BIOCHEMICAL CHANGES DURING GROWTH AND ENCYSTMENT OF THE CELLULAR SLIME MOLD *POLYSPHONDYLIUM PALLIDUM*

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ABSTRACT

The growth of the cellular slime mold, *Polysphondylium pallidum*, was studied on a semidefined medium in shaken suspension. When the medium contained large quantities of particulate material, growth was more rapid and the cellular size and protein content were smaller than when growth occurred on a medium containing less particulate material. The cellular levels of DNA, RNA, and protein; of lysosomal enzymes (acid phosphatase, acid proteinase); and of peroxisomal enzymes (catalase) were assayed during growth and the subsequent stationary phase that led eventually to encystment. Only DNA remained at a constant cellular level. Encystment of exponentially growing cells could also be initiated by washing them and introducing them into a soluble peptone medium. The rate of encystment was proportional to the osmolarity of this medium. The encystment process was followed with respect to the cellular levels of DNA, RNA, protein, carbohydrates, acid phosphatase, acid β-*N*-Ac-glucosaminidase, and catalase. The most dramatic change occurred in the cellular cellulose content, which increased by at least an order of magnitude by the time encystment was morphologically complete. It was concluded that the encystment of this slime mold in suspension exhibits a number of biochemical similarities to the development of this and other cellular slime molds on a surface.

INTRODUCTION

The cellular slime mold has received considerable attention in recent years as a model system for studies of differentiation in animal cells. Although most of the published work has utilized *Dictyostelium discoideum*, another species of slime mold, *Polysphondylium pallidum*, is an attractive organism for study. The amoebae of one strain of *P. pallidum* can be grown rapidly and in high yield on axenic media (18, 19, 20, 34). (*D. discoideum* may also be grown axenically [e.g. 42], but with a longer generation time.) When the amoebae of *P. pallidum* are placed under starvation conditions, each individual cell is capable of encystment (37), in addition to the sequence of cell aggregation and fruiting body formation which has received much attention in *D. discoideum*.

Encystment, leading to only one final cell type, is a simpler developmental process than fruiting body formation on a surface, which leads to two final cell types: spores and stalk cells. Also, since encystment occurs as a single-cell process in shaken suspension, the environment of each cell can be more closely controlled than is possible during development on a surface. Finally, since *P. pallidum* is capable of both encystment and fruiting body formation, one may ask to what extent these de-

velopmental sequences differ, and perhaps assess the importance of cell-cell contact in directing development in this organism.

It would also be of interest to compare in more detail the biochemistry of differentiation in these two types of cellular slime mold. They exhibit similar morphogenetic patterns (6), but at the same time, they possess different aggregating hormones (22) and different development-specific surface antigens (e.g. 13), that may be responsible for genus-specific aggregation during development (28). All of these considerations recommend *P. pallidum* as a model system for developmental studies.

This paper will present an analysis of some aspects of growth and encystment of *P. pallidum* in shaken suspension.

MATERIALS AND METHODS

Organism

The cellular slime mold *P. pallidum* WS-320 was used in this study. Fruiting bodies of this strain were kindly provided by Professor M. Sussman of Brandeis University, Waltham, Mass.

Media

The cells were grown on a medium (GM)[1] containing 2.5 g milk powder (Sanalac, Sanna Dairies, Inc., Madison, Wis.), 0.2 g soybean lecithin (RG lecithin, Central Soya, Chemurgy Division, Chicago, Ill.), 10.0 g proteose peptone (Difco Laboratories, Detroit, Mich.), and 0.2 g yeast extract (Difco Laboratories) in 800 ml of 12.5 mM phosphate buffer (pH 6.35), supplemented with 200 ml of standard medium (SM) (see below), at a final pH of 6.5 (34; Sussman, M. 1967. Personal communication.). The milk powder, the lecithin, and the remainder of the ingredients were prepared as three separate solutions for autoclaving. The three sterile solutions were mixed immediately before use, and 80,000 U of penicillin G and 100 mg of streptomycin sulfate (Strep-Dicrysticin, E. R. Squibb and Sons, New York) were usually added per 1000 ml of GM to assure sterility. The SM contained 10.0 g Bactopeptone (Difco Laboratories) 10.0 g glucose, and 1.0 g $MgSO_4.7H_2O$ in 1,000 ml of 10 mM phosphate buffer, pH 6.5 (modified from 33). This medium was autoclaved and supplemented with antibiotics when long incubations were required. The basic salt solution (BSS) contained 0.6 g NaCl, 0.75 g KCl, and 0.3 g $CaCl_2$ in 1,000 ml H_2O (5).

[1] *Abbreviations used in this paper are*: BSA, bovine serum albumin; BSS, basic salt solution; GM, growth medium; SM, standard or starvation medium.

Growth and Counting of Cells

An inoculum culture of amoebae was prepared by scraping a few fruiting bodies off a stock plate (prepared as described below) with a wire loop and suspending them in 10 ml of GM in a 40-ml test tube, all under sterile conditions. The tube was incubated at 24 ± 1°C on a New Brunswick rotary shaker (New Brunswick Scientific Co., Inc., New Brunswick, N. J.) at 250 rpm under constant illumination. When the growing amoebae reached a concentration of 2–8 × 10^6 cells/ml, 500 ml of GM in a 1,500-ml Erlenmeyer flask was inoculated to a final concentration of 1–2 × 10^4 cells/ml and was incubated on the shaker as above. Growth was followed by periodically making replicate cell counts (four to eight chambers) in an A. O. Spencer "Brightline" hemacytometer (American Optical Corp., Scientific Instrument Div., Buffalo, N. Y.) at a magnification of 320× using a Zeiss microscope with phase contrast optics.

Harvesting and Washing of Cells

The cells were usually harvested after about 36 h of incubation during the exponential phase of growth (usually between 1 × 10^6 and 5 × 10^6 cells/ml) by centrifugation at about 1000 *g* for 4 min in a refrigerated centrifuge (model B-20, International Equipment Company, Needham Heights, Mass.). The cells were washed by vigorously resuspending them in cold SM with a 10-ml blowout pipette. They were again collected by centrifugation at top speed in a table model clinical centrifuge at room temperature. This washing procedure was repeated until the cells and the wash supernate appeared free of particulate or granular material. The washed cells were then resuspended in a moderate volume of incubation medium (usually SM), the cell concentration was determined, and more medium was added to adjust the cell concentration as desired.

Maintenance of Stock Culture

New stocks of fruiting bodies were prepared by growing a flask culture of amoebae to early stationary phase, harvesting the cells by centrifugation, and plating them directly (without washing) onto nonnutrient agar in plastic petri dishes, all under sterile conditions. After several days of incubation at room temperature, the fruiting bodies formed. The dishes were sealed with tape and were stored inverted at 4°C. The spores of the fruiting bodies were viable in this form for at least 14 mo.

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Measurement of Cell Sizes

Dimensions of amoebae and cysts were measured under phase contrast optics in a Zeiss microscope, using a Bausch and Lomb eyepiece micrometer (Bausch & Lomb Inc., Rochester, N. Y.). Approximately 10–20 cells were measured in each case.

Homogenization of Cells

The cells were broken by sonication in approximately 5-ml aliquots, 1 min for amoebae, 5 or more min for cysts, (Branson Instruments Co., Stamford, Conn. or Measuring & Scientific Equipment, Inc., Westlake, Ohio sonicators). The suspensions were cooled during sonication by immersion of the vessel in a bath of ethanol and dry ice.

For the studies on lysosomes and peroxisomes, the cells were harvested and washed as described above, except that the final wash was ice-cold 0.25 M sucrose, in which the cells were resuspended as a 10% (vol/vol) suspension. This cell suspension was homogenized in an ice-cold Potter-Elvehjem homogenizer with a motor-driven Teflon pestle for 2–3 min. The unbroken cells were collected by centrifugation at top speed in a clinical centrifuge, resuspended in the sucrose solution, and homogenized again, and this procedure was repeated a third time. The two supernates and final homogenate were combined and the extent of cell breakage was determined by counting intact cells in a hemacytometer. Breakage in excess of about 80% was achieved by this method.

Alternatively, the cells were broken by a single passage under suction through a Millipore filter (Millipore Corp., Bedford, Mass.) with a pore size of 5 μm. Unbroken cells did not pass through the filter. Approximately 70% of the original acid and alkaline phosphatase and catalase activities of whole cells could be accounted for in the filtrate.

Fractionation of Homogenates

Cell homogenates in 0.25 M sucrose and at 4°C were fractionated as follows. The unbroken cells and large debris were sedimented by spinning the homogenates for 3 min at 800 *g* in a clinical centrifuge. This material was washed once in sucrose and was resuspended as Fraction 1. The supernates and the sucrose washes of the pellets were combined and spun in 40-ml plastic tubes at 1200 *g* for 10 min (model B-20 refrigerated centrifuge, International Equipment Co.). The pellets were washed once in sucrose and were resuspended as Fraction 2. The supernates and washes were combined and spun at 25,000 *g* for 30 min in the same centrifuge. These sediments were not washed, but were carefully drained, and then resuspended as Fraction 3. The supernates were spun at 81,000 *g* for 60 min in a type 39 angle rotor in a Beckman L-2 ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The pellets were resuspended as Fraction 3 *a*. The supernates of the 81,000 *g* spins were combined as Fraction 4. In one experiment the opalescent material which collected at the top of the tubes after the final spin was collected with a Pasteur pipette, and called Fraction 4 *a*.

Protein, DNA, RNA, and Dry Weight Determinations

The protein content of whole cells was determined by sedimenting a known number of washed cells in the clinical centrifuge. The cells were washed free of any remaining SM by resuspension in either ice-cold BSS or 0.2 M sucrose, using a Vortex mixer (Scientific Industries, Inc., Queens Village, N. Y.), followed by collection of the cells by centrifugation. The washed cell pellets or cell homogenates, were dissolved in 0.5 N NaOH at room temperature, and aliquots were assayed for protein according to the method of Lowry et al. (24), using bovine serum albumin (BSA), Fraction V (Nutritional Biochemical Corp., Cleveland, Ohio) as a standard.

The nucleic acids of sonicated cells were assayed after fractionation according to Schneider (31), using added BSA as a carrier, as recommended by Burton (8). The DNA content of each nucleic acid extract was assayed according to Burton (8), using purified calf thymus DNA (Sigma type V, Sigma Chemical Co., St. Louis, Mo.) as a standard. The RNA content of each extract was assayed by the orcinol method (31), using yeast RNA (Sigma type VI) as a standard.

Dry weight determinations were made by washing known numbers of cells in BSS and sedimenting them in small weighed test tubes, which were brought to constant weight in a 100°C drying oven.

Carbohydrate Fractionation and Analysis

Samples of homogenized cells were fractionated into three portions, (1) alkali-insoluble, (2) alkali-soluble and ethanol-insoluble, and (3) alkali- and ethanol-soluble, according to the methods of Ward and Wright (41) and White and Sussman (43, 44), with minor modifications. Each fraction was resuspended in water and its total carbohydrate content was estimated by the anthrone method, with glucose as a standard (2).

For determination of the carbohydrate composition of the fractions, each was hydrolyzed in acid. Fraction 1 was hydrolyzed according to the method of Saeman et al. (30) which involves an initial solubilization of the sample in 72% H_2SO_4 at room temperature, followed by dilution to 1 N H_2SO_4 and incubation at 100°C for 4–5 h. Fractions 2 and 3 were hydrolyzed in 1 N HCl or H_2SO_4 at 100°C for 2–3 h. The samples were neutralized with NaOH or

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$Ba(OH)_2$, after hydrolysis in HCl or H_2SO_4, respectively. The hydrolyzed samples were assayed for their content of free glucose with the Glucostat reagent (Worthington Biochemical Corp., Freehold, N. J.).

Both hydrolyzed and unhydrolyzed samples of Fraction 3 were passed sequentially through an anion exchange resin Bio-Rad AG1-X8, 100–200 mesh, formate form (Calbiochem, San Diego, Calif.) and a cation exchanger, Dowex 50W-X8, 50–100 mesh, hydrogen form, (J. T. Baker Chemical Co., Phillipsburg, N. J.), in order to remove salts. The neutral sugar composition of the lyophilized eluates was checked by paper chromatography on Whatman # 1 paper. The chromatograms were developed at room temperature with *n*-butanol:pyridine:water (80:80:40) for 15 h and the spots were visualized with alkaline $AgNO_3$ (39) and were compared with standards chromatographed simultaneously, including glucose, mannose, galactose, fucose, ribose, trehalose, and lactose.

Enzyme Assays

The following enzymatic activities were assayed essentially according to published methods at the pH indicated: acid phosphatase (pH 3.0; 26); acid β-*N*-Ac-glucosaminidase (pH 4.5; 23); acid proteinase (pH 3.0; 1); acid ribonuclease (pH 4.5; 11); alkaline phosphatase (pH 9.0; 26); and catalase (pH 7.2; 4). The samples were further treated with 0.1% (wt/wt) Triton-X-100 to solubilize the enzymes maximally, and assay conditions were determined that yielded product linearly with time (except in the case of catalase, which was shown to follow the expected pseudo-first-order kinetics). The rate of product formation was, in each case, proportional to the amount of enzyme added. Saturating or near-saturating substrate concentrations were employed, and the reactions were carried out at 25°C. Optical densities were determined in quartz cells with a 1-cm light path in a Beckman model DU spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.).

Units of enzyme activity were defined as follows: (*a*) acid phosphatase, β-*N*-Ac-glucosaminidase, and alkaline phosphatase: 1 unit equals the amount of enzyme releasing 1 μmol of product per min; (*b*) acid proteinase: 1 unit releases 1 μg of protein in acid-soluble form per min; (*c*) acid ribonuclease: 1 unit releases 1 μmol of acid soluble nucleotides per min; (*d*) catalase: 1 unit destroys 90% of the H_2O_2 in 1 min in an assay volume of 50 ml.

RESULTS AND DISCUSSION

Growth in Shaken Suspension

In general agreement with previous reports (34, 36), growth of the amoebae was exponential with a generation time of 3.5–5 h, and the final cell concentrations achieved were 5–10 × 10^6 cells/ml. Growth ceased to be exponential when most of the coagulated material (a consequence of autoclaving the milk powder) had disappeared from the medium. At that time remaining coagulated material appeared to be in close association with the surfaces of the cells. The cells entered stationary phase 15–20 h after exponential growth ceased, at which time no coagulated material was visible. The final cell concentration could be increased by a factor of 3 to 4 if additional milk powder and lecithin were added as growth began to slow, indicating that growth had been limited by the exhaustion of the nutrients in the medium, rather than by the accumulation of some inhibitor(s) of growth. After a number of hours in the stationary phase, the first cysts were detectable microscopically, and further incubation led to the encystment of virtually every cell in the culture within 30–50 h after entry into stationary phase. Exponentially growing amoebae were 8.55 ± 1.13 (SD) μm in diameter, and mature cysts measured 5.57 ± 0.66 μm. These values agree with those reported previously for amoebae (19) and cysts (37) of *P. pallidum*.

Previous observations of *P. pallidum* (18–20) and *D. discoideum* (3) growing in suspensions have shown that the physical state of the nutrient material determines to some degree both the rate and extent of growth, and the average cell size. The variability of that measurement is also affected. We have confirmed and extended those observations by showing that the extent of coagulation of GM, as controlled by the duration of autoclaving, appears to have a direct bearing on these matters (Table I). The most particulate medium (autoclaved for 45 min) produced the most rapid growth rate, the smallest cells during exponential growth (as indicated by dry weight and protein content), and the highest final cell yield (Table I). It was again noted that the least variation in cell size occurred when the medium was most particulate. The large cells seen in the less particulate media were often multinucleate suggesting that cytokinesis was being affected more than mitosis.

The continued growth of the cells in the different media listed in Table I suggests that selective destruction of essential nutrients by autoclaving can be ruled out as a controlling factor since growth appeared more normal in media which had been

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TABLE I

Effect of Duration of Autoclaving of Milk Powder on Growth of P. pallidum

Duration of autoclaving	Generation time	Cell yield	Protein	Dry weight
min	*h*	*1 × 10⁶ cells/ml**	*mg/1 × 10⁷ cells‡*	*mg/1 × 10⁷ cells‡§*
5	4.0	7.3	0.71	—
15	3.8	8.8	0.60	1.1
30	3.5	9.7	0.44	0.71
45	3.5	11	0.36	—

* At stationary phase.
‡ In exponential phase.
§ Determined in separate experiments.

TABLE II

Specific Activities of Several Enzymes from P. pallidum and D. discoideum

	A	B
Acid phosphatase	106	124
Acid ribonuclease	145	184
Acid proteinase	109	126
β-*N*-Ac-glucosaminidase	107	442 (35°C)*
β-Glucuronidase	0	—
Catalase	154	—
Alkaline phosphatase	11	5

A. *P. pallidum*, assayed at 25°C.
B. *D. discoideum*, assayed at 25°C., (23, 46).
* Assay temperature, when different from that indicated above.

autoclaved for longer periods. Rasmussen and Kludt (29) have suggested that protozoans generally have an absolute requirement for particulate material in the medium before nutrients may be ingested. In the case of *P. pallidum* amoebae, the appearance of larger cells may represent a specific response to a less particulate medium, since we have observed that an increase in cell size is accompanied by a very disproportionate increase in the phagocytic capabilities of these cells (15).

Presence of Lysosomes and Peroxisomes in Amoebae

Lysosomes (12) and peroxisomes (10) have been identified in numerous cell types. These organelles are defined by the physical characteristics of marker enzymes in homogenates of cells prepared in osmotically protective media. Among these characteristics are latency and sedimentability of the enzymes, and the sensitivity of these properties to disruption of membranes by freezing and thawing, or treatment with Triton-X-100 (10, 12). We have applied these criteria to indicate the presence of lysosomes and peroxisomes in the amoebae of *P. pallidum*.

The following enzymatic activities were demonstrated in homogenates of amoebae: acid phosphatase, acid proteinase, acid ribonuclease, acid β-*N*-Ac-glucosaminidase (marker enzymes for lysosomes, 12), catalase (a marker enzyme for peroxisomes, 10), and alkaline phosphatase (a marker enzyme for plasma membranes, e.g., 27, 40). The specific activities of these enzymes are shown in Table II.

The intracellular distributions of some of these enzymes were determined in three separate experiments by fractionating cell homogenates by differential centrifugation, according to the procedures described in Materials and Methods. The results of one representative experiment are shown in Fig. 1. About 90% of the enzyme activities and protein in the homogenates were recovered in the fractions. Each marker enzyme sedimented in a reproducible fashion relative to the other enzymes assayed. The three acid hydrolases sedimented in a similar (although not identical) pattern, which was clearly distinguishable from the sedimentation patterns of catalase and alkaline phosphatase. The latter two enzymes resembled each other in their sedimentation patterns, but to a much lesser extent than did the group of acid hydrolases. Those enzymes in Fraction 4 (material not sedimented after 30 min at 25,000 *g*) were also nonsedimentable at 81,000 *g* for 60 min. The possibility, that these nonsedimentable enzymes were associated with opalescent material (presumably lipidic) that collected at the top of the tube after the final centrifugation, was examined in one experiment by isolating this material as Fraction 4 *a* (see Fig. 1). The results suggest that the acid hydrolases may be associated with this material to some extent, certainly far more than are catalase and alkaline phosphatase.

The effects of Triton-X-100 and freezing and thawing cycles on the sedimentability of the marker enzymes and total protein were examined in several experiments. Homogenates were simply exposed to 0.1% (wt/vol) Triton-X-100 on ice for

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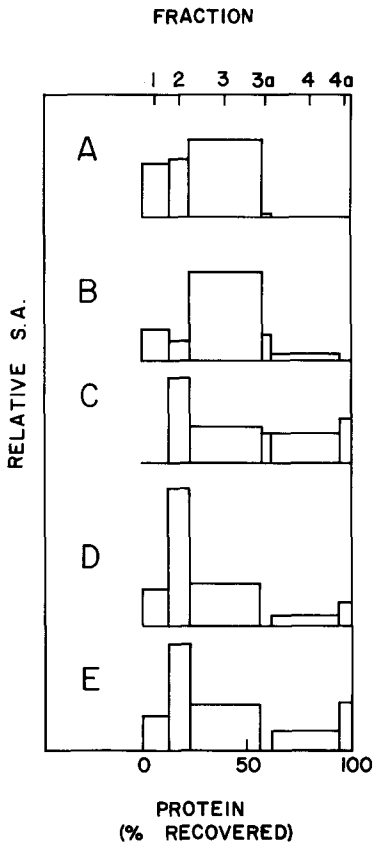

FIGURE 1 Distribution of marker enzymes among subcellular fractions of *P. pallidum* amoebae. Washed vegetative amoebae were suspended in ice-cold 0.25 M sucrose, homogenized, and fractionated by differential centrifugation. Homogenization in 0.25 M sucrose; Fraction 1 centrifuged at 800 *g* for 3 min; Fraction 2 at 1200 *g* for 10 min; Fraction 3 at 25,000 *g* for 30 min; Fraction 3 *a* at 81,000 *g* for 60 min. The supernatant fraction is 4. Fraction 4 *a* is the floating opalescent material that lies above supernatant Fraction 4. (See Materials and Methods for details.)

Each fraction was analyzed for its content of protein and several enzymes, and the relative specific activity (percent enzyme recovered per percent protein recovered) was plotted against the % protein recovered in each fraction. A = catalase; B = alkaline phosphatase; C = acid proteinase; D = acid ribonuclease; E = acid phosphatase.

about 15 min, or were frozen (at −20°C) and thawed four times, before centrifugation. Results are shown in Table III. Substantial portions of each of the enzymes were sedimentable. Treatment of the homogenates with Triton led to an extensive solubilization of the acid hydrolases and alkaline phosphatase, while catalase and protein were affected to an intermediate extent. Freezing and thawing of the homogenate had no effect on alkaline phosphatase, an intermediate effect on catalase and total protein, and solubilized major fractions of the acid hydrolases.

When homogenates of amoebae were prepared by suction filtration through Millipore filters and assayed at 0°C in the presence and absence of Triton-X-100, the following latencies of marker enzyme activities were obtained: acid phosphatase, 94%; catalase, 61%; and alkaline phosphatase, 85%.

These results conform generally with the known properties of marker enzymes for lysosomes and peroxisomes (10, 12). The behavior of alkaline phosphatase is at least consistent with a membranous localization. There is good agreement with the previously reported specific activities of these enzymes in amoebae of *D. discoideum* (23, 46), and with the evidence that those cells also contain lysosomes (46) (Table II).

Changes in Cellular Levels of Various Biochemical Entities during Growth and Encystment

Amoebae were grown on GM containing milk powder autoclaved for either 15 or 30 min. Aliquots of cells were harvested periodically during the growth cycle and were analyzed for their content of DNA, RNA, protein, and the activity of acid phosphatase, acid proteinase, and catalase. The results are shown in Fig. 2.

The data indicate that even during exponential growth there were significant changes in the cellular levels of most of the variables assayed. During this period and during the lag phase before the entry into stationary phase, the levels of the two acid hydrolases and of catalase increased while the level of RNA decreased. In Experiment 2, there was no consistent change in the protein content of the cells during the growth and lag phases, while in Experiment 1 there was a marked rise in the protein content of the cells, at the same time as morphologically larger cells became apparent. During the stationary phase there were substantial decreases in all of the components measured, although the cellular level of acid phosphatase in Experiment 1 continued to rise during the early portion of stationary phase, before finally declining.

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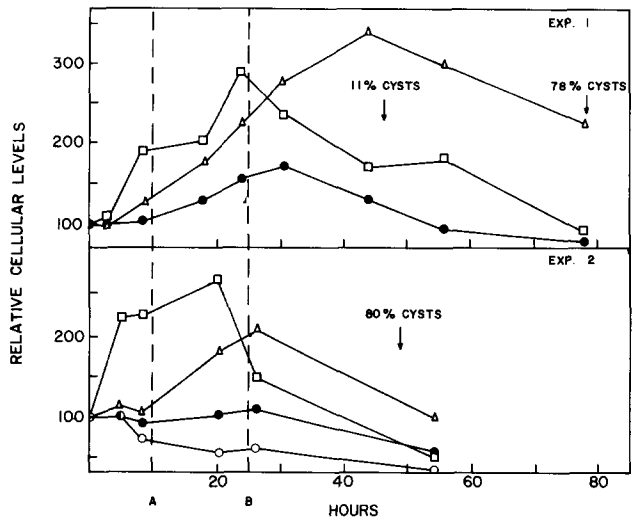

FIGURE 2 Changes in cellular levels of various biochemical entities during growth. Cells harvested at various points during the growth cycle were analyzed for the substances indicated in the legend below. Cellular levels were expressed as a percentage of the levels in the first sample. Experiments 1 and 2 represent data from cells grown on GM containing milk powder autoclaved for 15 and 30 min, respectively. The initial cellular levels, based on 1×10^7 cells, were as follows: Experiment 1, protein = 0.44 mg, acid phosphatase = 34 milliunits, and catalase = 36 milliunits; Experiment 2, protein = 0.54 mg, RNA = 0.11 mg, acid phosphatase = 38 milliunits, catalase = 32 milliunits. DNA and acid proteinase were also analyzed in Experiment 2. There were no consistent changes in the cellular DNA levels, while the acid proteinase levels changed in a pattern resembling that of acid phosphatase.

At the time of the first measurements in each experiment the cells were in exponential phase (ca. 1×10^6 cells/ml). The end of exponential phase and the beginning of the stationary phase (leading eventually to encystment) are indicated approximately as A and B. For Experiment 1, the numbers of cells at A and B, respectively, were 2.5×10^6 and 6×10^6 cells/ml; for Experiment 2, the numbers were 6.5×10^6 and 10×10^6 cells/ml. A and B thus demarcate a lag phase of about 13 h between the end of exponential growth and the cessation of growth. Legend: ●, protein; ○, RNA; □, catalase; △, acid phosphatase.

These biochemical changes are probably due to specific adaptations by the cells to changes in their environment. The most obvious change was a depletion of the available nutrient material in the medium, which led eventually to encystment. The changes occurring during the latter portion of exponential growth indicate that the cells were already responding to changes in the supply of nutrients. Increases in the level of acid phosphatase and acid proteinase, marker enzymes for lysosomes (see above), suggest that one adaptation might be an increase in autophagy, an intracellular digestive process involving lysosomes (12), which very likely is responsible for the substantial decreases in cellular materials eventually seen during starvation (see Fig. 2 and below). The increase in the cellular levels of catalase, a marker enzyme for peroxisomes (see above) suggests that this organelle may be involved in some fashion with adaptations to changing conditions in late exponential phase of growth, but not with adaptation to starvation, since the levels decreased rapidly as a consequence of induced starvation (see below). The increase in cellular protein levels during the later stages of growth in Experiment 1, but not in Experiment 2, was a consequence of the physical state of the medium, as discussed above. Similar changes in the cellular protein content of *D. discoideum* amoebae during the late stages of growth in suspension culture have been observed (32).

Encystment in SM

Encystment in the growth flask was apparently a consequence of starvation due to the exhaustion of nutrient material in the growth medium, since the addition of more milk powder and lecithin allowed growth to continue. Encystment could also be induced by washing exponentially growing amoebae, and incubating them in SM at 23–25°C.

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The first cysts became visible after about 15 h, 50% encystment occurred after 30 h, and it was essentially complete by 45 h, as shown in Fig. 3.

It is interesting that the amoebae recognized incubation in SM as constituting starvation, since SM contains 1% Bactopeptone and 1% glucose. Although it is possible that these materials could not support growth, it is also possible that one or more critical substances were unable to enter the cells. The latter possibility is strongly suggested by the apparent requirement of these cells (see above and [21]) and protozoans in general (29) for high molecular weight or particulate material for growth and by the fact that high concentrations of SM actually stimulated the rate of encystment (see below).

Increase in Cell Number during Starvation in SM

Although the amoebae did eventually encyst when incubated in SM, they did not immediately cease dividing. The increases in cell concentration averaged 43% in 11 experiments. There was at the same time no change in the cellular protein content per volume of medium over at least the first 15 h of starvation, so the average protein content per cell decreased, as detailed below.

TABLE III

*Sedimentation Characteristics of Marker Enzymes and Protein after Breaking Cells**

Enzyme	Sedimented enzyme (% ± SD of total enzyme)		
	No treatment	Frozen + thawed‡	Triton-X-100§
Acid phosphatase	71 ± 6 (4)‖	17	3
Acid ribonuclease	74 ± 13 (3)	11	7
Acid proteinase	65 ± 4 (4)	20	0
Catalase	89 ± 9 (4)	58	45
Alkaline phosphatase	93 ± 8 (4)	90	10
Protein	55 ± 4 (4)	46	38

* Homogenate prepared in tissue grinder (See Materials and Methods).
‡ Frozen and thawed four times under conditions described in text.
§ Treated with 0.1% (wt/vol) Triton-X-100 on ice for about 15 min.
‖ Number of determinations. All other figures in the table represent single determinations. Sedimentation at 25,000 *g* for 30 min.

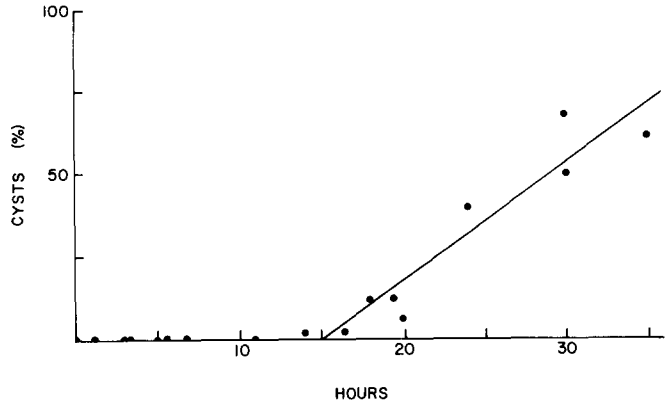

FIGURE 3 Time course of encystment in the starvation medium. The time course of encystment of amoebae in the starvation medium (SM) was followed by periodically counting the percentage of cysts by phase contrast microscopy.

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This increase in total cell number in the face of a constancy of total cell protein content can be explained in several ways. The presence of nutrient material adsorbed to the cell surface or present in food vacuoles may have satisfied requirements for a small increase of "true" cell protein and cell number. Alternatively, the cells may have been fulfilling a requirement to finish ongoing nuclear synthesis and to divide, so that all cells in the population contained single genomes before encysting. This phenomenon is seen in bacteria which complete already initiated rounds of DNA replication before responding to a signal to cease DNA synthesis (25). A residual increase in cell number has also been observed when growing *D. discoideum* amoebae were allowed to develop on a non-nutrient surface (7, 35).

Effect of Osmolarity on Encystment

While the encystment process is primarily a response to starvation, its extent in this organism has been shown to be dependent on the osmolarity of the suspending medium (36). The following experiments have confirmed this finding and have shown that the *rate* of encystment is also affected by the osmolarity of the medium.

When washed amoebae were resuspended in SM containing varying concentrations of sucrose, or in different concentrations of SM, the rate of encystment was stimulated in the media with higher osmolarities, as shown in Table IV. A medium of low osmolarity appeared to suppress encystment almost entirely. These findings can be rationalized by considering the dehydrated appearance of the mature cyst: a high osmolarity in the medium would tend to dehydrate the cells and thus increase the rate and extent of encystment to the extent that dehydration is an obligatory portion of the process.

TABLE IV

Effect of Osmolarity on Encystment (as percent cells encysted)

Experiment	Starvation medium	Hours starved 6	8	20	30	40
I	10% SM*	—	0	0	0	0
	55% SM	—	0	0	6	35
	100% SM	—	0	6	68	97
II	55% SM plus					
	0 mM sucrose	—	0	0	6	35
	45 mM sucrose	—	0	5	47	80
	90 mM sucrose	—	0	20	90	97

* The osmolarity of SM is approximately 150 mosM.

Biochemical Aspects of Encystment in SM

The encystment process was initiated as described above. Periodically, aliquots of the suspension (containing known numbers of cells) were removed, the cells were collected by centrifugation, washed when appropriate, and then frozen. Subsequently the aliquots were resuspended in distilled water, and assayed for acid phosphatase, acid β-*N*-Ac-glucosaminidase, catalase, and several carbohydrates. Separate aliquots of washed cells were analyzed directly for DNA, RNA, protein, and dry weight.

The results of these experiments (with the exception of those on carbohydrates, which will be discussed below) are shown in Fig. 4 as a function of incubation time in SM. Some of the variation in the absolute values was undoubtedly due to the time of harvest of the cells during exponential growth (which varied), since the levels of at least some of these substances change during exponential growth, as discussed above.

The cellular DNA content remained essentially constant, at 5.1 μg DNA/1 × 10^7 cells, throughout the starvation period of 30–40 h, which culminated in nearly complete encystment of the cells, as shown in Fig. 3. The cellular levels of dry weight, protein, RNA, and catalase all fell during this period. At the same time, acid phosphatase activity rose and then returned to approximately the original value. Another acid hydrolase, β-*N*-Ac-glucosaminidase, appeared to parallel this behavior. As expected, these changes are similar to those we have seen to accompany starvation and eventual encystment in GM, as described above. These results also resemble in general those reported for the development of *D. discoideum* and *P. pallidum* on a non-nutrient surface, with the obvious exception that development on a surface results in the formation of multicellular aggregates consisting of spores supported on a stalk (6). The average decrease in protein content that we noted was considerably less than that normally seen during the surface development of *P. pallidum* and *D. discoideum* (36, 43). This was probably due to the presence in SM of glucose, which to some extent satisfied the requirement of the cells for energy production or for the synthesis of development-

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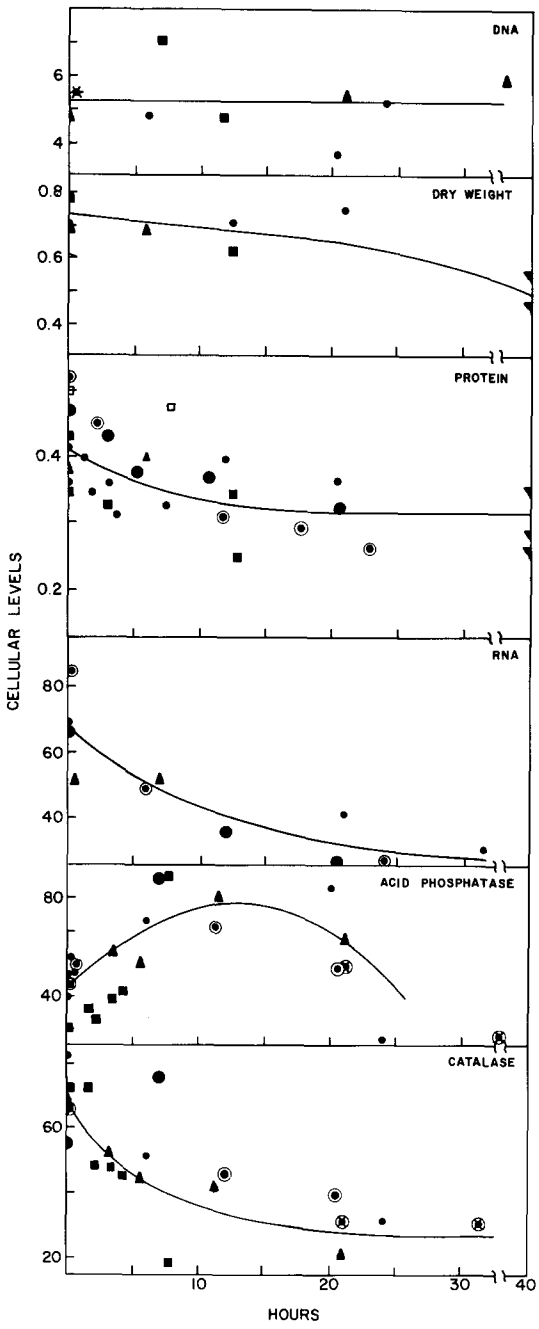

FIGURE 4 Effect of starvation on the cellular levels of various biochemical entities. Starving amoebae were assayed periodically for the cellular levels of the substances indicated in the figure, as described in Materials and Methods. The cellular levels of the substances per 1×10^7 cells were expressed as follows: DNA, μg; dry weight, mg; protein, mg; RNA, μg; acid phosphatase, milliunits; and catalase, milliunits. The data are taken from the following numbers of independent experiments: DNA, four (the star represents five replicate determinations); dry weight, four; protein, six; RNA, four; acid phosphatase, six; catalase, six. The various symbols indicate measurements in different experiments. The 40-h points for dry weight and protein were obtained from independent measurements of mature cysts. The curves drawn indicate only the overall trend of the data.

specific saccharides (see below) and thereby reduced the need for endogenous protein catabolism. It is evident from our data that catalase was inactivated more rapidly than total protein, suggesting that the organelle which contains this enzyme, presumably the peroxisome (10), is also more rapidly degraded.

The initial increase in the two acid hydrolases studied suggests again (see below) that the loss of cellular material may have been due to autophagy. Increases in the cellular levels of these two enzymes have also been reported in *D. discoideum* developing on a surface (23, 46). However, Wiener and Ashworth (46) followed changes in the levels of three other acid hydrolases during development of *D. discoideum* on a surface: acid proteinase, acid ribonuclease, and amylase. The cellular levels of all three of these enzymes fell during development by 50–70%, suggesting that the levels of all these presumably lysosomal enzymes are not coordinately controlled. It should also be pointed out at this point that the presence of a lysosomal hydrolase in a cell homogenate does not necessarily mean that it was *active* in the intact cell, since such enzymes are membrane bound (12). Thus one cannot argue that an increase in the cell content of a lysosomal hydrolase necessarily implies an increased activity of that enzyme, or of lysosomes, in vivo.

Changes in the cellular levels of carbohydrates during encystment were followed, as described in Materials and Methods, by dividing the carbohydrates of the cells into three fractions and analyzing each for its total carbohydrate content and monosaccharide composition.

The cellular levels of Fraction 1 (alkali-insoluble material) increased in quantity during encystment by a factor of about 14, as shown in Fig. 5. When samples of Fraction 1 material prepared from cysts of *P. pallidum* were analyzed for their sugar composition, virtually all of the anthrone-positive material could be accounted for as glucose, as shown in Table V. In addition to its insolubility

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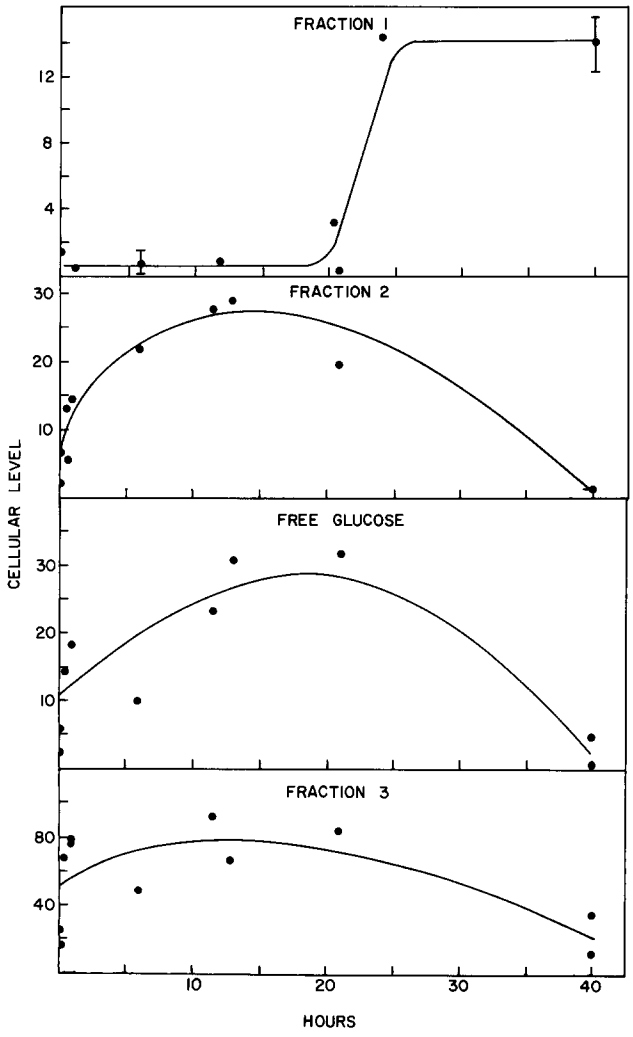

FIGURE 5 Changes in the carbohydrate composition of *P. pallidum* during encystment. Starving amoebae were assayed periodically for the following carbohydrate fractions, as described in Materials and Methods: Fraction 1 (alkali-insoluble), Fraction 2 (alkali-soluble and ethanol-insoluble), and Fraction 3 (alkali- and ethanol-soluble). The quantity of each fraction was expressed in terms of μg of glucose equivalents of anthrone-positive material per 1×10^7 cells. The amount of free glucose in Fraction 3 was determined with the Glucostat reagent, as described in Materials and Methods. Where replicate determinations on several cell samples were made, standard deviations are indicated by bars.

in hot alkali, the tentative identification of this material as cellulose was further strengthened by experiments showing its resistance to hydrolysis by 5 N HCl for 24 h at 25°C (only 8% of the material was released as glucose, as would be predicted for cellulose [21]) and its partial sensitivity to cellulase (26% of the material released as glucose in one experiment, using a commercial enzyme (Worthington CSE-II)).

The cellular levels of Fraction 2 (alkali-soluble and ethanol-insoluble) increased in quantity during starvation and then decreased to very low levels in the cysts, as shown in Fig. 5. This material as isolated from both amoebae and from cysts could be entirely accounted for as glucose after mild acid hydrolysis (see Table V).

Fraction 3 (alkali- and ethanol-soluble) was found to contain significant amounts of material reacting as carbohydrate, which rose and fell during encystment in a pattern resembling that of

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TABLE V

Sugar Composition of Carbohydrate Fractions from P. pallidum

Cell type	% glucose*		
	Fraction 1‡	Fraction 2§	Fraction 3‖
Amoebae¶		101 ± 10 (7)	76 ± 4 (9)
Cyst	111 ± 26 (9)	108 ± 27 (2)	86 ± 65 (3)

* The percentage of the anthrone-positive material which could be accounted for as authentic glucose after acid hydrolysis. The values were corrected for the destruction of standards. The standard deviations and the number of determinations (in parentheses) are given.

‡ Alkali-insoluble carbohydrate.

§ Alkali-soluble and ethanol-insoluble carbohydrate.

‖ Alkali- and ethanol-soluble carbohydrate.

¶ Included in this category are amoebae which had been starved up to 13 h.

Fraction 2, as shown in Fig. 5. Roughly 20–40% of this material could be accounted for as free glucose, which also rose and fell during encystment. Mild acid hydrolysis of aliquots of Fraction 3 showed that only 76–86% could be accounted for as glucose, as shown in Table V. In the case of the cysts, at least, this was probably due in part to the resistance of trehalose (which is found in cysts, see below) to mild acid hydrolysis (16). The non-glucose material also included ribose (found in amoebae and cysts, see below) and probably amino acids, both of which will react with anthrone and thus contribute to the apparent total carbohydrate content of the fraction, to a minor degree (see above).

The soluble carbohydrates of amoebae and cysts were analyzed qualitatively by paper chromatography, both before and after mild acid hydrolysis, as described in Materials and Methods. In agreement with the above analyses, glucose was the predominant sugar in all cases. A spot corresponding in mobility to authentic trehalose was observed in the cyst sample, and was reduced in intensity, but not abolished, by mild acid hydrolysis. Spots corresponding to ribose and to several unidentified sugars (which were not fucose or mannose) were seen in all samples.

The apparent presence of cellulose in cysts agrees with previous analyses of *P. pallidum* cysts (38). The patterns of the changes in the individual fractions also resemble those seen during the development of *D. discoideum* on a surface (43, 44). However, the absolute levels of Fractions 2 and 3 were much lower in experiments with *D. discoideum* (43, 44). This may be due in part to the presence of glucose in the starvation medium of *P. pallidum*, resulting in high intracellular levels of glucose and glucose polymers. This is supported by the observation that the glycogen content of growing *D. discoideum* amoebae reflects the glucose content of the medium (3). However, *P. pallidum* amoebae starving on a surface have been shown to have a higher glycogen content than *D. discoideum* amoebae exposed to the same conditions (14), suggesting that species differences may also be involved.

The compositions of the three fractions agree generally with the values reported for *D. discoideum* developing on a surface (43, 44). However, there appear to be minimal quantities of non-glucose polysaccharides analogous to those reported for *D. discoideum* (45) and *P. pallidum* (14) developing on a surface, since all of the material in our Fraction 2 could be accounted for as glucose (see Table V). We did not assay directly for other monosaccharides. The structure of the cyst wall of *P. pallidum* has been reported as appearing much simpler than the spore wall of the same species (17). The development of *D. discoideum* on a surface is accompanied by the accumulation of about 13 μg trehalose per 1×10^7 cells (9). Thus encystment of *P. pallidum* corresponds at least qualitatively in this respect to fruiting body formation of *D. discoideum*.

CONCLUSION

It is evident from the data presented here and in previous reports that encystment in *P. pallidum* is a complex developmental process involving (*a*) decreases in the dry weight, protein, and RNA

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content of the cells, (*b*) increases in the levels of polysaccharides (cellulose and glycogen) and probably the disaccharide trehalose, and (*c*) changes in the cellular levels of several enzymes. This encystment process thus resembles development on a surface by *D. discoideum* and *P. pallidum* in a number of biochemical aspects. More detailed studies would enable one to ask how cell-cell interactions (on a surface) alter the pattern of biochemical development seen in single cells in suspension. In addition, such data would allow one to begin examining the ways by which *P. pallidum* and *D. discoideum* accomplish similar morphogenetic feats, perhaps by differing biochemical mechanisms.

This work was supported by U. S. Public Health Service Grant no. A1-03260 from the National Institute of Allergy and Infectious Disease. Dr. Sherwood Githens was a recipient of a predoctoral fellowship from the National Institute of General Medical Sciences.

Received for publication 23 October 1972, and in revised form 4 May 1973.

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