

The Cyst Wall Composition of *Hartmannella glebae* (41815)

J. M. UPADHYAY, S. CROW, AND A. COX

Department of Biological Sciences, Loyola University, New Orleans, Louisiana 70118

Abstract. In this study cyst walls of *Hartmannella glebae* were isolated and quantitatively analyzed. They were fractionated into alkali-insoluble and alkali-soluble fractions. The alkali-insoluble fraction appeared to be cellulose which represented 4.2% of the total weight of the wall. The major component of the alkali-soluble fraction consisted of proteins (64.7%). It also contained a glucose polymer (probably a precursor of cellulose) and lipids. The amino acid composition of the wall was also determined.

The chemical composition of the cyst walls of protozoans has been studied by several authors. Chitin has been claimed to be present in the cyst membranes of *Pelomyxa illinoisensis* (1). Tibbs (2) showed that the cyst walls of *Colpoda steinii* are primarily protein in composition. Cellulose is reported to make up one-third of the cyst wall of *Acanthamoeba* sp. (3). Werth and Kahn (4) in their studies on the walls of *Naegleria grüberi* showed that the walls had a relatively high protein content, readily extractable and tightly bound lipid components, as well as an alkali-insoluble material that appeared to be cellulose. According to Neff and Neff (5), the cyst wall of *A. castellanii* contained 33% protein and 35% carbohydrate (almost entirely cellulose).

This paper reports on quantitative aspects of the cyst wall of another soil amoeba, *Hartmannella glebae*.

Materials and Methods. *Culture and growth.* *H. glebae* was maintained monoaxenically in conjunction with *Enterobacter aerogenes* in our laboratory. For the preparation of the cysts of amoebae, several petri dishes containing brain heart infusion (BHI) agar were inoculated with a culture of amoebae. The petri dishes were incubated for 4 days at 30°C. *Enterobacter aerogenes* was also grown on BHI agar and incubated overnight at 35°C. Both the cysts and bacteria were collected and suspended in sterile water. A 2-ml amount of the mixture was used to inoculate BHI agar in large Pyrex dishes (3-qt size). These were incubated at 30°C for approximately 10 days to allow the encystment to be completed and to yield mature cysts.

The cysts were suspended in water and separated from bacteria by several differential centrifugations at 300g for 5 min. They were

further purified by filtering through Whatman No. 1 filter paper in a large Büchner funnel and washing with water several times to remove the remaining bacteria. This procedure was very effective in removing the bacterial cells. The cysts were examined in a phase-contrast microscope for purity and no bacterial cells were found. Representative cysts are shown in Fig. 1.

The cyst walls were ruptured in a Mickle disintegrator using an equal volume of No. 4 Ballotini beads. The disintegration was continued until the phase-contrast microscope examination showed complete rupturing. The cyst walls were subjected to differential centrifugation at 1200g for 5 min and washed several times with 0.1 M phosphate buffer, pH 7.0. They were suspended in 0.1 M phosphate buffer, pH 7.6, and digested at 37°C with trypsin (100 µg/ml) for 6 hr. After washing, they were digested with ribonuclease (100 µg/ml) in 0.1 M phosphate buffer, pH 7.6, at 37°C for 6 hr. The walls were washed several times with water and lyophilized. The purified walls are shown in Fig. 2.

Amino acids were determined by Roland Coulson, LSU Medical Center, New Orleans, Louisiana in a Modified Technicon Amino Acid Analyzer (6), after hydrolysis of 10-mg walls in 5 ml of 6 N HCl for 24 hr at 105°C in evacuated sealed tubes.

Aminosugars. Ten milligrams of cyst walls were heated in 6 N HCl in evacuated sealed tubes at 105°C for 14 hr. Excess HCl was removed in vacuum over P₂O₅ and NaOH. The total hexosamine content was estimated by the method of Randle and Morgan (7).

Sugars. Total carbohydrate was determined by the anthrone method (8).

Identification of monosaccharides was done

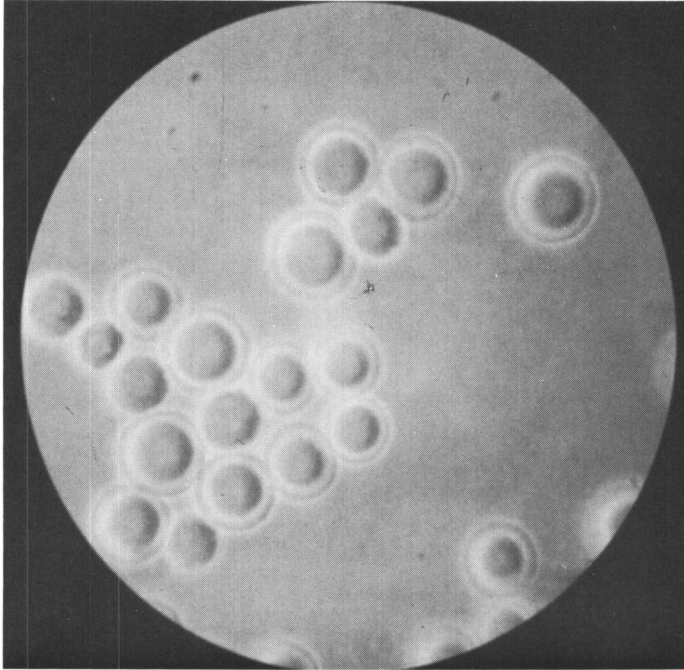


FIG. 1. Phase microscopy of purified cysts of *H. glebae*. $\times \sim 1370$.

by thin-layer chromatography on silica gel G (Merck) of the hydrolysates prepared from cyst walls or alkali-insoluble fraction in 2 *N* H₂SO₄

at 100°C for 2 hr. The hydrolysate was neutralized with Ba(OH)₂. The solvents used were: *n*-butanol:acetone:H₂O (4:5:1), ethyl ace-

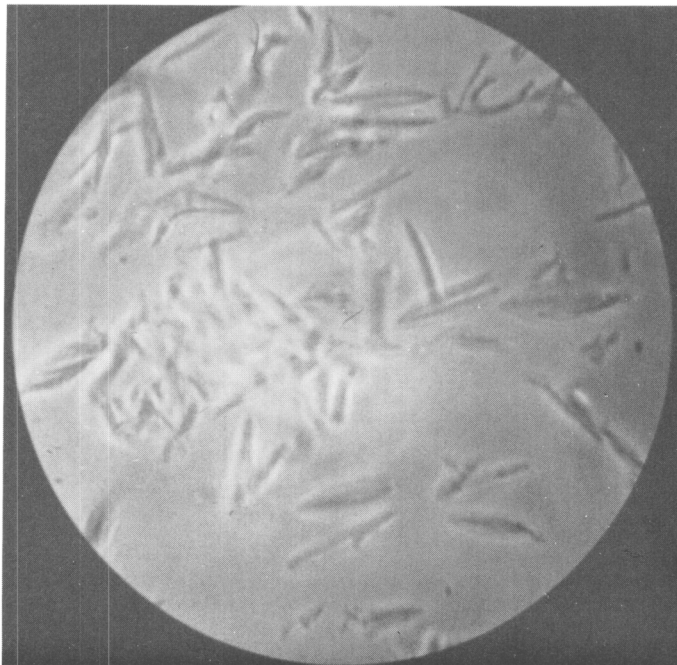


FIG. 2. Phase microscopy of cyst walls of *H. glebae*. $\times \sim 1370$.

tate:pyridine:acetic acid:H₂O (5:5:1:3), *n*-butanol:acetic acid:H₂O (4:1:1) and phenol:H₂O (3:1). Sugars were detected with aniline phthalate or ammoniacal AgNO₃. Glucose was assayed by the Glucostat method (Worthington Biochemical Corp., Freehold, N.J.).

Proteins. The protein content of the walls was determined by the method of Lowry *et al.* (9) using crystalline albumin (Sigma) as a standard.

Phosphorous. Total phosphate was estimated by the method of Fiske and Subbarow (10).

Lipids. The amount of total lipids was determined gravimetrically by the method of Toama and Raper (11).

Results. Essentially the method of Toama and Raper (11) was used for the fractionation of the cyst walls. The procedure is outlined in Fig. 3. A 76.7-mg sample of the cyst walls was extracted with 5 ml of 5% NaOH for 12 h at room temperature in a test tube (2 × 15 cm) with continuous shaking. The insoluble material was centrifuged and treated in the same manner as above for the second time

followed by a third treatment with 5 ml of 15% NaOH for 12 hr. The alkali-insoluble material was collected and washed several times with distilled water, lyophilized, and weighed. It constituted 4.2% of the cyst wall. The direct anthrone determination gave a value of 100% using glucose as a standard. Thin-layer chromatography of 2 *N* H₂SO₄ hydrolysates showed the presence of only one spot which was glucose. The fraction was nitrogen free and almost entirely dissolved in Schweitzer's reagent. From these findings it has been tentatively identified as cellulose. Tomlinson and Jones (3) found the cyst wall of *Acanthamoeba* sp. to contain 28% cellulose and Neff and Neff (5) reported the cyst wall of *A. castellanii* possessing 35% cellulose.

The protein content in the whole cyst wall, as determined from the alkali-soluble fraction, was 64.7%. It corresponds well with the protein value (66.25%) calculated from the data on amino acid analysis in which nitrogen content of each amino acid was multiplied by 6.35 to estimate the total protein weight.

The amino acid composition of the cyst

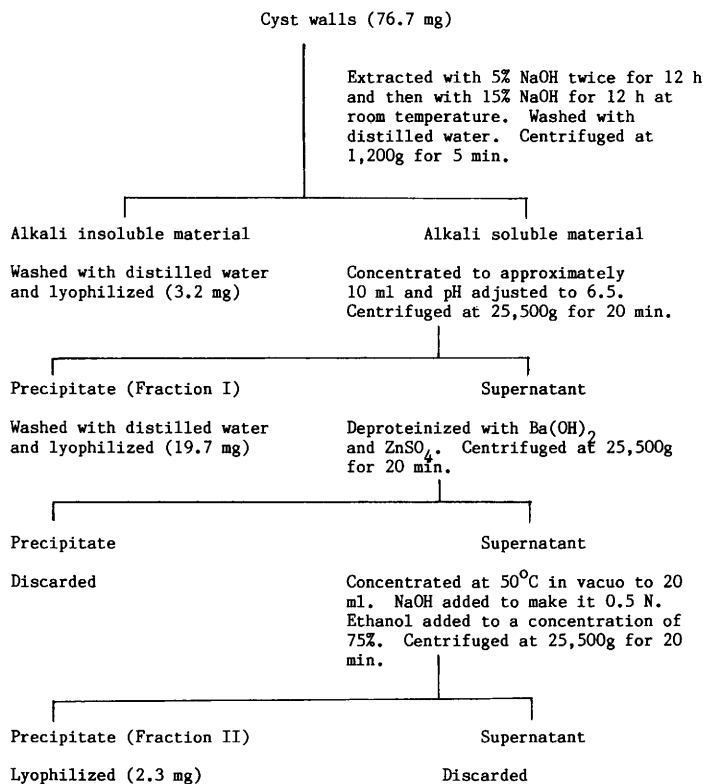


FIG. 3. Fractionation scheme of cyst walls of *H. glebae*.

walls is presented in Table I. Glycine content was the highest (14.6%). Valine, serine, and threonine have high values, each representing more than 10% of the total amino acids. No aminosugars were detected.

The total carbohydrate content in the walls was 8.2%; total lipids 5%; ash, 1.3%; and phosphorous (as H_2PO_3), 0.08%.

The alkali-soluble material was fractionated further using the method of Potter and Weisman (12) and Toama and Raper (11). The supernatant and the washings (98 ml) after alkali treatment of the walls were concentrated to approximately 10 ml *in vacuo*. The pH was adjusted initially with 12 *N* and then with 1 *N* HCl to 6.5. The precipitate was formed. It was kept overnight at 4°C after which it was centrifuged at 25,500*g* for 20 min and washed three times with water. This fraction (Fraction I) was lyophilized (19.7 mg).

After removal of the precipitate (Fraction I) the centrifugate was deproteinized by the addition of $Ba(OH)_2$ and $ZnSO_4$ (13). The $Zn(OH)_2$ and $BaSO_4$ precipitate was removed by centrifugation at 25,500*g* for 20 min. This process removed 90% of the Folin-reactive material. The supernate was concentrated at 50°C *in vacuo* to 20 ml. Enough NaOH was added to the concentrate to make it to 0.5 *N*. Ethanol was then added to a concentration of 75% to precipitate the polysaccharide fraction

(11, 14). The mixture was left overnight at 4°C. The precipitate (Fraction II, 2.3 mg) was formed. It was removed by centrifugation at 25,500*g* for 20 min and the supernate was discarded.

Fraction I was found to contain carbohydrate (5.55% anthrone value). The sugar chromatogram of the H_2SO_4 hydrolysate showed only one spot which was identical to the glucose standard. Also it was positive for glucose when tested with the glucostat reagent. It is thought to be a glucose polymer which may be a precursor of cellulose as suggested by Potter and Weisman (15). The 6 *N* HCl hydrolysate (24 hr) of this fraction also showed six amino acid spots. It is not known whether it represents the presence of a peptide in this fraction. No attempt was made to identify them. Aminosugars were not detected. It also contained lipids which were confirmed by staining with an alcoholic solution of Sudan black.

Fraction II contained 10% nonremovable Folin-reactive material and lipids. The sugar chromatogram of this fraction showed a weak spot which corresponded to glucose and another pink spot which indicated the presence of pentose but did not correspond with sugars ribose, arabinose, and xylose.

Discussion. Comparative data on the chemical composition of the cyst walls from *H. glebae*, *A. castellanii* (5), *N. grüberi* (4), and another unrelated organism, the cellular slime mold *Polysphondylium pallidum* (11) are summarized in Table II. Compared to a high percentage of cellulose found in the cyst walls of *A. castellanii* and *P. pallidum*, the walls of *H. glebae* contained only 4% alkali-insoluble material that appears to be cellulose, but the percentage of protein (64.7%) was much higher.

Neff and Neff (5) identified galactosamine in the cyst walls of *A. castellanii* whereas no aminosugars were found in the cyst walls of *H. glebae*.

Of the macromolecules studied during encystment in amoebae glycogen is most rapidly degraded. Only 10–20% of glycogen found in trophozoites is found in cysts of *A. castellanii* (16, 17). Potter and Weisman (12, 15) reported the presence of alkali-soluble and alkali-insoluble material as β -glucans (cellulose) in the cyst walls of *A. castellanii*. It is suggested by Weisman (17) that the alkali-soluble glucan

TABLE I. AMINO ACID COMPOSITION OF THE CYST WALLS OF *HARTMANNELLA GLEBAE*

Amino acid	$\mu\text{mol/mg}$	Percentage of total
Aspartic acid	0.638	9.15
Threonine	0.751	10.75
Serine	0.751	10.75
Glutamic acid	0.448	6.43
Proline	0.533	7.65
Glycine	1.020	14.60
Alanine	0.515	7.38
Cystine	0.010	0.14
Valine	0.839	12.00
Methionine	0.010	0.14
Allo-isoleucine?	0.001	0.01
Isoleucine	0.230	3.29
Leucine	0.506	7.24
Tyrosine	0.077	1.10
Phenylalanine	0.424	6.08
Ornithine	0.002	0.03
Lysine	0.029	0.41
Histidine	0.012	0.17
Arginine	0.184	2.64

TABLE II. THE CHEMICAL COMPOSITION OF CYST WALLS OF A VARIETY OF AMOEBAE

Component	Percentage dry weight			
	<i>Hartmannella glebae</i>	<i>Acanthamoeba castellanii</i> (Ref. 5)	<i>Naegleria grüberi</i> (Ref. 4)	<i>Polysphondylium pallidum</i> (Ref. 11)
Total carbohydrate	8.20	35	ND ^a	55
Cellulose	4.20	35 ^b	14	24
Protein	64.70	33	40	31
Total lipids	5.00	4.5–6.5	13	21
Phosphate (as H ₂ PO ₃)	0.08	2.1	ND	ND
Ash	1.30	7.8	ND	ND

^a ND, not determined.

^b Neff and Neff assumed 35% total carbohydrate to be almost entirely cellulose because it agrees with Tomlinson's measurement of cellulose which was 33% in *Acanthamoeba* sp. (3).

may be a precursor for the formation of cellulose (alkali-insoluble fraction). The glucose polymer found in the alkali-soluble fraction of the cyst walls of *H. glebae* is suspected to be a similar β -glucan. In a cellular slime mold, *Dictyostelium discoideum* cellulose and cell wall-bound glycogen are present only in insignificant quantities in the myxamoebae (14). The accumulation of cellulose and glycogen represents the major changes that occur in the gross chemical composition of the cell from the vegetative to the microcyst stage (11). Both cellulose and glycogen-like material were found in the cyst walls of another slime mold, *P. pallidum* by these authors.

We thank Dr. E. L. Beard for reading the manuscript. We are also grateful to Dr. Robert A. Weisman for reviewing the manuscript and making comments and suggestions. This research was supported by the Edward G. Schlieder Educational Foundation, New Orleans, Louisiana, undergraduate research grants from the American Heart Association-Louisiana, Inc., and the American Cancer Society, New Orleans, Louisiana.

- Sachs IB. The chemical nature of the cyst membrane of *Pelomyxa illinoisensis*. *Trans Amer Microsc Soc* **LXXV**:307–313, 1956.
- Tibbs, J. The cyst wall of *Colpoda steinii*. *Biochem J* **98**:645–651, 1966.
- Tomlinson G., Jones EA. Isolation of cellulose from the cyst wall of a soil amoeba. *Biochim Biophys Acta* **63**:194–200, 1962.
- Werth JJ, Kahn AJ. Isolation and preliminary chemical analysis of the cyst wall of the amoeba-flagellate *Naegleria grüberi*. *J Bacteriol* **94**:1272–1274, 1967.
- Neff RJ, Neff RH. The biochemistry of amoebic encystment. *Symp Soc Exp Biol* **23**:51–81, 1969.
- Coulson, RA, Hernandez T, Buyers V. A simple two-column system for the rapid analysis of plasma amino acids. *Anal Biochim* **10**:159–162, 1965.
- Rondle CJM, Morgan WTJ. The determination of glucosamine and galactosamine. *Biochem J* **61**:587–589, 1955.
- Umbreit WW, Burris RH, Stauffer JF. *Manometric techniques*. Minneapolis, Burgess, 4th ed, p210, 1964.
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**:265–275, 1951.
- Fiske C, SubbaRow T. The colorimetric determination of phosphorous. *J Biol Chem* **66**:311–338, 1925.
- Toama MA, Raper KB. Microcysts of the cellular slime mold *Polysphondylium pallidum*. *J Bacteriol* **94**:1150–1153, 1967.
- Potter JL, Weisman RA. Differentiation in *Acanthamoeba*: β -glucan synthesis during encystment. *Biochim Biophys Acta* **237**:65–74, 1971.
- Somogyi M. Determination of blood sugars. *J Biol Chem* **160**:69–73, 1945.
- White GJ, Sussman M. Polysaccharides involved in slime-mold development. I. Water-soluble glucose polymer(s). *Biochim Biophys Acta* **74**:173–178, 1963.
- Potter JL, Weisman RA. Correlation of cellulose synthesis in vivo and in vitro during the encystment of *Acanthamoeba*. *Dev Biol* **28**:472–479, 1972.
- Bowers B, Korn E. Fine structure of *Acanthamoeba*. II. Encystment. *J Cell Biol* **41**:786–805, 1969.
- Weisman RA. Differentiation in *Acanthamoeba castellanii*. *Ann. Rev Microbiol* **30**:189–219, 1976.

Received July 12, 1983. P.S.E.B.M. 1984, Vol. 175.

Accepted December 13, 1983.