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Microcysts of the Cellular Slime Mold *Polysphondylium pallidum*[1]

II. Chemistry of the Microcyst Walls

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Methods are described for obtaining large masses of myxamoebae, for inducing these to form microcysts, and for the isolation of the microcyst walls from other cell components. The walls were fractionated into two parts, one alkali-soluble, the other alkali insoluble. The alkali-insoluble fraction is a type of cellulose and constitutes 28% of the microcyst wall by weight. The alkali-soluble fraction contains a glycogenlike material, lipids, and proteins. A possible mechanism of microcyst wall synthesis is discussed.

In the preceding communication (13), the precise environmental factors that influence microcyst formation in *Polysphondylium pallidum* were described. During the process of encystment, the myxamoebae form rigid walls. The chemical nature of these walls has not been investigated previously, except for the tentative demonstration of cellulose (3) by staining reagents such as congo red and chloroiodide of zinc. Accordingly, a study of the chemistry of the cyst wall was undertaken in order to understand the biochemical events underlying the process of encystment.

MATERIALS AND METHODS

Growth and preparation of the myxamoebae. The method of cultivation previously described (13) was impractical for growing large quantities of myxamoebae. Accordingly, another method was devised. *Escherichia coli* strain B/r was grown in 500-ml Erlenmeyer flasks, each containing 100 ml of a growth medium: Tryptose (Difco), 5 g; yeast extract (Difco), 5 g; dextrose, 1 g; $K_2HPO_4 \cdot 3H_2O$, 1 g; and tap water to 1,000 ml. The *p*H was adjusted to 7.0 and the medium was autoclaved for 20 min at 121 C. The flasks were inoculated with *E. coli* B/r and were incubated on a rotary shaker at 30 C. After 19 to 24 hr of incubation, the bacteria were centrifuged, washed twice with water, resuspended in phosphate buffer (0.015 M at *p*H 6.2) at a concentration of 0.5×10^{10} to 1.0×10^{10} bacteria/ml, and autoclaved. Portions of the autoclaved suspension of bacteria (100 ml in 500-ml Erlenmeyer flasks) were then inoculated with 2×10^4 to 3×10^4 myxamoebae/ml of *P. pallidum* Pan-17, and the cultures were incubated at 30 C on a rotary shaker at 250 rev/min. After 32 to 36 hr, the myxamoebae had reached the stationary phase at a population density of 4×10^6 to 6×10^6 myxamoebae/ml. The myxamoebae were centrifuged, washed with distilled water, and then transferred to a KCl solution, the microcyst-inducing medium, at a concentration of 5×10^7 cells/ml.

Induction of microcyst formation. The optimal KCl concentration for microcyst production was 0.12 M and the maximal percentage of microcysts was 86 ± 4% after 24 hr. This level of KCl is different from that (0.08 M) reported previously (13), and resulted from the different methods employed for growing the myxamoebae. The optimal concentration of KCl needed for encystment, and the percentage of microcysts subsequently formed, were thus shown to be influenced by nutritional and environmental conditions during cell growth. After encystment, the microcysts were washed with water, resuspended in KCl (0.12 M), and returned to the shaker for 2 days to ensure complete maturation.

Preparation of the cyst walls. The microcysts were suspended in 1.0 M KCl at a density of 1.5×10^9 microcysts/ml, then ruptured in a Sonifier at maximal power (8 amp) for two successive periods of 3 min each. Rupturing of the microcysts was done with a Branson Sonifier, Model S 125 (Branson Instrument Inc., Danbury, Conn.). The ruptured cysts were subjected to differential centrifugation and washed several times in double-distilled water at $50 \times g$ for 5 min and at $350 \times g$ for 20 min in a swinging-bucker centrifuge. The fraction of cyst walls subjected to chemical analysis was that obtained between 50 and $350 \times g$. The purity of the microcyst wall preparation was above 99%. Microcysts and cell-free microcyst walls, as seen with a phase-contrast microscope, are shown in Fig. 1.

Reagents. A standard cellulose, cellulose II, was

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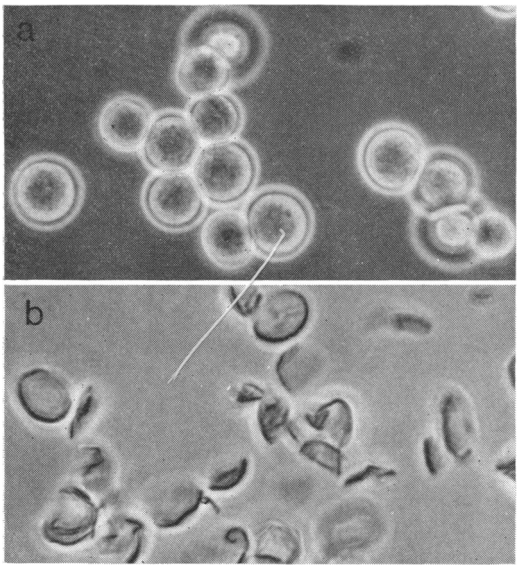

FIG. 1. *(A) Microcysts of Polysphondylium pallidum, Pan-17, formed from pregrown myxamoebae in KCl (0.12 M) solution in shaken flasks. Phase microscopy.* × *1,620. (B) Microcyst walls prepared by sonic treatment and differential centrifugation. Phase microscopy.* × *1,620.*

prepared from pure cellulose (2) according to the method of Corbett (5). α-Amylase and β-amylase were obtained from Calbiochem, Los Angeles, Calif. Schweitzer's reagent (10) was prepared to contain 13.5 g of copper per liter final concentration.

Analytical procedures. Nitrogen content was determined by the Dumas method (12). Protein content was determined by the Folin reagent method (1).

Total carbohydrate was determined by the anthrone method (16). Descending paper chromatography was conducted at 25 C with *n*-butanol-pyridine-water (6:4:3) on Whatman no. 1 paper (9). The indicator spray was ammoniacal silver nitrate (15). Glucose was determined by the Glucostat method (Worthington Biochemical Corp., Freehold, N.J.). Hexosamine content was determined by a modification of the Elson-Morgan reaction (19). Amylase treatment was carried out according to a modification of the Bernfeld method (14). Samples were prepared for infrared analysis according to the KBr-pellet technique as described by Baker (2). Through the courtesy of S. Bailey, Department of Geology, University of Wisconsin, X-ray diffraction exposures were made with Cu K α radiation for 16 hr.

Acid hydrolysis of the cyst walls, or of the alkali-insoluble fraction, was accomplished by dissolving 5 mg of the material in 0.1 ml of 72% H_2SO_4 at room temperature for 24 hr; water was then added to bring the acid concentration to 1.0 N. The material was further hydrolyzed by heating it in a water bath at 95 C for 6 hr under an atmosphere of nitrogen. Hydrolysis of the alkali-soluble fraction was accomplished by heating it in 1.0 N HCl at 95 C for 2 hr. The hydrolyzed material was lyophilized and redissolved in water twice, to remove excess HCl.

Extractable lipids were determined gravimetrically on 20 mg of microcyst walls. Extraction of the material was carried out in a Soxhlet apparatus, with a solvent mixture of ethyl alcohol and ether(1:1) for 24 hr, followed by chloroform and methanol (1: 1) for 24 hr. To determine total lipids, the sample was acidified by HCl to 6.0 N, and then heated at 95 C for 6 hr. The lipids were extracted by shaking several times with ether in a separatory funnel, after which the ether extract was dried and the percentage of lipids was determined gravimetrically. To detect the presence of lipids, a staining method based upon Sudan Black was used (4). The material was spotted on Whatman no. 1 paper, and the paper was immersed in an alcoholic solution of Sudan Black (100 mg of Sudan Black dissolved in 100 ml of 50% alcohol, boiled, and filtered) for 1 hr. The paper was then washed several times in 50% alcohol. The lipid material was stained blue.

RESULTS

Fractionation of the microcyst walls. After it was lyophilized for 15 hr, the wall material (60 mg) was extracted with alkali (5 ml of 5% NaOH solution) for 12 hr in a test tube (15 × 125 mm). The insoluble material was centrifuged and a similar alkali treatment was carried out, followed by a third treatment with 5 ml of 15% NaOH for 12 hr. The alkali-insoluble material was separated and washed by centrifugation five successive times, each with 3 ml of double-distilled water. The alkali-insoluble material was then lyophilized and weighed.

The washing solutions from the alkali-soluble fraction were added to the alkali extract and together these constituted the alkali-soluble fraction. This was concentrated in vacuo to half its volume and acidified by HCl (6.0 N) to *p*H 6.5. A precipitate was formed. After it was left overnight at 4 C, the precipitate was centrifuged and washed twice with water. This precipitate (F_1) can be obtained, also, by adding alcohol to 40% to the alkali-soluble fraction.

After removal of the precipitate (F_1), the supernatant liquid was deproteinized by the addition of $Ba(OH)_2$ solution 0.3 N and an equimolar amount of $Zn(SO_4) \cdot 7H_2O$ (11), whereupon $Zn(OH)_2$ and $BaSO_4$ precipitates were formed. After centrifugation of the latter precipitates, the supernatant liquid was concentrated in vacuo to 20.0 ml. The process of deproteinization removed 95% of the Folin reactive material. To the concentrated supernatant liquid, NaOH was added to a concentration of 0.5 N, and alcohol to 75%. The mixture was left overnight in a refrigerator and a precipitate (F_2) was formed. This was removed by centrifugation and the supernatant liquid was discarded.

General chemistry of the cyst wall. The total carbohydrate of the wall, as determined by the direct anthrone method without prior hydrolysis, is 55%, with glucose as a standard. The carbohydrate content determined after acid hydrolysis was 33%. The low percentage obtained after hydrolysis indicates that a considerable degradation had occurred during the acid hydrolysis.

The percentage of nitrogen was 5.85%. The percentage of protein in the whole microcyst wall, as determined from the alkali-soluble fraction, was 31%, with human serum albumin as standard. It was found that the preparation should be neutralized before determination of the percentage of protein in the alkali-soluble fraction, because the color reaction disappears rapidly.

The total lipids was 21%, of which 11% represented extractable lipids and 10% represented bound lipids. It is not known whether the lipid was derived entirely from the microcyst walls or whether it came in part from adherent membranes.

The microcyst wall contained no hexosamines, as was determined on the alkali-soluble fraction by the modified method of the Elson-Morgan reaction.

Alkali-insoluble fraction. The alkali-insoluble fraction constituted 24% of the cyst wall. Direct anthrone determination gave a value of 100%, with glucose as a standard. The fraction was nitrogen-free, completely soluble in Schweitzer's reagent, and precipitable by glacial acetic acid at *p*H 6.5.

Identification of the monosaccharides constituent was accomplished after acid hydrolysis of the fraction. The hydrolysate was treated with $Ba(OH)_2$ and was then centrifuged. The supernatant fluid was passed through an ion-exchange column containing Dowex 50 resin in the hydrogen form. The eluate was concentrated and identified by paper chromatography. Only one spot was detected and this corresponded to authentic glucose. This was further confirmed by comparing the quantitative anthrone value with that of the Glucostat reagent.

The alkali-insoluble fraction was resistant to the effects of α- and β-amylases, as compared with soluble starch.

A fingerprint of the alkali-insoluble fraction was compared with that of cellulose II by using X-ray diffraction (Fig. 2) and infrared spectroscopy (Fig. 3). The two products appear to be similar.

Pure cellulose will give 85% of alkali-insoluble material if it is treated with alkali in the same manner as the microcyst walls; it is thus concluded that the wall contains 28% cellulose.

Alkali-soluble fraction. Subfraction F_1 was found to be composed of carbohydrates and lipids. The lipid part was absent when the microcyst walls were defatted before fractionation. The carbohydrate part gave 90% anthrone value, with glucose as a standard. The sugar chromatogram of the hydrolysate showed only one spot, which corresponded to glucose and was confirmed by the Glucostat reagent. It is thus concluded that the carbohydrate part is a glucose polymer and may be a glycogen-like material.

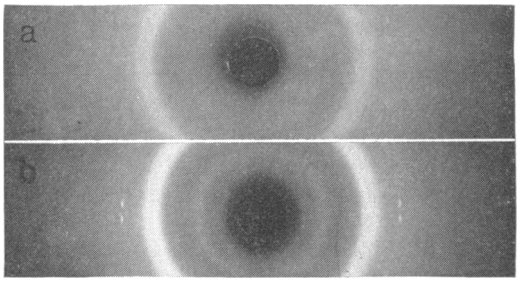

FIG. 2. *Comparison of the X-ray diffraction patterns of the alkali-insoluble fraction (A) and of standard cellulose (B).*

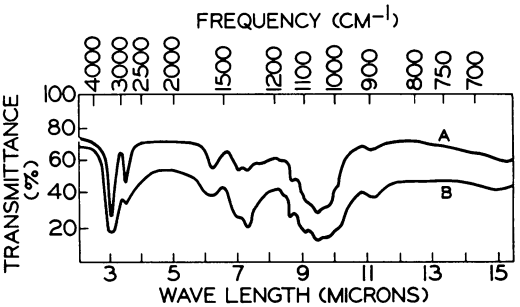

FIG. 3. *Comparison of the infrared spectrum of the alkali-insoluble fraction (A) with that of standard cellulose (B).*

Subfraction F_2 was found to be mainly lipoidal. Associated with this fraction is the 5% nonremovable Folin reactive material. The sugar chromatogram of this subfraction showed a weak spot which corresponded to glucose and another spot which was not identified. Color tests such as the Elson-Morgan reaction, carbazole, and secondary cysteine reaction (7), indicated the absence of hexosamines, galactose, and uronic acids. The lipid part was present, whether or not the microcyst walls were previously defatted.

DISCUSSION

A clean preparation of the microcyst walls was obtained as shown by the phase-contrast microscope (Fig. 1b). The wall constitutes about 35% of the microcyst by weight, and this has been fractionated into two parts, one alkali-soluble, the other alkali-insoluble.

The alkali-insoluble fraction is a type of cellulose as evidenced by chemical tests, chromatographic and enzymatic techniques, X-ray diffraction, and infrared spectroscopy. It constitutes 28% of the wall by weight. It appears that cellulose is present in a low crystalline order, as evidenced from the X-ray diffraction pattern. A similar situation was reported earlier for the cellulose of *Dictyostelium* (8). The original shape of the microcyst wall is dependent upon the alkali-insoluble fraction, indicating that cellulose represents the "backbone" of the cyst wall. It is probably present in an intermeshed complex with other wall constituents.

The alkali-soluble fraction contains a glucose polymer, lipids, and proteins. The glucose polymer is probably a glycogen-like material. Previous reports have indicated presence of a glycogen-like material in the sorocarps of several species of the cellular slime molds (18), and in *Dictyostelium discoideum* it was formed in vitro from uridine diphosphate glucose and cell husk extract (17). It is not known whether protein and lipids occur as lipoproteins, because 5% of the protein was still associated with the lipid fraction after deproteinization.

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. Cellulose and cell wall bound glycogen are present only in insignificant quantities in the myxamoebae (18). Proteins and lipids are present particularly in the membrane of the amoeboid cells. However, it is not known if the classes of lipids present in the myxamoebae (6) and those in the microcysts are the same. Quantitative analyses would be needed to establish similarities and differences.

It was shown by Ward and Wright (17) that the synthesis of the cell-wall glycogen is catalyzed by an enzyme (polymerase) which is bound to the cell wall. It is thus conceivable that, in microcyst wall synthesis, the membrane acting as a template becomes part of the wall and then serves as the site of activity for the enzymes synthesizing glycogen and cellulose.

The mechanism by which osmotic pressure is believed to induce microcyst formation will be discussed in the third paper of this series.

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