of *Rhizobium* added to nonsterile soil (2). The number of surviving bacteria was quite large, however, an observation that is surprising since rhizobia are susceptible to attack by protozoa and these predators have been found in almost every soil examined (8). Competition for available food, parasitism by indigenous bacteriophages, and attack by *Bdellovibrio* do not appear to be major factors in the decline of rhizobia in nonsterile soils (2; S. O. Keya and M. Alexander, Soil Biol. Biochem., in press).

The present investigation was designed to determine whether protozoa can, in fact, eliminate rhizobia when the bacteria are provided in solution culture. The report is part of a larger study to establish why bacteria not known to possess resistant stages can survive in soil containing predators, viruses, and bdellovibrios and are able, in culture at least, to live and multiply at the expense of bacterial cells. A large literature exists on protozoan feeding on bacteria and other microorganisms, including mathematical models of the interacting populations (11), but experimental evidence to explain why bacteria are not eliminated by their predators is largely lacking.

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### MATERIALS AND METHODS

An axenic culture of *Tetramitus rostratus* was obtained from the Culture Collection of Algae and Protozoa, Cambridge, England. Uniprotozoan cultures of *Hartmanella* sp., *Naegleria* sp., and *Vahlkampfia* sp. were isolated from Valois silt loam, Lote K-25 C soil from Colombia, and water collected from Beebe Lake, Ithaca, N.Y., respectively. The protozoa were classified by using the keys of Jahn and Jahn (5) and Kudo (6).

To isolate the protozoa, 1.0 ml of a thick suspension of *Rhizobium meliloti* M<sub>5</sub>V<sub>1</sub>-S was spread on the surface of sterile water agar containing 0.5% NaCl, and about 1 g of soil or 2 drops of lake water was then added to the center of the bacterial lawn. The plates were incubated at 30 C for 5 to 7 days, and samples taken from the edges of cleared areas which appeared on the lawn were examined under the light microscope for the presence of protozoa. Material from a cleared area containing protozoa was diluted in sterile distilled water, and 0.5-ml portions were mixed with 5.5 ml of 0.6% molten agar containing a thick suspension of *R. meliloti* and 500 µg of streptomycin sulfate per ml. The mixture was poured over a layer of solidified agar (1.5% agar and 0.5% NaCl) contained in a petri dish. The plates were incubated for 3 days at 30 C, after which inocula were taken from the highest dilution to give isolated zones of clearing; these were again diluted, and the whole process was repeated 3 to 4 times. Single zones of clearing were examined each time to test whether the cultures were uniprotozoan.

*R. meliloti* was grown for 2 to 3 days at 30 C in 500-ml Erlenmeyer flasks containing 300 ml of yeast

extract-mannitol broth (2) supplemented with 1.0 mg of filter-sterilized streptomycin sulfate per ml. The flasks were incubated on a rotary shaker operating at 120 rpm, and the cells were harvested by centrifugation. The pellets were suspended aseptically in sterile distilled water and centrifuged again, the process being repeated three times. The pellet was then suspended in sterile distilled water to give a thick suspension. Dilutions for the experiments were made from this suspension.

The protozoa were cultivated in 250-ml Erlenmeyer flasks rinsed with a 1% solution of silicone concentrate obtained from Cutin Scientific Co., Houston, Tex. This chemical coated the walls of the flask and minimized bacterial or protozoan adherence to the vessel's sides. Each flask contained 25 ml of sterile carbon- and nitrogen-free (CNF) solution and about  $10^9$  *R. meliloti* cells/ml derived from a 3-day bacterial culture. CNF solution contained 0.02%  $MgSO_4 \cdot 7H_2O$ , 0.1%  $CaCl_2 \cdot 2H_2O$ , and 0.1% NaCl in distilled water. After incubation for 2 to 3 days at 26 C on a rotary shaker operating at 70 rpm or a reciprocating shaker operating at 70 strokes/min, the cells were collected by centrifugation aseptically at  $5,000 \times g$  for 5 min. The pellet was suspended aseptically in the CNF solution and recentrifuged at  $5,000 \times g$  for 5 min. A thick suspension of the pellet was made in the sterile CNF solution, and dilutions of this suspension were used for the experiments.

Yeast extract-mannitol agar medium supplemented with 1.0 mg of streptomycin sulfate per ml was employed to count the rhizobia. Protozoa were enumerated by a modification of Singh's (9) method. About 10 to 12 ml of sterile 1.5% agar containing 0.5% NaCl was poured into 150-mm sterile petri dishes. Ten sterile glass rings (10-mm deep and 20-mm internal diameter) were arranged inside each petri dish containing the molten medium. The agar was allowed to solidify, after which the agar inside each glass ring was inoculated with about  $10^8$  *Klebsiella pneumoniae* cells from a 24-h-old culture. Tenfold serial dilutions of the protozoan suspensions were made, and 0.2 ml of each dilution was added to each glass ring. The petri dishes were incubated upright at 30 C for 7 to 10 days, after which the area enclosed within each ring was examined under the low power of a microscope for the presence or absence of protozoan cysts or trophozoites. The most-probable-number table of Cochran (1) was used to estimate the abundance of protozoa. *K. pneumoniae* was grown on a rotary shaker at 30 C in nutrient broth.

To determine the ability of *R. meliloti* to survive in the presence of protozoa, 1.0-ml portions of a bacterial suspension and 1.0 ml of a protozoan suspension were added to 250-ml Erlenmeyer flasks, each containing 23 ml of sterile CNF solution. The flasks had previously been rinsed in 1% silicone concentrate followed by distilled water and then dried in an oven. The flasks were incubated at 26 C on a rotary shaker operating at 70 rpm, or they were allowed to stand at 30 C. Counts of the organisms were made on duplicate samples at selected times, and the values presented are the means of the duplicate determinations.

To test if the rhizobia surviving in the presence of protozoa were inherently resistant to predation, 1.0 ml

of a thick suspension of washed rhizobium cells and 1.0 ml of a protozoan suspension, both from 3-day-old cultures, were added to 1,200 ml of CNF solution contained in 3-liter Erlenmeyer flasks. The vessels were incubated at 30 C without shaking, except immediately before samples were taken, and counts of rhizobium were made periodically for 15 days. On day 15, each flask was gently agitated and then allowed to stand for 2 h, after which about 800 ml of the partially cleared fluid above the sedimented particles was passed through three layers of sterile cheesecloth. The resultant filtrate was centrifuged aseptically at  $18,000 \times g$  for 20 min, the pellet was resuspended in sterile CNF solution, and the latter was centrifuged at  $8,000 \times g$ . The pellet was then suspended in 50 ml of CNF solution, and 10 ml of this mixture was added to 15 ml of CNF solution contained in 250-ml Erlenmeyer flasks. The flasks were left standing at 30 C, and counts of both rhizobia and protozoa were made at regular intervals.

## RESULTS

As a prerequisite to the study of the interaction between protozoa and the bacterium, the survival of *R. meliloti* in CNF solution in the absence of the predator was assessed. The results showed that *R. meliloti* did not readily lose viability in this solution. Thus, daily counts of suspensions initially containing  $1.8 \times 10^7$ ,  $1.8 \times 10^8$ , and  $1.8 \times 10^9$  cells/ml showed essentially no increase and only a slight fall with time, the counts at 7 days being  $1.8 \times 10^7$ ,  $6.0 \times 10^7$ , and  $1.1 \times 10^8$  bacteria/ml, respectively.

The growth of protozoa fed with *R. meliloti* was studied using three species of amoebas. In each instance, the numbers of rhizobia declined up to a point below which their density did not fall further (Fig. 1). Similarly, the protozoan numbers increased initially and then stabilized at a reasonably constant value. The decline in numbers of bacteria occurred at the time the protozoa were actively growing, and from about  $7.5 \times 10^6$  to  $50 \times 10^6$  bacteria/ml were not consumed by the protozoa. The results suggest that the growth of the amoebas ceases when the bacterial numbers fall to a critical level.

A study was then performed to establish the effect of prey density on growth of one of the protozoa, *Hartmanella* sp., and to determine why the predator would cease growing as the prey density fell. For this purpose, CNF solution was inoculated with  $1.3 \times 10^8$  *Hartmanella* sp. cells/ml and  $1.8 \times 10^7$ ,  $1.8 \times 10^8$ , or  $1.8 \times 10^9$  rhizobia/ml. It is evident from Fig. 2 that the final numbers of amoebas were proportional to the numbers of prey provided. It is also evident that more than  $10^6$  bacteria/ml survived. In this instance, the number of survivors was greater with many bacteria in the inoculum. From these data, it is possible to calculate

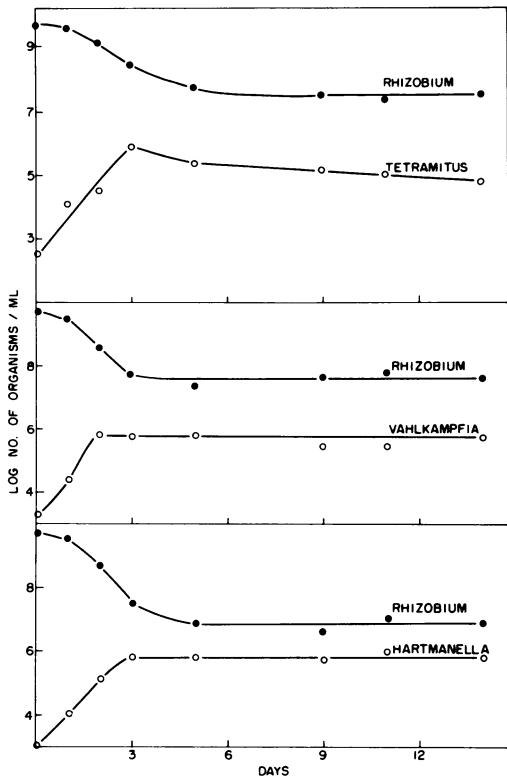


FIG. 1. Growth of three protozoan species on *R. meliloti* in CNF solution.

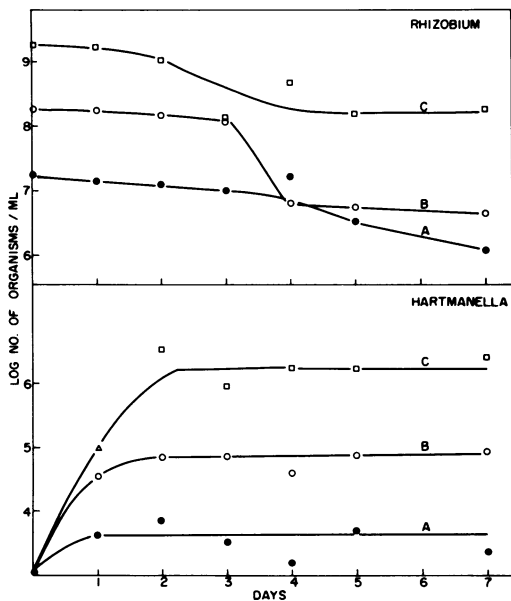


FIG. 2. Development of *Hartmanella* sp. and decline of *R. meliloti* provided in CNF solution initially containing (A)  $1.8 \times 10^7$ , (B)  $1.8 \times 10^8$ , and (C)  $1.8 \times 10^9$  rhizobia/ml.

that each protozoan consumed from 600 to 3,600 cells, the lower values coming from calculations made with cultures initially having the larger number of bacteria.

In a similar study, different levels of *R. meliloti* were fed to *Naegleria* sp. in CNF solution. In this instance, too, higher numbers of *Naegleria* sp. were attained with increasing bacterial abundance (Fig. 3). The numbers of cells of *R. meliloti* which survived at various intervals are also presented in Fig. 3, the density of survivors rising with increasing inoculum levels but always being above  $2 \times 10^5$ /ml. From the data in the figure, it can be estimated that *Naegleria* sp. consumed 800 to 7,500 bacteria per protozoan cell.

To establish further the role played by bacterial food in the growth of protozoa,  $1.8 \times 10^7$  *R. meliloti* cells/ml were fed to  $1.1 \times 10^8$  *Hartmanella* sp./ml of CNF solution. Counts of protozoa and bacteria were made periodically for 7 days. On day 7, the suspension in one group of flasks was supplemented with additional *R. meliloti* cells to a final density of  $1.9 \times 10^9$ /ml, whereas the other group did not receive fresh bacterial cells. The flasks were again incubated on a rotary shaker, and counts of both rhizobia and protozoa were made regu-

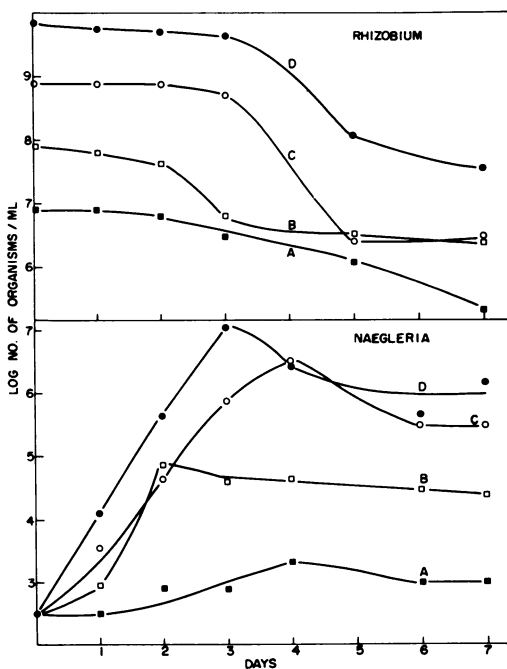


FIG. 3. Growth of *Naegleria* sp. and decline of *R. meliloti* in CNF solution initially containing (A)  $7.5 \times 10^6$ , (B)  $7.5 \times 10^7$ , (C)  $7.5 \times 10^8$ , and (D)  $7.5 \times 10^9$  rhizobia/ml.

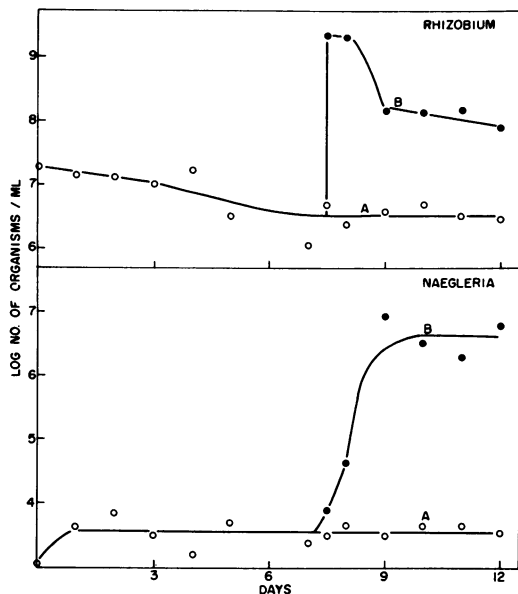


FIG. 4. Replication of *Naegleria* sp. in solutions containing *R. meliloti* with and without additional prey cells being added on day 7. Numbers in cultures without (A) and with (B) supplemental bacteria.

larly. The results show that during the first 7 days there was little growth of the protozoa (Fig. 4), probably owing to the paucity of prey. However, the numbers of protozoa rose abruptly in the suspension which received supplemental bacteria, whereas no further amoebal growth occurred in the samples which were not inoculated with fresh rhizobia on day 7. When more prey cells were added, the protozoa clearly consumed the bacteria, as shown by the decline in rhizobia.

In another experiment,  $1.8 \times 10^9$  *R. meliloti* cells/ml were supplied to  $1.1 \times 10^5$  *Hartmanella* sp./ml of CNF solution. The protozoa were allowed to grow for 7 days, and the cultures were then divided into two groups, one receiving additional *R. meliloti* cells and the other receiving none. The flasks were again incubated on the rotary shaker. Only in the flasks to which more bacteria were added did the predator increase in numbers; at the same time, the bacterial density in the supplemented but not in the unsupplemented flasks declined (Fig. 5). On day 11, additional *R. meliloti* cells were introduced into the flasks which received fresh bacteria on day 7, and this addition of bacteria (to  $8.5 \times 10^9$ /ml) resulted in further growth of the protozoa and a fall in bacterial density.

Microscopic examination of the culture was conducted in an experiment similar to the

preceding one, except that the original culture initially contained  $2.0 \times 10^9$  *R. meliloti* and about  $10^5$  *Hartmanella* sp./ml. The amoeba multiplied for 4 days and then ceased growing. Examination of the culture showed that most of the amoebas had encysted by day 5, and nearly all were in the cyst form by day 7. Within 12 h after supplementing the liquid with  $8.0 \times 10^9$  *R. meliloti*/ml, many trophozoites reappeared and amoebal replication was reinitiated, although the protozoa remained in the cyst form in the unamended cultures.

To establish whether a certain ratio of protozoa to bacteria was necessary to allow for growth of protozoa or for a decline in the numbers of bacteria, two levels of *Naegleria* sp. were fed with  $1.8 \times 10^6$  *R. meliloti*/ml. The data in Fig. 6 show that bacterial numbers were not reduced when the solution had  $10^5$  *Naegleria* sp./ml, but the predator ate some of the bacteria when the initial number of animals was  $10^7$ /ml. The lack of appreciable increase in predators is not unexpected, because the density of bacteria would not support many protozoa.

The coexistence of many bacteria with the protozoa may result from the lack of feeding on cells intrinsically resistant to attack. To test

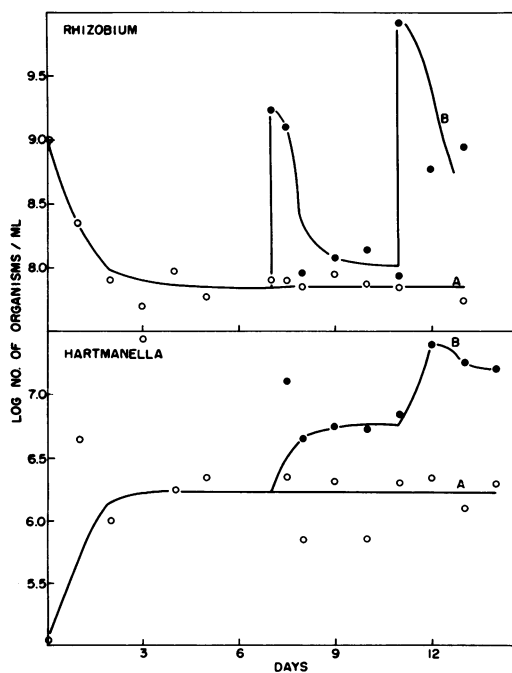


FIG. 5. Growth of *Hartmanella* sp. on *R. meliloti* in solutions with or without additional prey being added on days 7 and 11. Cells in cultures without (A) and with (B) supplemental bacteria.

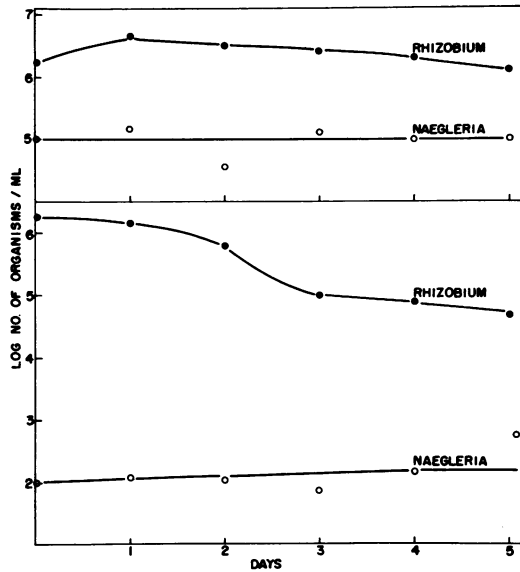


FIG. 6. Changes in the numbers of *Naegleria* sp. and *R. meliloti* in solutions initially containing  $10^8$  or  $10^3$  amoebas/ml.

this hypothesis, *R. meliloti* and *Naegleria* sp. were inoculated into CNF solution. The numbers of the prey fell from an initial value of  $3 \times 10^8$  to  $3 \times 10^7$ /ml on day 12, after which no further decline was noted. On day 15, the cultures were treated to obtain a centrifuged suspension with most of the protozoa removed, as described above. The pellet was then suspended in fresh CNF solution, and portions of the suspension were added to CNF solution. The flasks containing the solutions were incubated without shaking except on days samples were taken for counts. The numbers of *R. meliloti* in this culture declined from  $4.0 \times 10^8$  to  $7.9 \times 10^6$ /ml, whereas the protozoa increased slightly in abundance (Fig. 7). Thus, the results suggest that the cells which survived in the culture up to day 15 were not intrinsically resistant to attack by protozoa since 98% of the survivors were destroyed.

As a further test of the possibility of a predator-prey ratio governing the extent of protozoan growth and bacterial destruction, constant numbers of rhizobia ( $7.5 \times 10^7$ /ml) were fed to variable numbers of *Naegleria* sp. The protozoa at all the different levels multiplied and achieved nearly identical values (Fig. 8). Similarly, the final numbers of bacteria which survived when fed to the different prey levels were almost identical. This suggests that the amount of available food determines the extent of protozoan growth, and that after the

predators have achieved a certain density they will not grow again until more food is supplied. The results obtained when a constant number ( $7.5 \times 10^7$ /ml) of *R. meliloti* was fed to variable

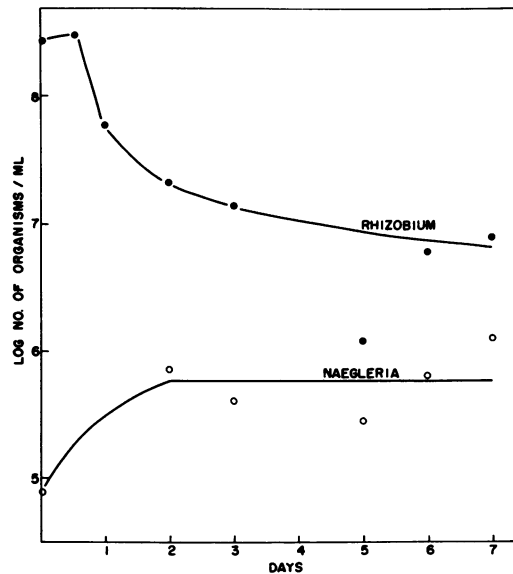


FIG. 7. Feeding of *Naegleria* sp. on cells of *R. meliloti* harvested from a protozoan-rhizobium culture in which the bacterial numbers had fallen to a stable value.

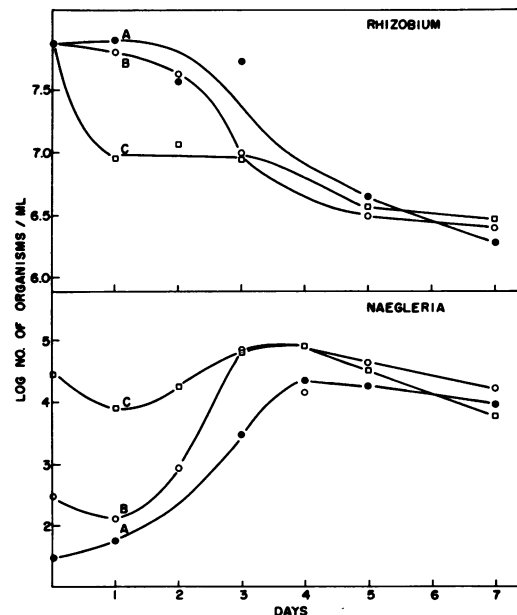


FIG. 8. Changes in the abundance of *Naegleria* sp. and *R. meliloti* in solutions with  $7.5 \times 10^7$  rhizobia/ml and 20 (A), 200 (B), and 20,000 (C) amoebas/ml.

numbers ( $2.0 \times 10^2$ ,  $2.0 \times 10^3$ , and  $2.0 \times 10^4$ /ml) of *Hartmanella* sp. were quite similar to those observed with *Naegleria* sp.

When cells of *R. meliloti* killed by steaming for 20 min were supplied to *Naegleria* sp. in CNF solution, the protozoa grew and achieved densities similar to, although somewhat lower than, those obtained when consuming viable bacterial cells (Table 1). Both living and dead bacteria were provided at a density of  $7.5 \times 10^7$ /ml. Although counts of the killed cells were not made, the cultures were still turbid at the end of the incubation period, suggesting that many cells were still present. This was confirmed by the finding of intact *Rhizobium* cells in the pellet when the suspension was centrifuged at  $8,000 \times g$  for 5 min. Thus, the protozoa could not eat all the heat-killed cells, although the dead bacteria obviously cannot mutate to yield individuals resistant to predation.

### DISCUSSION

The present findings are consistent with the view that a critical density of bacteria is necessary for protozoa to initiate or to maintain active feeding. High bacterial densities will sustain the amoebas, but, once sufficient bacteria are consumed that their density falls to the critical value, feeding by the predators slows and then ceases. Protozoa have been implicated in the decline of *Xanthomonas* in soil, and here too the predators reach a maximum level and do not multiply further despite the presence of abundant xanthomonads (4). Similar observations had been made in the *Rhizobium-Bdellovibrio* association; in this instance, the vibrio attacked and had a major impact only on large rhizobial populations, but it left many surviving rhizobia, the density of survivors being similar to the critical level needed for *Bdellovibrio* to initiate attack on a fresh host population (Keya and Alexander, Soil Biol. Biochem., in press). The critical density may be

determined by the distance between individual prey cells. Thus, as the number of prey per unit volume falls, the distance between survivors increases, and the predator will no longer be able to reduce appreciably the numbers of rhizobia when it uses up more energy in seeking food than it obtains from the bacteria consumed.

According to Pimentel (7), predation can serve as a means for the natural selection of survivors that are inherently more resistant to attack than the original prey. Data obtained in this study, however, do not support the hypothesis that the survivors are not eliminated owing to their intrinsically greater resistance, either because the original bacterial population contained cells of differing edibility or because of the proliferation of a mutant resistant to the amoebas. The evidence arguing against the prior existence or the emergence of individuals not consumed by the protozoa comes from studies showing that (i) the survivors, when concentrated by centrifugation, are easily consumed, and (ii) populations containing  $10^6$  or  $10^7$  cells/ml of bacteria not previously exposed to the animals were not detectably affected, whereas denser populations were reduced in abundance.

The predator that eliminates its food source might be expected to have a tenuous existence inasmuch as it destroys its nutrient source (10). However, once active feeding ceases, the amoebas must themselves have a means of enduring until conditions favor the multiplication of bacteria they can utilize. The cysts permitting such survival were observed to appear once the prey for *Hartmanella* sp. became sparse. By contrast, a predator which completely destroys its prey and does not develop a resistant structure may die out. Thus, the complete destruction of *Paramecium* by *Didinium* in culture resulted in the extinction of the predator, too (3). The simultaneous presence in soil of edible bacteria and protozoa suggests that coexistence rather than extinction is characteristic of predator-prey relationships among the indigenous species.

TABLE 1. Growth of *Naegleria* sp. on live and heat-killed cells from 3-day cultures of *R. meliloti*

Days	No. of <i>Naegleria</i> sp./ml	
	Live prey	Killed prey
0	$3.0 \times 10^2$	$3.0 \times 10^2$
1	$9.0 \times 10^2$	$1.9 \times 10^3$
2	$7.4 \times 10^4$	$1.0 \times 10^4$
3	$1.4 \times 10^4$	$1.0 \times 10^4$
5	$4.4 \times 10^4$	$1.1 \times 10^4$
7	$1.7 \times 10^4$	$7.0 \times 10^3$

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