

# EVIDENCE FOR LYSOSOMAL ENZYMES IN *ACANTHAMOEBA* AND THEIR ACTIVITY CHANGES DURING ENCYSTMENT<sup>1</sup>

SCOTT M. MARTIN<sup>2</sup> AND THOMAS J. BYERS, Graduate Program in Zoology and Department of Microbiology, The Ohio State University, Columbus, Ohio 43210

**Abstract.** Assays on cell-free homogenates of *Acanthamoeba castellanii* reveal that the three hydrolases, acid phosphatase (APase), acid deoxyribonuclease, and acid ribonuclease (RNase), possess pH optima of 5.0, 4.8, and 5.2, respectively. These enzymes exhibit an enhanced sedimentation at 20,000 x g when sucrose is in the homogenizing buffer. Treatment of homogenates with Triton X-100 increases total enzyme activity. These results suggest that the enzymes are particle-bound in lysosomes. During encystment there is a differential decrease in the activity per cell of all three enzymes, with RNase decreasing most rapidly and APase least rapidly. The specific activity of APase increases during encystment even though its activity per cell gradually decreases.

OHIO J. SCI. 77(1) 28, 1977

*Acanthamoeba castellanii* is a free-living soil and freshwater amoeba that can be cultured axenically. Its life cycle consists of a vegetatively multiplying trophozoite which can, under unfavorable environmental conditions, differentiate into a cyst form. During encystment there is a prominent decrease in the cellular contents of DNA, RNA, protein, lipid, and glycogen; these endogenous degradations are thought to supply both energy and precursors for the synthesis of the cyst wall (Neff and Neff, 1969; Griffiths, 1970). Most likely lysosomal enzymes are involved in this degradation since such enzymes act as catabolic hydrolases in a wide variety of eukaryotic differentiative processes (Dingle and Fell, 1969). This viewpoint is strengthened by the electron micrographs of Bowers and Korn (1969) which reveal the appearance of autophagic vacuoles during encystment in *A. castellanii*. These autophagic vacuoles stain positively for the lysosomal enzyme, acid phosphatase (APase), and contain mitochondria, various cytoplasmic debris, and glycogen particles. In order to learn more about the role that

lysosomal enzymes play during differentiation in *A. castellanii*, we have assayed the activities of three lysosomal markers, APase, acid deoxyribonuclease (DNase), and acid ribonuclease (RNase), during starvation-induced encystment.

## MATERIALS AND METHODS

The amoeba used in this study was obtained from Robert J. Neff (designated as *Acanthamoeba* sp., strain I-12) who isolated it from soil at Pacific Grove, California (Neff, 1957). Page (1967) concluded that the sarcodine should correctly be called *Acanthamoeba castellanii* (Douglas, 1930). The amoeba was cultured axenically at 30°C in a growth medium devised by Neff *et al* (1964) that was prepared according to Byers *et al* (1969). Detailed procedures for counting the cells electronically and for normalizing the growth data are discussed by Byers *et al* (1969) and Martin (1973). The staining solution of Mattar and Byers (1971) was used for differentially counting encysting cells in a hemacytometer.

Trophozoites were harvested for encystment experiments after they had grown for 72 hrs and had reached a concentration of 1.5 to 2.5 x 10<sup>5</sup> cells/ml, or after 168 hrs of growth, when they had attained a density of 4.5 to 6.0 x 10<sup>5</sup> amoebae/ml (fig. 1). They were collected aseptically by centrifugation at 1000 x g, washed in 0.15 M NaCl, and inoculated into a nutrient-free salt solution to induce a synchronous encystment. The encystment medium of Neff *et al* (1964) was used, but its preparation was modified (Martin, 1973) to eliminate the necessity of bubbling autoclaved encystment medium with CO<sub>2</sub> to dissolve the precipitate of carbonates which forms during sterilization. The

<sup>1</sup>Manuscript received February 6, 1976, and in revised form July 29, 1976 (#76-13).

<sup>2</sup>Present address: Department of Biology, University of Missouri at Kansas City, 5100 Rockhill Road, Kansas City, Missouri 64110.

72 hr amoebae were inoculated at  $1.2 \times 10^6$  cells/ml into 150 ml of encystment medium in Roux bottles, which were sealed with aluminum foil, positioned horizontally, and incubated at 30°C without aeration. The 168 hr amoebae were induced to differentiate in aerated cultures of encystment medium by a modification (Martin, 1973) of the method of Neff *et al* (1964). The encystment-sampling apparatus consisted of a 2 l aspirator bottle which was connected

phenol/min, using disodium *p*-nitrophenyl phosphate (Sigma Chem) as substrate. The substrate for DNase was a sodium salt of salmon sperm DNA (Calbiochem), while that for RNase was yeast soluble RNA (Type III, Sigma Chem). For both nucleases, one unit of enzyme activity was defined as that amount of acid-soluble oligonucleotide formed which caused an increased absorbance of 1.0 per hr at 260 nm.

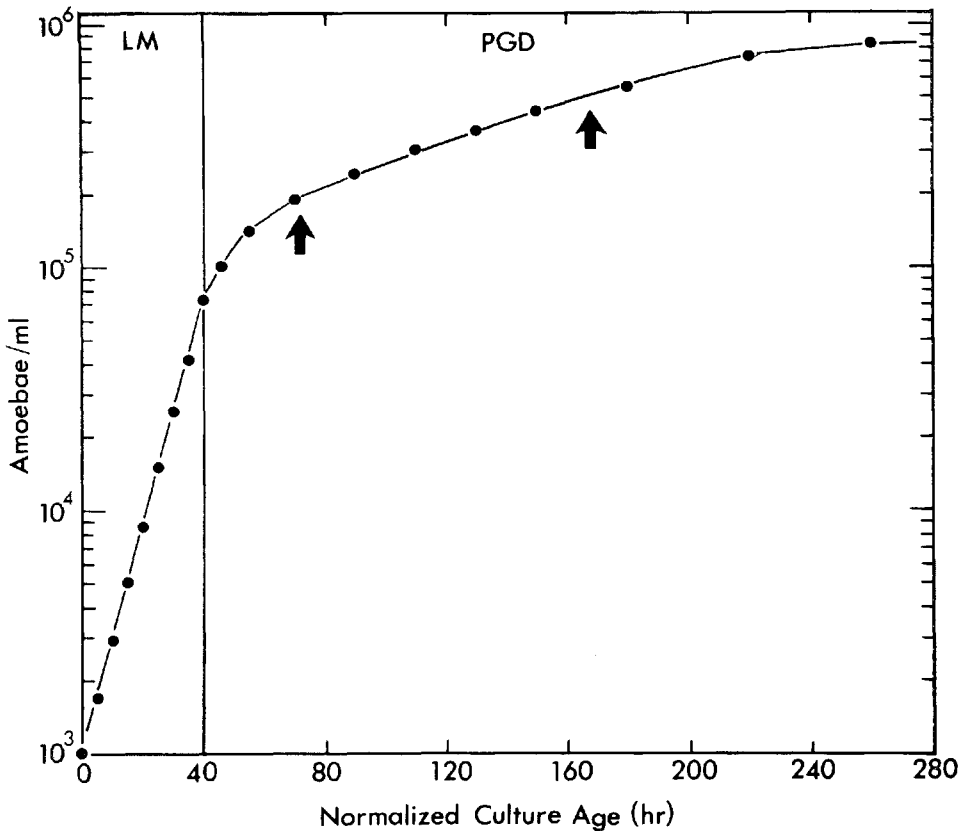


FIGURE 1. Normalized growth curve for trophozoites of *A. castellanii*. The points shown here for logarithmic multiplication (LM) were calculated by using the average growth rate constant for 20 growth curves. The mean generation time during LM was  $6.5 \pm 0.4$  hr. The points during population growth deceleration (PGD) represent average values for selected growth periods. Arrows indicate when amoebae were harvested for use in encystment experiments.

to an aeration train and a sampling port. Cultures were kept at 30°C and were gassed with 4 cu ft/hr of air. Amoebae were inoculated at  $6.0 \times 10^6$  cells/ml into 1.5 l of encystment medium contained in the 2 l aspirator bottle.

Procedures for harvesting and homogenizing the amoebae, as well as the assays for protein, APase, DNase, and RNase, are detailed in Martin (1973). One unit of APase activity was defined as the formation of 1  $\mu$ mole of *p*-nitro-

## RESULTS AND DISCUSSION

### EVIDENCE FOR LYSOSOMES

There are three biochemical criteria for the existence of lysosomes in cell-free homogenates (Dingle and Fell 1969). These criteria are acid pH optima of the constituent hydrolases and sedimentation at 20,000 x g in 0.25 M sucrose

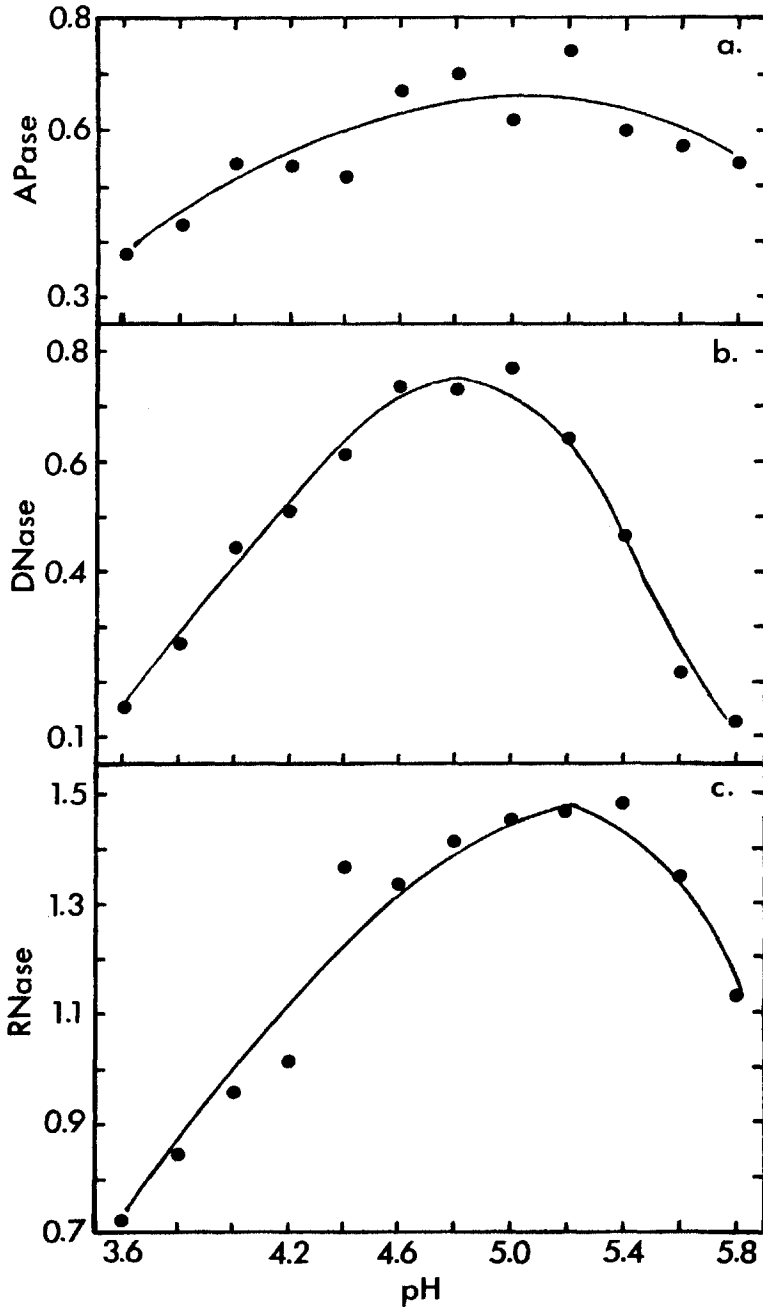


FIGURE 2. pH-Activity profiles for three lysosomal enzymes. All enzyme assays were performed in 0.1 M acetate buffers of differing pH using a crude extract prepared from a 72 hr growth culture. The ordinate for APase is the absorbance for *p*-nitrophenol at 410 nm after terminating the enzyme reaction by addition of alkali. The ordinates for DNase and RNase represent the absorbances at 260 nm for the supernatants obtained after pelleting precipitated unreacted substrate by centrifugation.

attesting to a particle-bound nature, and latency, or the enhancement of enzyme activity upon treatment with procedures that rupture lysosomal membranes. The enzymes which we assayed in this study fulfill these three criteria and thus serve as markers for lysosomes in *A. castellanii*.

The pH optima for APase, DNase, and RNase in cell-free homogenates of *A. castellanii* are 5.0, 4.8, and 5.2, respectively (fig. 2). This is in agreement with Müller (1969) who reported that extracts of *Acanthamoeba* sp. (Neff strain) contained APase and RNase, having pH optima of 5.0 and 5.5; he did not assay DNase.

One line of evidence favoring a particle-bound nature of the acid hydrolases we assayed is that inclusion of 0.25 M sucrose in the homogenizing buffer enhances the sedimentation of these enzymes at 20,000 x g (table 1). About 50% of the acid

observed that several acid hydrolases, including APase and RNase, possessed the same relatively broad and unimodal distribution pattern, with a peak at a density of 1.17. This equilibrium density differed from that obtained for malate dehydrogenase, a marker for mitochondria, and that for urate oxidase and catalase, which are markers for peroxisomes. These data also attest to the particle-bound nature of the acid hydrolases and to a homogeneous population of lysosomes. RNase sediments at 20,000 x g, however, independently of APase and DNase during encystment (Martin, 1973). Furthermore, we found that during growth about 13% of all the RNase activity, but only 2% of the total APase activity, sedimented at 100,000 x g but not at 20,000 x g. These observations suggest the existence of a heterogeneous population of lysosomes in *A. castellanii*.

TABLE 1

*Sucrose enhancement of lysosomal enzyme sedimentation in A. castellanii homogenates.\**

Culture Age (hr)	Cells/ml (x 10 <sup>5</sup> )	Sucrose Conc. in Buffer	% APase in Pellet	% DNase in pellet	% RNase in Pellet
48.0	1.48	0.00 M	26.5	18.9	10.8
		0.25 M	54.3	53.9	33.3
168.0	5.81	0.00 M	52.4	61.7	48.6
		0.25 M	83.2	79.3	82.1

\*Amoebae of each culture age were collected by centrifugation at 1000 x g and divided into two equal groups. One group was homogenized in a 0.05 M Tris-HCl (pH 8.0)-0.01 M mercaptoethanol buffer that lacked sucrose; the other was homogenized in buffer containing 0.25 M sucrose. Cells were broken in a Potter-Elvehjem homogenizer and homogenates were centrifuged at 20,000 x g for 30 min. Enzyme activities were determined in both the pellet and supernatant fractions in order to calculate the % activity in the pellet.

hydrolase activity sediments in an homogenate prepared from a 48 hr culture, whereas 80% of the activity is pelleted in a cell-free extract of 168 hr amoebae. The fact that APase, DNase, and RNase sedimented similarly regardless of culture age (table 1 and Martin, 1973) suggests that they are contained within identical cellular compartments. Müller (1969) subjected cell-free extracts of growing cultures of *Acanthamoeba* sp. to isopycnic centrifugation in sucrose gradients and

The observation of latency is another line of evidence favoring the particle-bound nature of the acid hydrolases in *A. castellanii*. Latency was effected by treating homogenates with a final concentration of 0.1% Triton X-100, a detergent known to rupture lysosomal membranes (Wattiaux and de Duve, 1956). The latency of both APase and DNase in whole homogenates was about 40% regardless of the culture age from which extracts were prepared (table 2). These

data are consistent with the viewpoint that at least these two hydrolases are contained in the same cellular subunit and are harmonious with the observation that APase and DNase exhibit similar sedimentation profiles throughout growth and encystment (Martin, 1973). In another experiment, we determined the percent latency of APase as a function of time at

encystment was eventually attained. Not all of the inoculum cells actually form cysts, however. Due to lysis, there is a cell loss in the cultures of 20–30% (Martin, 1973). Although the encystment conditions that we used gave identical rates of differentiation for the 72 and 168 hr cultures (fig. 3), the amoebae of these two ages differed sig-

TABLE 2  
Percent latency of lysosomal enzymes in homogenates  
of *A. castellanii* trophozoites.\*

Culture Age (hr)	Cells/ml (x 10 <sup>6</sup> )	Percent Latency	
		APase	DNase
98.2	3.28	46.8	37.8
101.8	2.67	43.1	—
149.5	4.79	43.1	40.1
170.5	5.55	41.0	41.3
186.6	9.32	35.7	32.3
261.3	10.70	—	42.0

\*Homogenates of amoebae, prepared with a Potter-Elvehjem homogenizer, were divided into two portions. Release of latent activities was effected in one portion by 0.1% Triton X-100 with a subsequent incubation at 4°C for 60 min prior to performing the enzyme assays. Non-latent activities were determined in the remaining portion of freshly prepared homogenate by assaying the enzymes as quickly as possible. Latency was calculated as that percentage of the total hydrolase activity that was undetectable in the absence of Triton treatment.

4° in 0.1% Triton X-100. The APase was assayed in the pellet obtained upon centrifuging a cell-free homogenate of *A. castellanii* at 20,000 x g. Since the homogenizing buffer contained 0.25 M sucrose, this pellet should contain unbroken lysosomes (de Duve, 1971). After a 15 min exposure to Triton X-100 the latency of APase reached a plateau of about 35%.

#### CHANGES DURING ENCYSTMENT

Although the 72 hr amoebae were encysted in unaerated monolayer cultures of encystment medium and the 168 hr amoebae were encysted in vigorously aerated suspension cultures of the same medium, the encystment kinetics appeared to be identical (fig. 3). Synchronous differentiation commenced sometime between the 8th and 15th hr of incubation, and by 24 hrs, over 90% of the amoebae formed cysts; essentially 100%

in at least four parameters (Martin, 1973). The percent of the hydrolase activity that sediments at 20,000 x g was less in the younger cells. The RNase activity per amoeba was twofold higher in the 72 hr cells. The 72 hr amoebae exhibited a greater tendency to clump during encystment and 168 hr cells differentiated more slowly than the 72 hr amoebae in unaerated monolayer cultures of encystment medium.

Alterations in lysosomal enzyme activity and protein per amoeba for the 168 hr cells encysting in aerated suspension cultures of encystment medium are shown in fig. 4. The data are relatively similar in profile to those for 72 hr cells differentiating in unaerated monolayer cultures of the same medium. There was a selective decrease in the cellular activity of all three hydrolases during incubation in encystment medium (fig. 4a). The re-

duction in activity for the nucleases commenced even before the appearance of thick-walled cysts, which occurred between 8 and 15 hrs (fig. 3). The decline in RNase was particularly pronounced. After 6 hrs in encystment medium, about 60% of the RNase activity had been lost, and after 40 hrs of incubation over 90% of the RNase activity was depleted. In contrast, it took 70 hrs for the amoebae to

per cell during encystment may merely reflect a disposal of enzymes not needed by dormant cysts. The electron micrographs of Bowers and Korn (1969), which demonstrated a depletion of food vacuoles during encystment in *A. castellanii*, support this viewpoint. The dramatic decline in RNase activity per cell, which occurred before the appearance of thick-walled cysts (figs. 3, 4a), possibly re-

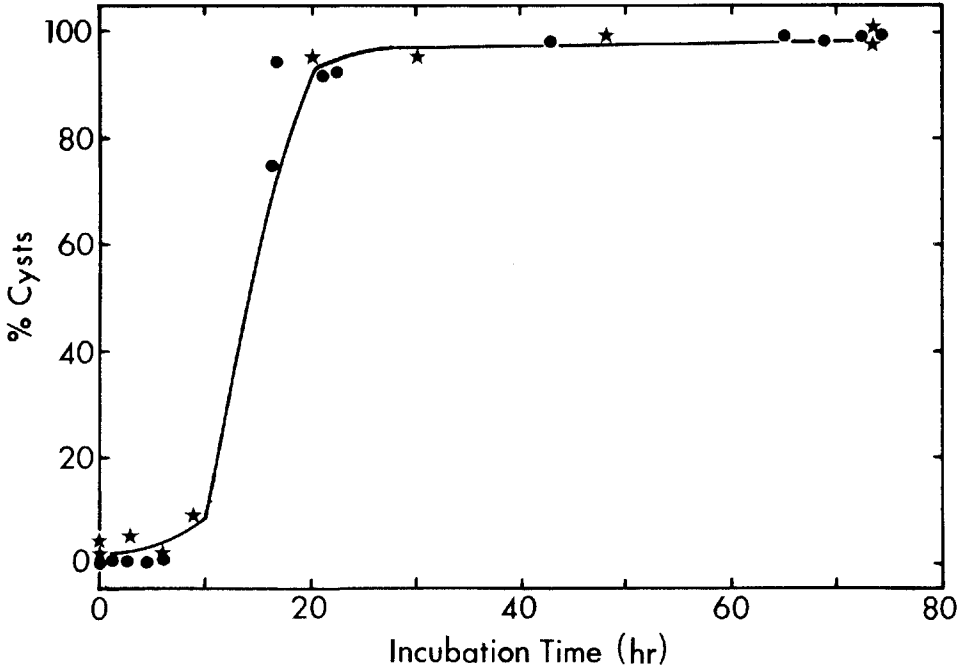


FIGURE 3. Encystment kinetics of *A. castellanii* in nutrient-free encystment medium. 72 hr amoebae encysting in un-aerated monolayer cultures ( $\bullet$ ). 168 hr cells encysting in aerated suspension cultures ( $\star$ ).

lose 60% of their cellular protein (fig. 4b). This decline in total protein during encystment agrees with the data of other investigators, who ascribed it to endogenous degradation (Neff and Neff, 1969; Griffiths, 1970).

The selective decrease during encystment in activity per cell of the three hydrolases might be caused by several factors: such as, accumulation of inhibitors, loss of activators, secretion into the ambient medium, or intracellular degradation of the hydrolases by protease enzymes. If APase, DNase, and RNase function primarily as digestive hydrolases in trophozoites, their decrease in activity

presents an adaptation by the cells to protect against degradation of RNA essential for encystment. Rudick (1971) presented evidence that at least some of the RNA required for encystment was synthesized during culture aging.

RNase specific activity was quickly reduced in a fashion similar to that for activity per amoeba (fig. 4a, c). The specific activity for DNase decreased at a slower rate. APase, however, actually was enriched in encysting amoebae even though its activity per cell was gradually decreasing (fig. 4a, c). After 20 hrs an increase of about 75% in APase/mg protein was attained; by 70

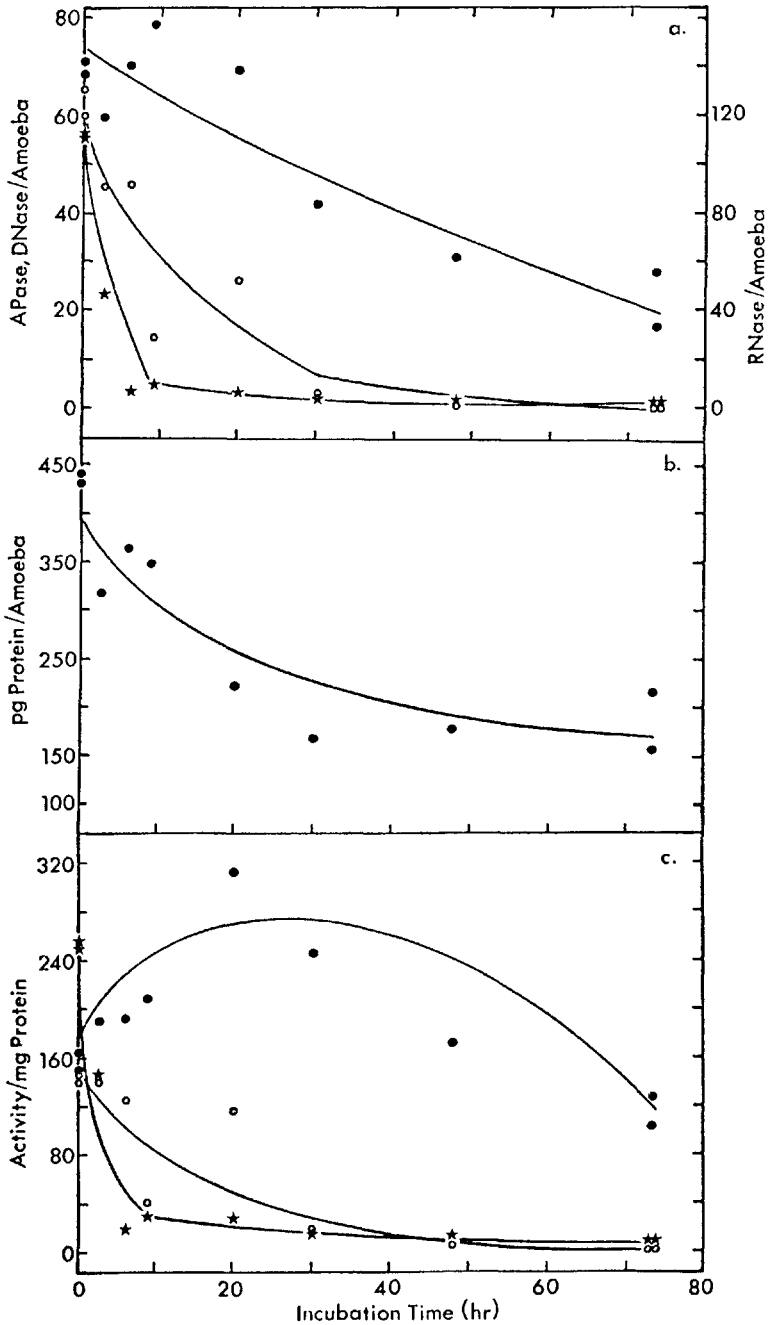


FIGURE 4. Changes occurring in 168 hr *A. castellanii* encysting in aerated suspension cultures of encystment medium. a. Activities/amoeba of lysosomal enzymes. Activity/cell of APase (●) is expressed as fmoles of *p*-nitrophenol formed/min, whereas DNase (○) and RNase (★) are expressed as  $\mu$  units of activity/amoeba. b. Cellular protein content. c. Specific activities of lysosomal enzymes. Specific activity of APase (●) is calculated as nmoles of *p*-nitrophenol formed/min/mg protein. Specific activity of DNase (○) and RNase (★) is expressed as units/mg protein.

hrs the specific activity of this enzyme subsequently dropped back to its initial value. The observation that APase was enriched during encystment supports the cytological findings of Bowers and Korn (1969) that this enzyme participates in autophagic processes during differentiation in *A. castellanii*. Perhaps APase plays an important role in supplying free phosphate for use either in an energy-generating system or else as a precursor for synthesizing the phosphoprotein and cellulose of the cyst wall. Moreover, it cannot be ruled out that the enrichment of APase activity during encystment is caused by the synthesis of novel APases.

If encysting amoebae conserve APase to provide free phosphate for physiological activities, addition of phosphate to encystment medium should promote a decline in the activity of this enzyme because amoebae could acquire phosphate from the medium, if no permeability barriers were established. APase activity per cell, as well as cellular protein content and percent cysts, however, were unaffected by adding final concentrations of 0.25, 0.50, or 0.75 mM  $\text{KH}_2\text{PO}_4$  to encystment medium (Martin, 1973). These data suggest that APase levels are not regulated by exogenous phosphate during differentiation.

When 168 hr amoebae differentiated in unaerated monolayer cultures of encystment medium, they exhibited changing patterns in lysosomal enzyme activities and cellular protein content that were essentially identical to those shown in figure 4 (Martin, 1973). Preliminary evidence suggests that the same is true for 32 hr log phase amoebae induced to encyst by adding either ethidium bromide or erythromycin to growth medium (Martin, 1973). We believe that in *A. castellanii*, the changing levels in lysosomal enzyme activities and cellular pro-

tein content are characteristic features of encystment and are independent of environmental conditions or age of the cells.

#### LITERATURE CITED

- Bowers, B. and E. D. Korn. 1969. The fine structure of *Acanthamoeba castellanii* (Neff strain). II. Encystment. *J. Cell Biol.* 41: 786-805.
- Byers, T. J., V. L. Rudick and M. J. Rudick. 1969. Cell size, macromolecule composition, nuclear number, oxygen consumption and cyst formation during two growth phases in unagitated cultures of *Acanthamoeba castellanii*. *J. Protozool.* 16: 693-699.
- de Duve, C. 1971. Tissue fractionation: past and present. *J. Cell Biol.* 50: 20D-55D.
- Dingle, J. T. and H. B. Fell, eds. 1969. Lysosomes in biology and pathology, vols. 1, 2. North-Holland Pub., Amsterdam. 1256 p.
- Griffiths, A. J. 1970. Encystment in amoebae. *Adv. Micro. Physiol.* 4: 105-129.
- Martin, S. M. 1973. Lysosomal activity during population growth and encystment in the soil amoeba, *Acanthamoeba castellanii*. Unpub. Ph.D. thesis. Ohio State University, Columbus. 143 p. (Accession no. 74-3245, Xerox University Microfilms).
- Mattar, F. E. and T. J. Byers. 1971. Morphological changes and the requirements for macromolecule synthesis during encystment of *Acanthamoeba castellanii*. *J. Cell Biol.* 49: 507-519.
- Müller, M. 1969. Lysosomal hydrolases in *Acanthamoeba* sp. *J. Protozool.* 16: 428-431.
- Neff, R. J. 1957. Purification, axenic cultivation, and description of a soil amoeba, *Acanthamoeba* sp. *J. Protozool.* 4: 176-182.
- and R. H. Neff. 1969. The biochemistry of amoebic encystment. *Symp. Soc. Exp. Biol.* 23: 51-81.
- , S. A. Ray, W. F. Benton and M. Wilborn. 1964. Induction of synchronous encystment (differentiation) in *Acanthamoeba* sp. *Meth. Cell Physiol.* 1: 55-83.
- Page, F. C. 1967. Re-definition of the genus *Acanthamoeba* with descriptions of three species. *J. Protozool.* 14: 709-724.
- Rudick, V. L. 1971. Relationships between nucleic acid synthetic patterns and encystment in aging unagitated cultures of *Acanthamoeba castellanii*. *J. Cell Biol.* 49: 498-506.
- Wattiaux, R. and C. de Duve. 1956. Tissue fractionation studies. 7. Release of bound hydrolases by means of Triton X-100. *Biochem. J.* 63: 606-608.