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Resistance of Cysts of Amoebae to Microbial Decomposition

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Cyst walls of *Acanthamoeba rhysodes*, *A. palestinensis*, *A. castellanii*, and one other strain of *Acanthamoeba* contain 36 to 45% protein and 20 to 34% carbohydrate. More than half of the protein in the walls of *A. palestinensis*, *A. castellanii* and *Acanthamoeba* sp. is accessible to and hydrolyzed by protease, and 67 to 69% of the carbohydrate of *A. palestinensis* and *A. rhysodes* walls is hydrolyzed by cellulase. The extent of hydrolysis of walls of the other amoebae by these enzymes is appreciably less, and chitinase and β-1,3-glucanase have no detectable effect. Protease solubilizes 10% or less of the weight of intact cysts, and no solubilization is observed with cellulase. Walls of *A. palestinensis* are extensively degraded in soil, the activity is less with *A. rhysodes*, and little attack on the other amoebae occurs. When added to soil, the protozoa excyst and grow for short periods, the trophozoites then die, and chiefly cysts persist thereafter.

Protozoa are major components of the soil community, and recent evidence suggests that they are chief contributors to the decline in populations of certain bacteria introduced into the soil (5, 6). Nevertheless, little is known about their persistence, growth, and factors affecting their excystation in this ecosystem. Such information is important not only for an understanding of the ecology of these microorganisms but also to be able to predict the longevity and population fluctuations of pathogenic amoebae entering soil with fecal matter, raw sewage, or municipal sludge.

It is generally believed that protozoa persist in soil in the form of cysts (2, 3), yet the behavior of these cysts in the natural habitat has scarcely been studied. On the other hand, some investigations have been performed using trophozoite inocula (16, 20). Because the cyst is presumably the persistent structure, a study was initiated to evaluate the susceptibility of cysts to microbial and enzymatic degradation in soil and in vitro.

MATERIALS AND METHODS

Organisms. Axenic cultures of *Acanthamoeba castellanii*, *Acanthamoeba* sp., *A. rhysodes* (synonymous with *Hartmannella rhysodes*), and *A. palestinensis* (synonymous with *Mayorella palestinensis*) were obtained from Sharon Mohrlok, Michigan State University, East Lansing, Mich. The organisms were grown at 30°C in 200 ml of peptone medium (1) contained in 500-ml Erlenmeyer flasks incubated on a shaker operating at 250 rpm. After 4 to 5 days of incubation, the cultures were harvested aseptically by centrifugation, and the cells were washed once in sterile encysting medium (11). The protozoa were then suspended in 200 ml of encysting medium contained in 500-ml Erlenmeyer flasks and incubated on the shaker for an additional 2 days during which time 80 to 90% of the cells had encysted as determined by microscopic examination. The cysts were collected by centrifugation, washed once in a sterile 0.5% NaCl solution, and suspended once more in a 0.5% NaCl solution. The cysts were enumerated by using a Levy-Hausser hemacytometer, and the values presented represent the mean of six replicates.

Cyst wall isolation. To disrupt the cysts, a suspension containing 150 to 450 mg (dry weight) of cysts and 0.45- to 0.50-mm-diameter glass beads in a cold 0.5% NaCl solution was mixed in the sample flask of a Bronwill cell homogenizer in a 1:1:1 (vol/vol/vol) ratio. The homogenizer was operated at 4,000 rpm. Agitation was accomplished in short periods with intermittent rest periods to maintain the temperature at about 5°C. Cell breakage appeared complete as observed microscopically. The glass beads were removed by filtration through four thicknesses of cheesecloth, and the cyst walls were isolated by a modification of the method of Mahadevan and Tatum (8). Sodium laurylsulfate was added to the filtrate to a concentration of 1.0%. The mixture was stirred for 12 h at 4°C and then centrifuged at 10,000 × *g* for 3 min. The pellets were washed 20 to 25 times in distilled water and then twice in a 0.5% NaCl solution. Microscopic examination indicated that the walls were free of cytoplasm.

Enzyme studies. Chitinase and beef pancreas protease were obtained from ICN Life Sciences Group, Cleveland, Ohio. Purified cellulase derived from *Trichoderma viride* and purified β-1,3-glucanase de-

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rived from Basidiomycete 806 were obtained from E. T. Reese. The reaction mixtures contained 1.0 ml of enzyme preparation, 1.0 ml of a 0.01% merthiolate solution, 3.0 to 5.0 mg of walls, and buffer to a final volume of 5.0 ml. The buffers were: 0.50 M citrate buffer (pH 5.0) for cellulase and β-1,3-glucanase; 0.20 M phosphate buffer (pH 7.0) for chitinase; and 0.10 M tris(hydroxymethyl)aminomethane buffer (pH 7.8) for protease. The incubations were performed with shaking at 30°C for β-1,3-glucanase and at 37°C for the other enzymes.

Walls incubated with chitinase were assayed for *N*-acetylhexosamine formation according to the method of Reissig et al. (11). The product of protease hydrolysis was determined by the procedure of Spies (18). Walls incubated with cellulase and β-1,3-glucanase were assayed for the release of soluble carbohydrates by the procedure of Trevelyan and Harrison (19) and for glucose formation by the glucostat reagent (Worthington Biochemical Corp., Freehold, N. J.) using the method of the manufacturer.

Chemical determinations. Protein was measured according to the Folin phenol method (7). Samples (5 to 10 mg) of cyst walls were analyzed for their content of carbohydrates according to the method of Trevelyan and Harrison (19) and for reducing sugars by the procedure of Somogyi (17).

Tracer studies. The organisms were grown for 2 days in 300 ml of the peptone medium on a rotary shaker, the cells were harvested aseptically by centrifugation, and the protozoa were suspended in 300 ml of glucose-free peptone medium containing 0.1 mCi of [U-^{14}C]glucose (California Bionuclear Corp., Sun Valley, Calif.). The specific activity of the sugar was 240 mCi/mmol. The flasks were incubated on the rotary shaker at 30°C for an additional 2 days, and the cells were then collected by centrifugation, washed once in encystment medium, and resuspended in 300 ml of fresh encystment medium contained in a 500-ml Erlenmeyer flask. The flasks were incubated for an additional 2 days on the shaker at 30°C, during which time 80 to 90% of the cells encysted. The cysts were harvested, and their walls were isolated as described previously.

To study decomposition of the walls in soil, 1.4, 2.2, 3.1, and 2.9 g of labeled walls of *A. castellanii*, *Acanthamoeba* sp., *A. rhysodes*, and *A. palestinensis*, respectively, or 5.0 μCi of [U-^{14}C]glucose were separately mixed with 10 g of air-dried Williamson silt loam. The moisture was adjusted to 30% (wt/wt), and the soil was incubated at about 22°C. Air freed of CO_2 was passed over the soil at a rate of 10 to 15 ml/min, and the air emerging from the flask was passed through 95% ethanolamine, which was replaced at intervals. A portion of the ethanolamine that was removed was added to a scintillation vial containing a solution of 0.50% 2,5-diphenyloxazole and 10% naphthalene in *p*-dioxane. The radioactivity of the solution was determined in a Beckman liquid scintillation counter.

Survival in soil. A 1.0-ml portion of a 0.5% NaCl suspension of cysts was added to 2.0 g of Williamson silt loam contained in culture tubes (150 by 18 mm). The soil was adjusted to 30% moisture (wt/wt) and incubated at 25°C in a moist atmosphere. The protozoa in duplicate tubes were counted by a modification of a method of Singh (14). About 10 to 12 ml of sterile 1.5% agar containing 0.5% NaCl was poured into sterile, 150-mm-diameter petri dishes. Before the agar solidified, 10 sterile glass rings, 20 mm in diameter and 20 mm deep, were placed in each dish. After the agar solidified, 0.1 ml of a thick cell suspension from a 1-day-old *Enterobacter aerogenes* culture grown in peptone medium was placed on the agar within each ring, and 0.1-ml portions of successive, 10-fold soil dilutions were added to five of the rings, each dish thus containing five replications of two dilutions. After a period of 5 to 10 days of incubation at 30°C, the area in each ring was examined under the microscope for the presence of amoebae, and the number of protozoa was estimated by the most-probable-number technique. To obtain cyst counts, the trophozoites were killed before plating by treatment with 2% HCl for a period of 8 to 12 h as described by Cutler (4).

The data presented in the various studies represent the mean of at least three observations.

RESULTS

The walls isolated from cysts treated with the Bronwill homogenizer for 60 s appeared to be free of cytoplasmic contamination as determined by microscopic examination. Based on dry weight determinations, the percentages of cysts recovered as cyst walls ranged from 32 to 35% for the four organisms. The cyst walls of the protozoa contained 36 to 45% protein and 20 to 34% carbohydrate (Table 1).

Cellulase catalyzed the solubilization of 3.2 to 15.2% of the walls of the test species (Table 1). These percentages represent two-thirds of the carbohydrate fractions of *A. palestinensis* and *A. rhysodes* but less than one-sixth that of *Acanthamoeba* sp. and *A. castellanii*. Neither chitinase nor β-1,3-glucanase had an effect on the cyst walls of the four organisms; in these determinations, *N*-acetylhexosamine release was used to test for chitinase action, and glucose, reducing sugar, and soluble carbohydrate release were employed to assess the activity of β-1,3-glucanase.

The effect of protease on the cyst walls is also shown in Table 1. One-third or less of the walls of the microorganisms was solubilized by this enzyme. However, protease degraded more than half of the protein fraction of *A. castellanii*, *Acanthamoeba* sp., and *A. palestinensis* but little of that in *A. rhysodes* cyst walls.

When the intact cysts of the various organisms were incubated with cellulase, no hydrolysis products were detected. On the other hand, protease degraded components of the intact cysts, and 8.1, 10.0, 2.7, and 9.5% of the cyst weight of *A. castellanii*, *Acanthamoeba* sp., *A. rhysodes*, and *A. palestinensis*, respectively,

TABLE 1. *Composition and hydrolysis of the walls of four amoebae by cellulose and protease*

Protozoan	Wall components (%)		% Wall wt hydrolyzed by:		Protein hydrolyzed by protease (%)	Carbohydrate hydrolyzed by cellulase (%)
	Carbohydrate	Protein	Cellulase	Protease		
Acanthamoeba cattellanii	33.5	42.4	3.2	23.5	55.4	9.6
Acanthamoeba sp.	34.1	45.1*	5.1	32.0	71.0	15.0
Acanthamoeba rhysodes	20.1	40.7	13.9	10.0	24.6	69.2
Acanthamoeba palestinensis	22.8	36.2	15.2	30.1	83.1	66.7

were solubilized by this enzyme. After protease treatment, the cysts still did not take up the vital stain described by Singh (15). The intact cysts appeared morphologically unchanged after protease treatment.

The walls used in the previous experiments were prepared from cysts disrupted for 60 s. The disruption time was increased to 90 and 120 s to obtain a more finely divided wall preparation to serve as substrate. As indicated in Table 2, increasing the time of disruption reduced the quantity of wall constituents solubilized by the protease with three of the amoebae and the amount of carbohydrate digested by cellulase with two of the organisms. In view of the smaller amounts of the constituents solubilized enzymatically under identical conditions, presumably less substrate was available, and hence the substrate was removed by abrasion. It is clear from these data that the method and duration of disruption are critical for assessments of susceptibility of wall components.

The cyst wall residues (prepared from walls homogenized for 60 s) remaining after three successive 12-h incubations first with cellulase and then with protease were analyzed for protein and carbohydrate content to determine the relative proportions resistant to these enzymes. Table 3 shows the quantity of unavailable protein and carbohydrate remaining in the residues. The sums of the values for protein resistant and susceptible to protease are in general agreement with the total quantity of protein found in the walls by chemical analysis, the data being given in Tables 1 and 3. The same is true for the carbohydrates resistant and susceptible to cellulase and the total carbohydrate found by chemical analysis.

The release of $^{14}CO_2$ from ^{14}C-labeled cyst walls incubated in soil is shown in Fig. 1. The metabolism of [^{14}C]glucose was tested at the same time to show activity on a readily available substrate. The radioactivity added to soil was 4.3×10^7 cpm for glucose and 3.5×10^6 to 5.3×10^6 cpm for the four protozoa. The walls of *A. palestinensis* were decomposed reasonably quickly, and about 59% of the label was recovered at 72 h. The degradation of *A. rhysodes* walls was more gradual, and the maximum yield of $^{14}CO_2$ was about 35%. The cyst walls of the two other organisms were attacked slowly, and only 11 and 14% of the label were recovered as CO_2 from *A. castellanii* and *Acanthamoeba* sp., respectively, after 28 days.

TABLE 2. *Effect of disruption time on the susceptibility of protozoan walls to enzymatic digestion*

Protozoan	Disruption time (s)	% Wall hydrolyzed		
		By protease	By cellulase	
			Carbohydrate released	Glucose released
Acanthamoeba castellanii	60	23.7	3.0	1.1
	90	13.9	3.1	1.4
	120	3.7	3.7	1.3
Acanthamoeba sp.	60	31.5	5.0	2.5
	90	7.3	5.4	3.8
	120	5.9	5.0	1.9
Acanthamoeba rhysodes	60	9.9	13.7	6.0
	90	10.4	2.9	1.6
	120	8.1	3.0	1.6
Acanthamoeba palestinensis	60	29.8	14.8	5.0
	90	19.8	7.7	5.2
	120	5.8	7.6	5.2

TABLE 3. *Protein and carbohydrate content of cyst wall residues following hydrolysis by protease and cellulase*

Organism	Wt of original cyst wall that is enzyme resistant (%)		Wt of wall residue after enzymatic hydrolysis (%)	
	Protein	Carbohydrate	Protein	Carbohydrate
Acanthamoeba castellanii	17.0	34.1	23.2	46.5
Acanthamoeba sp.	12.1	30.5	19.2	48.5
Acanthamoeba rhysodes	27.5	6.7	36.1	8.8
Acanthamoeba palestinensis	7.0	6.1	12.8	11.2

To determine the population fluctuations of cysts and trophozoites of the amoebae in soil, samples of Williamson silt loam were amended with 5.5×10^5 to 8.5×10^5 cells per gram of the

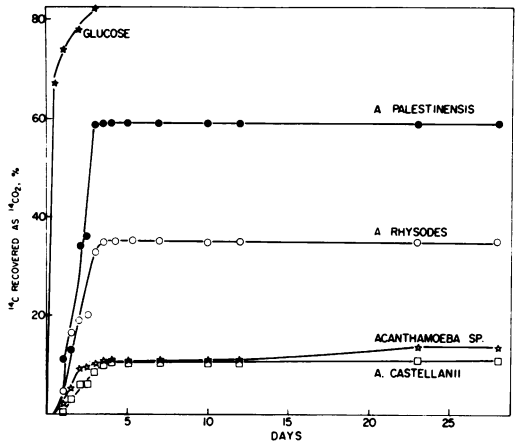

FIG. 1. *Formation of $^{14}CO_2$ from [^{14}C]glucose and walls of cysts of four amoebae added to Williamson silt loam.*

several amoebae. The count of native protozoa was 7×10^3 per gram and consisted only of cysts. At intervals, counts were made of cysts and total protozoa, the latter reflecting cysts plus trophozoites. The results in Fig. 2 indicate that the four amoebae grew after the initial excystation. Several days elapsed before appreciable excystation was evident, however. The number of trophozoites fell after several weeks, and the organisms remained largely, if not exclusively, in the cyst form during the latter part of the incubation period. It is noteworthy that the high cyst numbers persisted for the duration of the incubation period; for the introduced protozoa, the cyst count exceeded 10^5 per gram during the 77-day interval. By contrast, the numbers of cysts of indigenous protozoa never exceeded 10^4 per gram.

DISCUSSION

The present observations on the carbohydrate and protein content of the cyst walls of strains of *Acanthamoeba* sp. and *A. castellanii* are in general agreement with the findings of Neff and Neff (9). On the other hand, cyst walls of the related amoeba, *Naegleria gruberi*, are appreciably poorer in carbohydrate (21). The carbohydrate content of walls of *A. rhysodes* and *A. palestinensis* cysts is markedly different from the *Acanthamoeba* sp. and *A. castellanii* and is more close to that of *N. gruberi*.

Because *N*-acetylhexosamine was not released when the chitinase preparation was incubated with the walls, it is likely that chitin was not present. The absence of chitin is consistent with studies of the cyst walls of *Acanthamoeba* (R. J. Neff, W. F. Benton, and R. H. Neff, J. Cell Biol. 23:66A, 1964), *Didinium nasutum* (12), and *N. gruberi* (21). By contrast, Sachs (13) reported the presence of chitin in *Pelomyxa illinoisensis*. Similarly, the failure to observe glucose, reducing sugar, or soluble carbohydrate release in the presence of β-1,3-glucanase suggests that the walls either do not contain the glucan or that it exists at an inaccessible site. The ineffectiveness of cellulase against intact cysts indicates that cellulose ex-

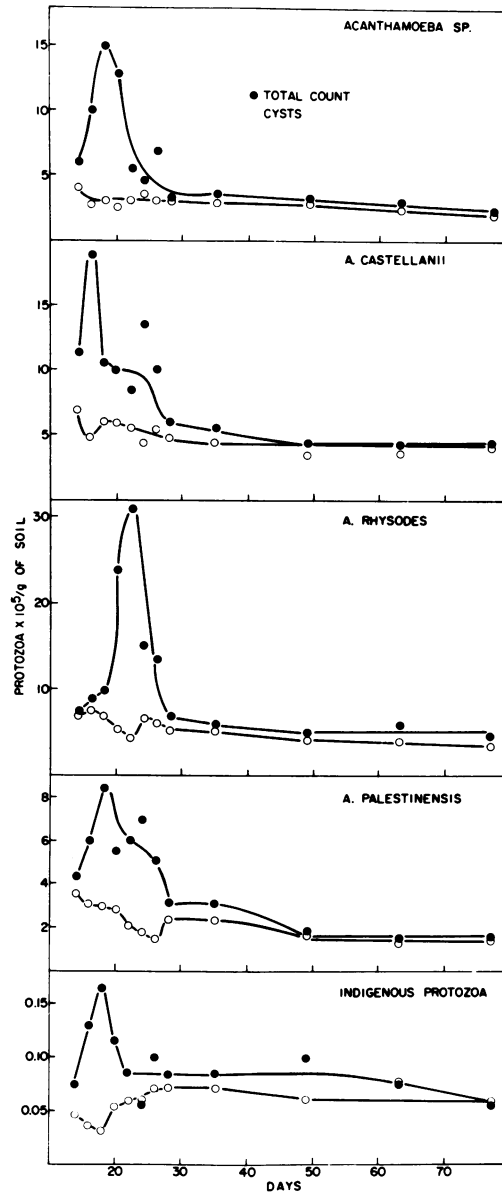

FIG. 2. *Changes in cyst and total protozoan counts in uninoculated Williamson silt loam and in soil receiving inocula of individual protozoa.*

ists as an inner and inaccessible layer of the wall, a view in line with the suggestion of Neff and Neff (9).

Inasmuch as the walls appear to account for one-third of the cyst weight and the percentage of the isolated wall material solubilized by protease was about three times greater than the percentage of the cyst weight solubilized by the same enzyme, it seems likely that the substrate for the enzyme was localized at the outside of the walls. These findings are in agreement with the proposal of Neff and Neff (9) that the cyst wall of *Acanthamoeba* sp. consists of an outer layer of protein.

The susceptibilities of the walls of the four protozoa to microbial degradation were quite different. However, these differences in resistance to microbial attack were not evident when the protozoa were added directly to soil, where the cysts of all the cultures persisted in large numbers for the 77-day incubation period. The explanation for the discrepancy is not readily evident, and further research is thus needed to explain the longevity of protozoan cysts in natural ecosystems.

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LITERATURE CITED

1. **Band, R. N.** 1959. Nutritional and related biological studies on the free-living soil amoeba, *Hartmannella rhysodes*. J. Gen. Microbiol. 21:80–95.
2. **Brzezinska-Dudziak, B.** 1954. Counting soil amoebae by a modified dilution technique of Singh (In Polish). Acta Microbiol. Pol. 3:121–124.
3. **Cutler, D. W.** 1919. Observations on soil protozoa. J. Agric. Sci. 9:430–444.
4. **Cutler, D. W.** 1920. A method for estimating the number of active protozoa in the soil. J. Agric. Sci. 10:135–143.
5. **Danso, S. K. A., S. O. Keya, and M. Alexander.** 1975. Protozoa and the decline of *Rhizobium* populations added to soil. Can. J. Microbiol. 21:884–895.
6. **Habte, M., and M. Alexander.** 1975. Protozoa as agents responsible for the decline of *Xanthomonas campestris* in soil. Appl. Microbiol. 29:159–164.
7. **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.
8. **Mahadevan, P. R., and E. L. Tatum.** 1965. Relationship of the major constituents of the *Neurospora crassa* cell wall to wild-type and colonial morphology. J. Bacteriol. 90:1073–1081.
9. **Neff, R. J., and R. H. Neff.** 1969. The biochemistry of amoebic encystment, p. 51–81. *In* H. W. Woolhouse (ed.), Dormancy and survival. Academic Press Inc., New York.
10. **Neff, R. J., S. A. Ray, W. F. Benton, and M. Wilborn.** 1964. Induction of synchronous encystment (differentiation) in *Acanthamoeba* sp., p. 55–83. *In* D. M. Prescott (ed.), Methods in cell physiology, vol. 1. Academic Press Inc., New York.
11. **Reissig, J. L., J. L. Strominger, and L. F. Leloir.** 1955. A modified colorimetric method for the estimation of N-acetylamino sugars. J. Biol. Chem. 217:959–966.
12. **Rieder, N.** 1973. Ultrastructure and histochemistry of the cyst wall of *Didinium nasutum* (Ciliata, Holotricha). Arch. Procesow Spalania 115:125–131.
13. **Sachs, I. B.** 1956. The chemical nature of the cyst membrane of *Pelomyxa illinoisensis*. Trans. Am. Microsc. Soc. 75:307–313.
14. **Singh, B. N.** 1955. Culturing soil protozoa and estimating their numbers in soil, p. 403–411. *In* D. K. McE. Kevan (ed.), Soil zoology. Butterworths Scientific Publications, London.
15. **Singh, B. N., T. Datta, and G. P. Dutta.** 1971. Factors inducing excystation in free-living amoebae. Indian J. Exp. Biol. 9:350–357.
16. **Skinner, C. E.** 1927. The effect of protozoa and fungi on certain biochemical processes when inoculated into partially sterilized soil. Soil Sci. 24:149–161.
17. **Somogyi, M.** 1952. Notes on sugar determination. J. Biol. Chem. 195:19–23.
18. **Spies, J. R.** 1957. Colorimetric procedures for amino acids, p. 467–477. *In* S. R. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 3. Academic Press Inc., New York.
19. **Trevelyan, W. E., and J. S. Harrison.** 1952. Studies on yeast metabolism. I. Fractionation and microdetermination of cell carbohydrates. Biochem. J. 50:298–303.
20. **Viswanath, G. K., and S. C. Pillai.** 1966. Occurrence and activity of protozoa in soil. Biochem. Rev. 37:7–15.
21. **Werth, J. M., and A. J. Kahn.** 1967. Isolation and preliminary chemical analysis of the cyst wall of the amoeba-flagellate *Naegleria grüberi*. J. Bacteriol. 94:1272–1274.