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CASTELLANII.

The Ohio State University, Ph.D., 1973
Zoology

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LYSOSOMAL ACTIVITY DURING POPULATION GROWTH
AND ENCYSTMENT IN THE SOIL AMOEBA,
ACANTHAMOEBA CASTELLANII

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
Scott McClung Martin, B.S., M.S.

The Ohio State University
1973

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ACKNOWLEDGEMENTS

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Laboratory space, equipment, and supplies were provided by both the Department of Zoology, and the Department of Microbiology, at The Ohio State University.

During most of the time spent on this degree, a half-time graduate teaching associateship in the Department of Zoology provided the author with financial support, and afforded him valuable experience in teaching.

While pursuing this doctoral degree, the author was deeply grieved by the unexpected death of his father, which was followed a few months later by the death of his brother. The clouded perspective which resulted, coupled by added responsibilities at home, often made it difficult to continue. The following quotations were found helpful:
Death of a loved one is the most severe test that you will ever face, and if you can rise above your griefs, and if you will trust in God, then you will be able to surmount any other difficulty with which you may be faced.

Harold B. Lee
(President, Church of Jesus Christ of Latter-day Saints)

In this sad world of ours, sorrow comes to all, and it often comes with bitter agony. Perfect relief is not possible, except with time. You cannot now believe that you will ever feel better. But this is not true. You are sure to be happy again. Knowing this, truly believing it, will make you less miserable now. I have had enough experience to make this statement.

Abraham Lincoln
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LIST OF ABBREVIATIONS

AEM - 10% HCl-95% ethanol-0.01 M MgCl₂
APase - Acid phosphatase
BSA - Bovine serum albumin
CM - Counting medium
DNA - Deoxyribonucleic acid
DNase - Acid deoxyribonuclease
Eb - Ethidium bromide
EDTA - Disodium dihydrogen ethylenediaminetetraacetate dehydrate
EM - Encystment medium
Ery - Erythromycin
GT - Generation time, or population doubling time, for LM amoebae
HB - Homogenizing buffer
LM - Logarithmic multiplication
NPP - Disodium p-nitrophenyl phosphate
OGM - Optimal growth medium
PGA - Perchloric acid
PGD - Population growth deceleration
RNA - Ribonucleic acid
RNase - Acid ribonuclease
TCA - Trichloroacetic acid
Tris - Tris(hydroxymethyl)aminomethane
UV - Ultraviolet light

.tri
INTRODUCTION

For the past several years, studies in this laboratory have been concerned with the molecular biology of encystment in the sarcodinid protozoan, *Acanthamoeba castellanii*. These studies have attempted to answer three basic questions: (1) What role does environmental change play in the regulation of encystment? (2) What intracellular events are most significant in the developmental phenomena involved in encystment? and (3) How are these events internally regulated?

It is anticipated that the knowledge gained from studying encystment in *A. castellanii* will contribute to understanding (1) the control of eukaryotic cytodifferentiation, and (2) the regulation of macromolecular syntheses. Furthermore, the knowledge garnered should also have application in understanding the epidemiology of amoebic pathogens, since there are several organisms in the same family that possess certain morphological and physiological similarities to *A. castellanii*, and which have been implicated in man and other animals as causative agents for the fatal disease, primary amoebic meningoencephalitis (Culbertson, 1971; Duma, 1972).

A significant reason for studying encystment in *A. castellanii* is the light it might shed on embryonic differentiation in eukaryotes. Both amoebic and embryonic systems appear to have at least two events in common: (1) an induction period, followed by (2) a period of differentiation, during
which specific new macromolecules may be synthesized. If all eukaryotic differentiative systems, including even neoplastic ones, have common mechanisms at the genomic and/or molecular level(s), one might expect first identification of them in the simpler systems, such as encystment in *A. castellanii*.

It is well known that lysosomal enzymes serve as active participants in a wide variety of eukaryotic differentiative processes (Table 1). It is further known that during encystment in *A. castellanii*, there is a rather massive decrease in the cellular contents of DNA, RNA, protein, lipid, and glycogen, and these changes are generally ascribed to endogenous degradation (Neff & Neff, 1969; Griffiths, 1970). Since it is well documented that lysosomal enzymes act as catabolic hydrolases in cells (de Reuck & Cameron, 1969; Dingle & Fell, 1969), there was reason to believe that such enzymes might play important roles in regulating cytodifferentiation in *A. castellanii*. To test the verity of this hypothesis, the activities of three lysosomal marker enzymes, acid phosphatase (APase), acid deoxyribonuclease (DNase), and acid ribonuclease (RNase), have been investigated throughout both population growth and encystment in *A. castellanii*.

The basic approach in this study was first to describe changes in the levels of lysosomal enzyme activities that occur during logarithmic multiplication (LM) and population growth deceleration (PGD), and then to induce encystment at appropriate times and examine the subsequent changes in enzyme activities. Three parameters of lysosomal enzyme activity were measured: (1) activity/amoeba; (2) activity/mg protein; and (3) % sedimentation at 20,000 $\times$ g in 0.25 M sucrose. The latter value measures the proportion of hydrolase activity which is particle-bound. Contents of total cellular
Encystment in *A. castellanii* is commonly induced by starving the cells in a nutrient-free salts solution (Neff, Ray, et. al., 1964; Griffiths & Hughes, 1968, 1969). Under such conditions, it is logical to expect that encysting amoebae would endogenously degrade macromolecules to supply energy and provide precursors for synthesizing the cyst wall, but is this degradation necessary for encystment? Does degradation still occur if encystment is induced in the presence of exogenous nutrients? Aeration is considered to be an essential requirement for rapid encystment (Neff, Ray, et. al., 1964), but in this study amoebae were found to differentiate synchronously and rapidly without aeration. Does aeration influence the patterns of intracellular degradation during encystment? Lastly, evidence has accumulated that cultures of *A. castellanii* in growth medium begin preparing for encystment during aging (Byers, et. al., 1969; V. Rudick, 1971). Do the patterns of endogenous degradation depend in any way on the age of cells when they are induced to encyst?

Experiments were performed in this study to begin answering these questions. The effect of cell age was tested by incubating both 3-day and 7-day PGD cells in nutrient-free encystment medium (EM), and also by chemically inducing encystment of mid-LM amoebae in growth medium. The influence of aeration was examined by incubating 7-day PGD cells in either aerated or unaerated EM. And the effect of exogenous nutrient supply was checked by chemically inducing encystment of mid-LM cells in optimal growth medium (OGM) with either ethidium bromide (Eb) or erythromycin (Ery).
Encystment in Protozoa

The encystment-excystment cycle occurs rather universally among Protozoa. These organisms have probably the most widespread geographic distribution of all the animal phyla. They also are short-lived and often lethally affected by slight changes in their environment. Without the ability to encyst, it is doubtful if Protozoa could successfully explicit the diversity of habitats which they do. Within the protective cyst, the dormant form may remain for many years until conditions favorable for emergence occur.

Protozoan cysts are basically of two types: (1) protective or resting; and (2) reproductive. There is often no clear distinction between the two, however. In the protective or resting cyst, a dormant animal is able to withstand certain adverse features of the environment which would normally kill the vegetative form. Within reproductive cysts, nuclear division occurs with the concurrent or ultimate formation of several offspring.

Some environmental factors listed by Crump (1950), van Wagendonk (1955), Manwell (1968), and Padilla & Cameron (1968) as possible inducers of encystment in Protozoa include (1) deficiency of food, (2) accumulation of excretory products of both bacterial and protozoan origin, (3) change in pH, (4) desiccation, (5) deplenishment of oxygen, (6) temperatures above or below optimal growth, (7) crowding, and (8) in the case of certain parasitic
forms, hormone levels of the host. Promotion of excystment has been ascribed to (1) osmotic phenomena, (2) high food and oxygen concentrations, and (3) suitable pH and temperature (Crump, 1950; van Wagendonk, 1955; Manwell, 1968).

Gould (1971) has listed six principal reasons for studying bacterial spores. With minor paraphrasing, these same reasons apply for studying protective cysts of Protozoa, and are as follows: (1) Many protozoan cysts are exceptionally resistant to adverse environmental conditions and to physical and chemical abrasives, even though they contain certain components also found in vegetative cells. An understanding of the mechanisms of resistance remains a challenging problem. (2) Protozoan cysts are dormant and often long-lived, and the phenomenon of dormancy in organisms is still little understood. (3) The breaking of the dormant state during excystment occurs by processes that likely are so rapid as to be aptly termed "trigger reactions." The changes underlying these germination trigger reactions are still unknown. (4) An excysting protozoan outgrows to produce a new vegetative cell. This germination comprises an ordered sequence of biochemical and cytological changes that are properly within the investigative domain of developmental biology. (5) When a vegetative cell transforms to a cyst, some vegetative genes probably become essentially suppressed while the cyst genes, which control the biosynthesis of novel proteins and other recognizable products and structures, become activated. In synchronously encysting cultures, many biochemical aspects of the differentiative process can be studied with precision. (6) Cysts represent "last-ditch" survivors of a population growing either in Nature or in the laboratory. Since many protozoans are pathogens of
man and other animals, detailed knowledge about their cysts might shed light on appropriate epidemiological controls.

The Acanthamoeba System

*A. castellanii* is a free-living soil and freshwater amoeba that can be cultured axenically in the lab (Neff, Ray, et al., 1964). It possesses a relatively uncomplicated life cycle, which consists of a vegetatively multiplying trophozoite that alternates, under unfavorable environmental conditions, with a cyst form. The wall of the cyst is double-layered and consists predominantly of cellulose and protein (Neff & Neff, 1969; Griffiths, 1970). Since encystment in *A. castellanii* involves a change in a single cell type, it is a cytodifferentiation in the best sense. Several reviews about encystment in this and related amoebae are available (Griffiths, 1970; Krishna Murti, 1971; Neff, 1971; Neff & Neff, 1969, 1972; Neff, Ray, et al., 1964). Encystment is the only specialized function that *A. castellanii* can perform, and it has the advantage for study of being uncomplicated by sexual processes; the multiplying trophozoites reproduce only by asexual fission, and so a population of cells is genetically homogeneous. The cyst stage is truly dormant, as evidenced by the fact that respiration of mature cysts is below the limits of measurement by Warburg respirometry (Neff & Neff, 1969), and by the lack of RNA and protein synthesis (Mattar, 1970; Mattar & Byers, 1971). Growth and division are also absent during the cyst stage. The mature cyst wall is tough and completely resists the usual homogenization techniques. Cysts will survive years of desiccation in shelf cultures, and will also survive vacuum desiccation (Neff & Neff, 1969).
Encystment in A. castellanii is a suitable system for analysis of cellular differentiation for (1) trophozoite and cyst forms are readily distinguishable by both structural and biochemical criteria, (2) the encystment process is reversible, and it can be separated from normal growth of vegetative cells, and (3) the development of a technique for induction of synchronous encystment (Neff, Ray, et al., 1964) allows the process to be followed under fairly well defined experimental conditions. An analysis of encystment in A. castellanii affords a comparison, in some ways, with the more complex developmental phenomena found in Bacillus bacteria and the slime mold Dictyostelium. In all three systems, there are definite biochemical similarities, such as the synthesis of cell-wall polysaccharide and the degradation of proteins. However, encystment in A. castellanii is not complicated by cell-cell interactions such as occur during bacterial sporulation, in which the spore develops from and remains within a mother cell, or slime mold morphogenesis, in which an aggregation of cells gives rise to a fruiting sporocarp. A single, isolated trophozoite of A. castellanii can be induced to encyst under appropriate conditions (Neff, Ray, et al., 1964).

The size, composition, and physiology of cells of A. castellanii have been studied during both LM and PGD phases of growth (Byers, et al., 1969; V. Rudick, 1969, 1971; M. Rudick, 1970), and some of the observed changes appear related to encystment. Cellular levels of DNA, RNA, and glycogen begin to change during late LM (Byers, et al., 1969; V. Rudick, 1969). DNA decreases about 50%, while RNA increases 75%. Glycogen decreases 50% during the RNA build-up, possibly to supply energy for the stimulated RNA synthesis, and then increases to a plateau above the level in LM cells. A
final decrease in glycogen follows an increase in the relative number of cysts in late PGD. The cyst fraction in aging cultures never exceeds a few percent of the total population, however, probably because some environmental factor inhibits cyst wall formation. It is known that the cyst wall of *A. castellanii* contains a substantial amount of cellulose (Neff & Neff, 1969; Griffiths, 1970), and the final glycogen drop in aging amoebae might be due to degradation to provide precursor molecules for synthesizing this polysaccharide.

Pulse-labeling experiments have shown that the rate of DNA synthesis decreases at the same time that DNA/amoeba is dropping by 50% (V. Rudick, 1969, 1971). This drop in DNA content was explained by demonstrating with hydroxyurea, an inhibitor of DNA synthesis (Adams & Lindsay, 1967), that the majority of LM amoebae can replicate once when DNA synthesis is inhibited and, therefore, must be in $G_2$ of the cell cycle, whereas PGD amoebae cannot multiply in the presence of hydroxyurea, and so must be in $G_1$. By agitating cells in conjunction with hydroxyurea treatment, encystment can be induced in growth medium (V. Rudick, 1969, 1971). Neff & Neff (1966, 1969, 1972) have shown that other inhibitors of DNA synthesis will stimulate encystment in *A. castellanii*.

Pulse-labeling experiments by V. Rudick (1969, 1971) have demonstrated that the rate of RNA synthesis increases 25% in late LM-early PGD while RNA/amoeba is increasing 75%; the rate of synthesis then decreases by 65%. The majority of this accumulated RNA was shown by acrylamide gel disc electrophoresis to be ribosomal (V. Rudick, 1971). By using actinomycin D at different times during the RNA build-up, it was shown that the ability of
amoebae to encyst when inoculated into starvation medium was partly dependent on the presence of this RNA (V. Rudick, 1969, 1971).

Since intracellular changes do occur in *A. castellanii* during aging, there was reason to suspect that levels of lysosomal enzyme activities might also alter during growth. Furthermore, it seemed logical that cells of different ages would vary in their competence to encyst. Indeed, Neff, Ray, et al. (1964) reported that for amoebae grown in suspension culture, stationary phase cells (2.8 x 10^6 cells/ml) required 90 min longer than log phase amoebae (2.6 x 10^5 cells/ml) to enter the cyst initiation phase (cysts = 10% of total) after they were inoculated into an aerated salts solution. However, once encystment in the older cells had been initiated, differentiation proceeded with rapid synchrony, so that both populations reached the 80% cyst level 10.5 hr after induction.

The Lysosome Concept

An historical account of the discovery and definition of the lysosome as a discrete cellular organelle can be obtained by consulting, in chronological sequence, the following review articles: (1) de Duve, 1959a; (2) de Duve, 1959b; (3) Novikoff, 1960; (4) Novikoff, 1961; (5) de Duve, 1963a; (6) de Duve, 1963b; (7) Novikoff, 1963; (8) de Duve, 1964; (9) Novikoff, et al., 1964; (10) de Duve, 1965; (11) de Duve & Wattiaux, 1966; (12) Vaes, 1966; (13) Tappel, 1968; and (14) de Duve, 1969. Independent biochemical and cytological studies by numerous investigators, foremost of whom were the Frenchman, Christian de Duve, and the American, Alex B. Novikoff, led to the recognition of lysosomes as a special class of
cytoplasmic particles. A now-voluminous scientific literature reveals that lysosomes are uniquely different from other cellular organelles because of (1) their polymorphism, and (2) the incredible variety of processes, both physiological and pathological, in which they are implicated. Some excellent source books for information about lysosomes have been compiled and edited by de Reuck & Cameron (1963), Dingle & Fell (1969), and Dingle (1972).

In brief, the lysosome is a sac-like structure, bounded by a unit membrane (Lucy, 1969), that contains a variety of hydrolase enzymes exhibiting acid pH optima. The term "lysosome" (meaning lytic, or digestive, body) was coined by de Duve and collaborators to make reference to this hydrolytic function of the constituent enzymes. It is now clear that lysosomes constitute an intracellular digestive system which is comparable, except for its discontinuity, with the digestive tract of higher organisms. The material digested within this lysosomal system may be either of exogenous or endogenous origin. In other words, the system may be associated with heterophagy or autophagy.

Lysosomes are not found in prokaryotes (de Duve & Wattiaux, 1966), but a rather extensive scientific literature attests to their ubiquity in animal cells (e.g., Tappel, et. al., 1963), and they are also present in some plant cells (Matile, 1969). The term "lysosome" refers to a variety of organellar forms that can be distinguished by both morphological and functional criteria. This variety has given rise to an abundant and sometimes confusing nomenclature. Ericsson, et. al. (1965) and de Duve & Wattiaux (1966) have compiled lists of names for lysosomes which have appeared in the literature, together with their synonyms and approximate definitions.
A selected list of biological functions in which lysosomal enzymes participate, as well as some disorders associated with hypo- or hyperactivity of lysosomes, is given in Table 1. This list was compiled by consulting the review articles and source books previously cited.

Some thirty different hydrolases have now been ascribed to lysosomes, and a few of the more well-known enzymes, with their substrates, are listed in Table 2. A more complete compilation of lysosomal enzymes, with a description of their properties and methods of assay, can be found in articles by Barrett (1969) and Tappel (1969) and the laboratory handbook by Dingle (1972).

According to the review articles and source books already cited, there are three biochemical criteria for the presence of lysosomes in a cellular extract: (1) acid pH optima of the constituent hydrolases; (2) sedimentation of these hydrolases at 20,000 X g in 0.25 M sucrose, thereby attesting to their particle-bound nature; and (3) latency, or the enhancement of hydrolase activity upon treatment of homogenates with procedures that rupture lysosomal membranes. In addition, the sole cytochemical criterion for the existence of lysosomes in a cell is a positive staining reaction for the enzyme, acid phosphatase (APase).

The formation and fate of lysosomes has been described in articles by Novikoff, et. al. (1964), de Duve & Wattiaux (1966), Cohn & Fedorko (1969), Daems, et. al. (1969), Hughes, et. al. (1970), and Beck, et. al. (1972). Some mention of lysosomal formation must be made here. The so-called primary lysosome is a somewhat ethereal organelle that contains newly synthesized acid hydrolases which have not yet reacted with their ultimate
Table 1. Selected functions involving participation by lysosomal enzymes

I. Functions concerned with cellular economy

A. Functions dependent on heterophagy or heterolysis
1. Heterotrophic nutrition by intracellular or extracellular digestion, especially the former
2. Defense against bacteria, viruses, other microorganisms, and toxic macromolecules
3. Invasion by lysis of obstructing structures

B. Functions dependent on autophagy or autolysis
1. Nutrition during times of starvation through piecemeal autodigestion
2. Cytodifferentiation
3. Mitosis
4. Organelle turnover
5. Self-clearance of a dead cell

II. Functions concerned with organismic economy

A. Functions involving programmed cellular breakdown
1. Fertilization and activation of the egg
2. Cell death during embryogenesis
3. Various developmental processes: insect and amphibian metamorphosis, mammary gland involution, sexual cycles, keratinization of the skin
4. Apocrine and holocrine secretion

B. Functions associated with food processing
1. Participation in digestion in the gastrointestinal tract

C. Functions involving breakdown of secreted macromolecules
1. Reabsorption in the kidney and urinary bladder
2. Absorption and digestion in various epithelial, mesothelial, and endothelial cells
3. Processing of secretory products in glands, such as the thyroid, pituitary, and adrenal
4. Resorption of bone and connective tissue fibers
Table 1. (Cont.)

D. Functions associated with scavenging, immunity, and detoxication

1. Destruction of aged erythrocytes, dead cells, and cellular debris by macrophages and reticuloendothelial cells

2. Destruction of bacteria, viruses, other microorganisms, foreign particles, and toxic macromolecules by leukocytes, macrophages, and reticuloendothelial cells

3. Processing of antigens

III. Disorders due to lysosomal malfunction

A. Disorders associated with an inadequate lytic activity

1. Aging effected by the accumulation of undigestible materials in lysosomes

2. Genetically inherited lysosomal diseases, such as glycogen storage disease

3. Mental retardation

B. Disorders associated with an injurious lytic activity

1. Cellular autolysis due to a lysosomal rupture caused by infection of some foreign agent, especially a virus

2. Tissue damage mediated by allergic reactions

3. Arthritic inflammation

4. Neuro-muscular degeneration due to sclerotic diseases

5. Carcinogenesis
Table 2. Some enzymes characteristic of lysosomes

<table>
<thead>
<tr>
<th>Name of Enzyme</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid phosphatase</td>
<td>Phosphate monoesters</td>
</tr>
<tr>
<td>Acid deoxyribonuclease</td>
<td>DNA</td>
</tr>
<tr>
<td>Acid ribonuclease</td>
<td>RNA</td>
</tr>
<tr>
<td>Acid proteinase (cathepsin)</td>
<td>Proteins</td>
</tr>
<tr>
<td>a-glucosidase</td>
<td>Glycogen</td>
</tr>
<tr>
<td>b-N-acetylglucosaminidase</td>
<td>Mucopolysaccharides</td>
</tr>
<tr>
<td>b-glucuronidase</td>
<td>Mucopolysaccharides</td>
</tr>
<tr>
<td>b-galactosidase</td>
<td>Mucopolysaccharides</td>
</tr>
<tr>
<td>Aryl sulfatase</td>
<td>Organic-linked sulfates</td>
</tr>
</tbody>
</table>
substrates. These structures are thought to arise either from the Golgi apparatus or the smooth endoplasmic reticulum; presumably, the original site of synthesis for their constituent enzymes is the rough endoplasmic reticulum.

Extracellular material to be digested is taken into the cell by some form of vesiculation of the plasma membrane, a process more generally referred to as endocytosis (Jacques, 1969; Beck, et al., 1971; Chapman-Andresen, 1972), and more specifically as either phagocytosis or pinocytosis, depending on the size of the material engulfed. The endocytic structure formed is called a heterophagic vacuole, or heterophagosome. Fusion of primary lysosomes with a heterophagosome results in formation of a secondary lysosome or, more simply, a food vacuole. In general, it is the secondary lysosome which is seen in cells because of its larger size, cytochemical reactivity, and characteristic density. Secondary lysosomes may fuse with each other to produce larger vacuoles. They might also accumulate sizable quantities of undigested or indigestible material, and are then termed residual bodies.

Many cells possess a mechanism for the bulk segregation and digestion of portions of their own cytoplasm, a process known as focal degradation (Swift & Hruban, 1964) or cellular autophagy (Ericsson, 1969). The circumscribed areas might contain mitochondria, rough and smooth endoplasmic reticulum, membranous arrays, microbodies, glycogen particles, or other cytoplasmic entities in variant stages of degradation. These structures are called autophagosomes, or autophagic vacuoles. They form when segments of cytoplasm become sequestered by either smooth endoplasmic reticulum or fused
Golgi vesicles and, hence, are initially bounded by a double unit membrane, the inner layer of which is resorbed. If acid hydrolases are present between the layers of the enclosing membranes, they will be discharged into the sequestered cytoplasm. Therefore, autophagic vacuoles might not obtain their constituent hydrolases by fusion with primary lysosomes, although such fusion can still occur.

**Lysosomes in Hartmannellid Amoebae**

Lysosomes in Protozoa function as both heterophagic and autophagic vacuoles. The digestive processes in these animals have been reviewed by Kitching (1956), Muller, et al. (1963), Hall (1965), Muller (1967), Manwell (1968), and Chapman-Andresen (1972).

Digestive, or heterophagic, vacuoles have been observed in electron micrographs of trophozoites of *A. castellanii* (Bowers & Korn, 1968; V. Rudick, 1969; Wetzel & Korn, 1969) and other hartmannellid amoebae (Vickerman, 1962). A Golgi complex can be seen in electron micrographs of both trophozoites (Bowers & Korn, 1968) and encysting cells (Bowers & Korn, 1969) of *A. castellanii*.

The only cytological evidence thusfar reported demonstrating involvement of both the Golgi and lysosomes in cytodifferentiation of a hartmannellid has been that of Bowers & Korn (1969) with their electron microscopic study of encystment in *A. castellanii*. They observed that early in encystment, the Golgi complex is enlarged and contains a densely staining material that appears to contribute to cyst wall formation. The cellular volume fraction of the Golgi increases about sixfold early in encystment, and
thereafter returns to its original volume fraction of about 0.5%. After amoebae have been in encystment medium for 8 hr, by which time they have synthesized the outer proteinaceous cyst wall, or exocyst, approximately 80% of the initial digestive vacuole system has disappeared. As encystment continues, this digestive system completely disappears as a recognizable component. Autophagic vacuoles are not seen when trophozoites are first put into encystment medium, but they appear very shortly and are found to contain mitochondria, sordy cytoplasmic debris, and glycogen particles. The contents of some of these autophagosomes are deposited in the developing cyst wall. Moreover, these autophagic vacuoles stain positively for APase, the marker enzyme for lysosomes. After 8 hr of encystment, by which time, as already mentioned, the exocyst has been formed, autophagosomes constitute about 9% of the cellular volume. By 18 hr, when the inner cellulosic cyst wall, or endocyst, has also been synthesized, these autophagosomes make up about 18% of the cell volume. They thereafter decrease in number, although they are still present in mature, 46 hr cysts.

Lasman (1967a, 1967b, 1969, 1972), Edelstein & Lichtenstein (1968), and Edelstein, et al. (1968) have investigated lysosomal hydrolases in Mayorella palestinensis, a hartmannellid found in Israel. Lasman (1967a) reported that the acid pH optimum for intracellular APase in M. palestinensis was 3.2, whereas that for the extracellular APase secreted by the organism was 4.8 (Lasman, 1967b). Two maxima of APase activity were demonstrated during the life cycle of the animal: (1) one from cultures in logarithmic growth; and (2) the other during the period of maximal cell encystation (Lasman, 1967a). On the other hand, the activity of extracellular APase
increased at a steady rate, reaching a maximum in old cultures (Lasman, 1967a, 1967b). Lasman postulated that the peak of activity during LM might reflect an increased APase requirement necessary for the high rate of synthesis of protein and nucleic acid occurring in rapidly dividing cells. He thought that the higher level of APase during encystation was associated with either the increased synthetic activities required for cyst wall formation, or else with the storage of reserves or resorption of organelles which also occur at this time. Lasman (1969) cytochemically stained cells of *M. palestinensis* for APase, and by this method confirmed his biochemical studies that maximal APase activity was found in either LM or encysting amoebae.

Lasman (1972) has also assayed for α- and β-glucosidase in both cell-free homogenates and the ambient medium during growth of *M. palestinensis*. He found that activity of these two enzymes increased in both compartments with culture aging. Edelstein & Lichtenstein (1968) and Edelstein, et. al. (1968) reported that lyophilized preparations of *M. palestinensis* hydrolyzed hemoglobin, with maximum cleavage occurring near pH 3.0. They suggested that this amoeba therefore contains an acid proteinase which functions during intracellular digestion.

Both pinocytic (Bowers, 1970; Bowers & Olszewski, 1972) and phagocytic processes (Korn & Weisman, 1967; Weisman & Korn, 1967; Wetzel & Korn, 1969; Ulsamer, et. al., 1969, 1971; Bowers, 1970; Rabinovitch & Stefano, 1970, 1971a, 1971b; Goodall & Thompson, 1971) have been investigated in *Acanthamoeba*. When latex beads were phagocytosed by trophozoites of *A. castellanii*, and the vesicles then isolated from the amoebae, they were
found to have associated APase and β-glucosidase activity (Wetzel & Korn, 1969).

Barnes, et. al. (1969) reported in an abstract that they had spectrophotometrically assayed APase in growing trophozoites, precysts, and cysts in a special strain of *A. castellani* which they developed in their lab (Jensen, et. al.; 1970). APase activity was fairly constant during logarithmic growth, but showed an apparent increase in stationary phase cells. APase activity was low in both precysts and cysts. Band & Mohrlok (1969) assayed APase in whole amoeba homogenates of *A. rhysodes*, and reported that this acid hydrolase increased in activity during starvation and encystation.

Griffiths & Bowen (1969) investigated in *Hartmannella castellanii* the activity of a latent, non-specific phosphatase having a pH optimum of 4.0. Latency of this enzyme could be released in extracts prepared in 0.6 M sucrose by treatment with the detergent Triton X-100, freezing and thawing, refrigeration, or carbon tetrachloride. When cellular extracts were centrifuged at 20,000 X g for 20 min, the presence in the homogenizing medium of either 0.3, 0.6, or 0.9 M sucrose substantially increased the sedimentation of APase compared to an extract containing no sucrose. When cell-free homogenates prepared in 0.6 M sucrose after 24, 48, and 72 hr of growth, and after 6 hr of encystment, were subjected to a 20 min, 20,000 X g centrifugation, approximately 80% of the total APase activity sedimented. This sedimentable APase exhibited a greater degree of latency than the apparently soluble form of the enzyme; freezing and thawing of the pelleted fraction produced an increase of 60–80% in the phosphatase activity, compared to 10–20% in the soluble fraction. Although their data are not completely convincing, but could simply be
due to experimental variability, Griffiths & Bowen (1969) reported that APase/mg protein increased by 34% during the first 3 hr of encystation, but by 6 hr had dropped back to the value obtained at the 0 hr of encystment. APase/amoeba steadily decreased during the first 6 hr of encystment, which was interpreted as being due to degradation. Inhibitors and promoters of encystation were found by Griffiths & Bowen (1969) to affect the cellular levels of APase activity, but did not affect sedimentability of the enzyme in 0.6 M sucrose. This sedimentation persisted throughout the initial degradative phase of encystment. Incubation of homogenates of *H. castellani* decreased the latency of the APase, but this activation could be modified by substances which are known to affect the encystment responses of the amoebae. Griffiths & Bowen (1969) concluded from their work that the degradative phase of encystment in *H. castellani* is due to activation of hydrolytic enzymes within sedimentable compartments of the cells, and is not the result of hydro­lases being released from these organelles. They hypothesized that agents which are capable of promoting encystment possibly function by inducing activation of lysosomal enzymes.

Müller (1967b, 1969a) and Müller & Møller (1969) reported that isopycnic centrifugation of *Acanthamoeba* sp. homogenates in sucrose gradients revealed that the distribution pattern of two peroxisomal enzyme markers, urate oxidase and catalase, were identical, but were different from the patterns observed for succinate dehydrogenase, a marker for mitochondria, and for APase, a marker for lysosomes. Müller (1969b) demonstrated that homogenates of *Acanthamoeba* sp. trophozoites contained ribonuclease, phosphatase, proteinase, α-glucosidase, β-N-acetylglucosaminidase, and β-glucuronidase activities, all
of which had acid pH optima. After isopycnic centrifugation of the extracts in sucrose gradients, all of these acid hydrolases exhibited 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 also for that of urate oxidase and catalase.

Bowen, et. al. (1969) observed that in vegetative amoebae of H. castellanii, 44% of the APase sedimented when extracts prepared in a 0.32 M sucrose medium were centrifuged the equivalent of 100,000 X g for 1 hr in a 15-60% sucrose gradient. Another lysosomal enzyme, b-N-acetylglucosaminidase, was only 28% sedimentable. After incubating the amoebae for 5 hr in a replacement medium (Griffiths & Hughes, 1968) to induce encystment, 69% of the APase, and 40% of the other enzyme, sedimented. After 15 hr of encystment, each of the two hydrolases became resolvable in the sucrose gradient as double peaks, whereas they each previously had only exhibited a single peak. The specific activity of b-N-acetylglucosaminidase increased during encystment, whereas that of APase decreased.

Morgan & Griffiths (1972) differentially centrifuged cell extracts of H. castellanii to obtain 10,000, 20,000, and 100,000 X g pellets, and a 100,000 X g supernatant, and then assayed each of these fractions for acid hydrolase enzymes. In vegetative cells, 30-40% of the enzyme activities was found in the 10,000 X g pellet, whereas after 15 hr of encystment, 60% was found in this fraction, even though there was no similar shift in the distribution of protein. Enzyme distributions were also studied by centrifugation at 100,000 X g for 1 hr in a zonal rotor by employing a 15-60%
Trio-sucrose gradient. In extracts from vegetative cells, acid p-nitrophenylphosphatase, b-N-acetylglucosaminidase, a-galactosidase, b-glucuronidase, and RNase all showed fairly simple distributions, whereas b-galactosidase, b-glycerophosphatase, and DNase exhibited more complex profiles. All the enzymes were 50-60% sedimentable and found mainly between densities of 1.16 and 1.20. In extracts from encysting cells, 75–90% of the acid hydrolases became sedimented, with the peak for lysosomal activities being shifted to a density of 1.185. Furthermore, in extracts from encysting cells, the hydrolase activities became concentrated over a narrower region of the gradient, thereby yielding a simpler and more uniform distribution. The acid hydrolase distribution patterns for both vegetative and encysting cells differed from the patterns obtained for cytochrome oxidase, a mitochondrial marker, and for catalase, a marker for peroxisomes. Morgan & Griffiths (1972) interpreted their results by suggesting that during encystment, lysosomes become larger and heavier due to the predominance of autophagic vacuoles.

Capsule Summary

The available literature on lysosomes in *A. castellanii* is primarily biochemical in scope, and involves the development of techniques for studying these organelles in cell-free extracts. The notable exception is the excellent electron microscopic study by Bowers & Korn (1969) in which it was observed that during starvation-induced encystment in this organism, there was a depletion of the vesicular heterophagic system and a pronounced appearance of autophagic vacuoles. No concerted effort has yet been made to relate lysosomal activity to encystment in *A. castellanii*, although the rather
massive decrease in cellular contents of macromolecules during differentiation (Neff & Neff, 1969; Griffiths, 1970) suggests the activity of catabolic hydrolases. The limited efforts along this vein by Barnes, et. al. (1969) and Griffiths & Bowen (1969) for A. (or H.) castellanii, and by Band & Mohrlok (1969) for the related A. rhyzodes, are incomplete because so few sample times were employed and only one lysosomal enzyme, APase, was assayed.
MATERIALS AND METHODS

The Organism

The free-living soil and freshwater amoeba used in this study was originally obtained from Dr. Robert J. Neff of Vanderbilt University, and was designated by him as *Acanthamoeba* sp., strain I-12. According to Dr. Neff (personal communication to Dr. Thomas J. Byers), this clone was characterized by the ability to encyst readily, and to form a thick cyst wall. Neff isolated this amoeba in 1955 from soil at Pacific Grove, California, by using an agar-surface migration method (Neff, 1957). He was able to cultivate the animal axenically, and with others, reported on its nutrition and metabolism (Neff, et al., 1958). Later, he and coworkers developed techniques for inducing a synchronous encystment by the organism (Neff, Ray, et al., 1964).

The proper binomial designation for the amoeba used in this study was obscured by a controversy regarding its taxonomic position. Presumably, the amoeba isolated from soil by Neff (1957) was first discovered by Castellani (1930) as a contaminant in a yeast culture; Douglas (1930) described this sarcodinid, and named it *Hartmannella castellani*. The genus *Hartmannella* had originally been defined by Alexeieff (1912a, 1912b). In 1931, Volkonsky reviewed the hartmannellid amoebae and established a new genus, *Acanthamoeba*; he renamed Douglas' amoeba *Acanthamoeba castellani*, and used it as the type species (Volkonsky, 1931). However, Adam (1964) argued for the retention of
Hartmannella castellanii since Volkonsky (1931), in his description of the genus *Acanthamoeba*, failed to use characteristics which would sufficiently separate it from other genera. The genus *Acanthamoeba* was then redescribed by Page (1967a, 1967b), who concluded that Neff's strain should correctly be called *Acanthamoeba castellanii* Douglas 1930. This name has been accepted for the identification of the organism used in this study.

The term "hartmannellid amoebae" referred to in the literature includes organisms having the generic names of *Acanthamoeba*, *Hartmannella* (which may be variously spelled), and *Mayorella* (Adam, 1961, 1964). These animals are often simply called "soil amoebae," although some of them are also found in freshwater or marine habitats (Kudo, 1965; Griffin, 1969) and as contaminants in mammalian tissue cultures (Jahnes, *et al.*, 1957; Culbertson, *et al.*, 1958; Moore & Hlinka, 1968). Moreover, some forms have been implicated as possible causative agents in man and other animals for the fatal disease, primary amoebic meningoencephalitis (Culbertson, 1971; Duma, 1972).

Reviewing the literature on the hartmannellid amoebae is frustrating, for two or three generic names might be used to refer to the same organism. This ambiguity was caused by the confused state of the taxonomy of these animals in the earlier literature. Furthermore, many of the pathogenic amoebae originally identified as being *Acanthamoeba* or *Hartmannella* are now considered to have been amoeboid–flagellates of the related genus *Naegleria* (Griffin, 1969, 1972; Duma, 1972). The possibility that some pathogenic forms do belong to Page's genera of *Acanthamoeba* or *Hartmannella* cannot be precluded, however.
Optimal Growth Medium

Stock and experimental cultures of *A. castellanii* trophozoites were grown axenically in the optimal growth medium (OGM) described by Neff, Ray, et al. (1964). The components of this medium are listed in Table 3. This medium supports a rapid growth of trophozoites, as measured by a short generation time (GT), and cells cultured in it will undergo a rapid and synchronous encystment when placed in starvation conditions (Neff, Ray, et al., 1964).

If OGM is autoclaved immediately after preparation, there is formed a nitrogen-rich precipitate (Neff, et al., 1958) that interferes with the electronic counting of amoebae during the subsequent culture period, as well as with protein assays on prepared amoebic homogenates. This precipitate problem was obviated in the following way: The medium was heated to 85-90 C and held there for 5-10 min. It was then cooled to room temperature or below by use of a Cold Finger (Forma Scientific, Inc.), or else overnight storage in a 4 C cold room. The precipitate-laden medium was then poured into a Millipore pressure vessel with attached filter holder, and was forced by pressure from a compressed air tank through a 0.8 μm pore membrane filter that was preceded by a fiberglass prefiltro (Type AP25, Millipore Corp.). Filtered OGM was then dispersed into culture vessels by using a Brewer automatic pipettor (Baltimore Biological Laboratory). The vessels were capped with a double layer of aluminum foil, and were then autoclaved at 121 C and 15 psi for 20 min. Ville & Ehret (1968) have shown that the ciliate *Tetrahymena*, when cultured in media autoclaved under different conditions, exhibits different growth rates and obtains different maximal cell densities.
Table 5 Components of optimal growth medium (OGM)\textsuperscript{a}

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Final Amt/Liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteose peptone (Difco Laboratories)</td>
<td>0.75%</td>
<td>7.5 g</td>
</tr>
<tr>
<td>Yeast extract (Difco Laboratories)</td>
<td>0.75%</td>
<td>7.5 g</td>
</tr>
<tr>
<td>Glucose (added to the incomplete, sterile OGM prior to inoculation)</td>
<td>1.50%</td>
<td>15.0 g</td>
</tr>
</tbody>
</table>

**Salts** (prepared as a 40X stock)

<table>
<thead>
<tr>
<th>Salt</th>
<th>Concentration</th>
<th>Final Amt/Liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>MgSO(_4) * 7H(_2)O</td>
<td>1.00 mM</td>
<td>246.0 mg</td>
</tr>
<tr>
<td>CaCl(_2) * 2H(_2)O</td>
<td>0.05 mM</td>
<td>7.4 mg</td>
</tr>
<tr>
<td>K(_2)HPO(_4)</td>
<td>2.00 mM</td>
<td>272.0 mg</td>
</tr>
<tr>
<td>Ferric citrate</td>
<td>0.10 mM</td>
<td>30.0 mg</td>
</tr>
</tbody>
</table>

**Vitamins** (prepared as a 2000X stock in 95% ethanol)

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>Concentration</th>
<th>Final Amt/Liter</th>
</tr>
</thead>
<tbody>
<tr>
<td>B(_1) hydrochloride</td>
<td>1.000 mg</td>
<td></td>
</tr>
<tr>
<td>d-biotin</td>
<td>0.200 mg</td>
<td></td>
</tr>
<tr>
<td>B(_{12})</td>
<td>0.001 mg</td>
<td></td>
</tr>
</tbody>
</table>

1.0 N KOH  
A sufficient quantity to make pH 7.0

\textsuperscript{a}After Neff, Ray, \textit{et. al.} (1964)
Therefore, since conditions of autoclaving might also have important effects on experimental results obtained with *A. castellanii*, the autoclaving procedures described here were repeated faithfully.

OGM prepared in this manner remained precipitate-free and usable for several weeks. The medium was stored at 30°C in order to acclimate it to the proper growth temperature (Neff, et al., 1958) prior to inoculation with amoebae. Insofar as possible, the medium was also stored in the dark, since Dolphin (1968, 1970) had learned that OGM was somewhat photodegradable.

Glucose was added to the other components of OGM as a sterile 30% w/v solution after autoclaving. It was added in 2.5 ml amounts to 47.5 ml of the incomplete OGM to obtain a final concentration of 1.5%. For some reason, glucose carmelizes when autoclaved in conjunction with the other compounds. Moreover, a growth-inhibiting reaction, at least for bacteria, occurs when glucose is heated in a phosphate-peptone medium (Meynell & Meynell, 1965).

**Culture Procedures**

Stocks of *A. castellanii* were grown in 50 ml of OGM in 250 ml Erlenmeyer flasks, and were maintained in continuous growth by subculturing every 2-4 days. Inoculations of amoebae were always performed aseptically under ultraviolet lamps in a hood, and the cells were shielded from irradiation by being beneath a protective layer of water or glass. The germicidal effects of UV on bacteria have been summarized by Davis, et al. (1968). The failure of cells of *A. castellanii* to exhibit any noticeable dire effects due to accidental exposure to UV during inoculation remains a puzzle. There are
two possible explanations: (1) a highly polyploid eukaryotic genome which does not readily display mutations, and (2) a rather active cellular content of catalase, an enzyme which would destroy intracellular amounts of \( \text{H}_2\text{O}_2 \) caused by UV irradiation. This author has found that cell-free homogenates of \textit{A. castellanii} are rather high in catalase activity.

The amoebae used in experiments were cultured either in (1) 50 ml of OGM in 250 ml Erlenmeyer flasks, (2) 150 ml of OGM in horizontally-positioned Roux culture bottles, or (3) 50 ml of OGM in Roux bottles. Generally, procedures 1 and 2 were employed. The depth of the medium was nearly identical in these two types of culture containers, and it was found by this author that the growth kinetics of \textit{A. castellanii} were the same, as well. Method 3 was used when encystment was induced in shallow growth cultures by addition to OGM of either ethidium bromide or erythromycin. Although cells reached higher densities in these shallow cultures, they multiplied with a GT very similar to that obtained with procedures 1 and 2. Cultures were always maintained at 30 C, the temperature optimal for growth of \textit{A. castellanii} (Neff, et al., 1958), in a dark incubator without aeration or agitation.

In culturing amoebae for growth studies and to obtain cells for encystment experiments, an effort was made always to inoculate with LM cells having a concentration of 4-7 x 10^4 cells/ml. Stock cultures of about 2-3 x 10^5 amoebae/ml were used to set up inocula. Several variables made it difficult to obtain inocula at the cell density desired for a particular time the next day: (1) differences in GT's due to variant physiological states of stock cultures; (2) slight differences in OGM made at different times; and (3) adherence of some cells to the inside walls of the 5 ml pipet used to set up
cultures of inocula, so that one was not actually inoculating amoebae at the concentration suspected.

This author wished to start cultures of inocula at a concentration of about \(6.5 \times 10^3\) cells/ml, and then allow them to grow 22-24 hr and, thus, go through about 3.5 generations. To obtain inocula at the desired concentration of \(4-7 \times 10^4\) amoebae/ml 22-24 hr after inoculation, it was necessary to set up a series of three inocula. One series was inoculated with stock at an initial concentration of \(6.5 \times 10^3\) cells/ml, thereby anticipating no cell loss or deviation from normal growth. A second series was inoculated at a 20% higher concentration, and a third series, at a 40% higher cell density. By the desired time the next day, one of these three series was always within the range of \(4-7 \times 10^4\) amoebae/ml.

Amoebae of the proper inoculum concentration were scraped off the flask walls with a rubber policeman, and the cell suspensions combined into one of the flasks. Amoebae were kept suspended by slow agitation with a magnetic stirrer. The cells/ml of the inoculum was accurately determined by cell counting, and the ml of inoculum required to obtain a starting density of about \(1 \times 10^3\) amoebae/ml in growth cultures was calculated. The appropriate quantity was injected into culture vessels by use of a 2 ml Mini-pet (Hanostat Corp.).

**Electronic Counting of Trophozoites**

Amoebae were fixed by adding 0.25 ml of formalin per 50 ml of culture. Since the cells adhered to glass surfaces even after fixation, they were scraped from the walls of the culture vessels with a rubber policeman. A
12 ml sample of cell suspension was centrifuged at 700 X g in a Safeguard centrifuge (Clay-Adams, Inc.) for 3 min to pellet the amoebae. The supernatant was aspirated off to 0.5 ml without disturbing the pellet, which was resuspended with a vortex mixer (Scientific Industries, Inc.) back to 12 ml with counting medium (CM). CM was a mixture of 0.5 % (v/v) formalin-0.6 % (w/v) NaCl. The suspension of amoebae was then counted with an electronic particle counter (Celloscope #101, Particle Data, Inc.) which was equipped with a 100 μ orifice and a 0.5 ml manometer section.

Neff (1964) reported problems with (1) clumping of cells, (2) nonrandom dispersion during counting, and (3) high background counts when an electronic counter was used for counting trophozoites of A. castellani. Clumping would cause coincidence; i.e., passage of a clump through the orifice of the electrozone tube would be counted as one large cell. In this study, coincidence was eliminated as a serious problem by adjusting the cell densities for counting so as not to exceed 2 x 10⁴ amoebae/ml. Above this level, the counting rate of the Celloscope was not a linear function of cell concentration. By filtering the CM through a 0.8 μ pore membrane filter, background was less than 30 counts/0.5 ml. To ensure that amoebae were randomly dispersed while being counted, they were gently agitated by hydropulsation provided by the vacuum pump of the Celloscope. Four counts/0.5 ml were obtained for each sample suspension, and the mean value used to calculate cells/ml.

Growth Curves

Since trophozoites of A. castellani adhere tenaciously to glass
surfaces, it was not possible to take a series of samples from the same
growth vessel without seriously disturbing the cultures at each sampling,
and thereby introducing a growth lag. Therefore, at each sample time, 2 or
3 culture vessels were fixed with formalin, and then discarded after their
cell concentrations were determined. The means for these replicate sample
counts were used in plotting growth curves and in computing the generation
times, or population doubling times, for LM amoebae.

Using the concentrations of amoebae/ml for the log-linear portion of
the growth curves, a least squares analysis was used to calculate the slope
of the linear regression line best fitting the points. The intercept of
this line was the calculated, or "true," \( N_0 \) of the growth equation
\[
N = N_0 e^{kt}
\]
whereas the slope of the line represents \( k \) of the growth equation in \( \text{hr}^{-1} \).
Furthermore,
\[
k = \frac{\ln 2}{\text{GT}} = 0.693 \frac{\text{GT}}{\text{GT}}
\]
It was therefore a simple matter to calculate the GT for a growth curve by
using its rate constant \( k \).

Normalization of Data

In order to facilitate the comparison of data from many experiments,
growth curves were normalized to starting cell concentrations of \( 1 \times 10^3 \)
amoebae/ml. In practice, the usual starting concentration was \( 0.8-2.0 \times 10^3 \)
cells/ml. Normalization was possible because (1) no lag growth phases were
observed, and (2) the obtained PGD cell concentrations were independent of
inoculum size. A normalized population growth curve for trophozoites of
\textit{A. castellanii} is shown in Fig. 1.
The normalizing time was that value in hr that either had to be added to, or subtracted from, each sampling time in order to make 0-time of the growth curve correspond to $1 \times 10^3$ amoebae/ml. This manipulation was employed to clarify the graphical presentation of data.

The normalizing time was determined by using the calculated, or "true" $N_0$ obtained from the regression equation for the log-linear portion of the growth curve. When this "true" $N_0$ was greater than $1 \times 10^3$ amoebae/ml, it was substituted as $N$ in the growth equation; the subsequent calculation of $t$ yielded the normalizing time, which would be added to each sampling time along the growth curve. If the "true" $N_0$ were calculated at less than $1 \times 10^3$ amoebae/ml, it was substituted as $N_0$ in the growth equation; the computed value of $t$ was then subtracted from each sampling time on the growth curve.

**Encystment Medium**

For most of the encystment experiments performed in this study, amoebae were induced to differentiate by transferring them from OGM to a starvation-type, nutrient-free salts solution, the components of which are listed in Table 4. This encystment medium (EM) was essentially the one described by Neff, Ray, et. al. (1964), but its preparation was modified by this author to eliminate the necessity of bubbling autoclaved EM with CO$_2$ to dissolve the precipitate of carbonates which forms during sterilization (Neff, Ray, et. al., 1964). To make 5 liters of EM, the ingredients of Solution A (Table 4) were dissolved in water in a final volume of 4500 ml, and the components of Solution B were dissolved in a final volume of 500 ml. In order to
Table 4. Components of encystment medium (EM)\textsuperscript{a}

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Final Amt/5 Liters</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Solution A</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KCl</td>
<td>0.1 M</td>
<td>37.2800 g</td>
</tr>
<tr>
<td>NaHCO\textsubscript{3}</td>
<td>0.04 M</td>
<td>16.8000 g</td>
</tr>
<tr>
<td>2-amino-2-methyl-1,3-propanediol (Eastman Kodak Company)</td>
<td>0.00032 M</td>
<td>0.1682 g</td>
</tr>
<tr>
<td><strong>Solution B</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CaCl\textsubscript{2} \cdot 2H\textsubscript{2}O</td>
<td>0.0004 M</td>
<td>0.2942 g</td>
</tr>
<tr>
<td>MgSO\textsubscript{4} \cdot 7H\textsubscript{2}O</td>
<td>0.008 M</td>
<td>9.8600 g</td>
</tr>
</tbody>
</table>

\textsuperscript{a}After Neff, Ray, et. al. (1964)
get the divalent salts into solution readily without heating, the Ca compound was dissolved first. Solutions A and B were autoclaved separately. After cooling to 30 C, the two solutions were mixed on a magnetic stirrer, and then aseptically dispensed into Roux bottles with a Brewer automatic pipettor in quantities of 142.5 ml. The pH after mixing Solutions A and B was 8.6-9.0, the optimum for cyst formation in A. castellanii (Neff, Ray, et. al., 1964).

EM was sometimes made in a slightly different manner. The components of Solution A were dissolved in a final volume of 4500 ml, and then prior to autoclaving, were dispensed in 135 ml quantities with the Brewer pipettor into Roux bottles, which were capped with aluminum foil. The divalent salts were dissolved in a final volume of only 250 ml. After autoclaving and cooling to 30 C, 7.5 ml of Solution B was aseptically delivered into each Roux bottle containing sterile Solution A by use of a 10 ml Mini-pet (Manostat Corp.). The pH of these EM culture vessels was checked before inoculating with amoebae. If the pH was slightly above 9.0, it was brought within the range of 8.6-9.0 by adding 1-3 drops of concentrated HCl. A pH of 9.2 or above will kill the cells in a few hr (Neff, Ray, et. al., 1964).

**Inducing Encystment in Starvation Medium**

In this study, encystment was induced in starvation medium by (1) incubating both 3-day (71-74 hr) and 7-day (163-169 hr) PGD cells in unaerated, stationary cultures of EM in Roux bottles, or (2) incubation of 7-day PGD amoebae in aerated, suspension cultures of EM in aspirator bottles. Three-day PGD cells had a concentration of $2-3 \times 10^5$ amoebae/ml, while 7-day
cultures had a density of $4-6 \times 10^5$ cells/ml.

When amoebae were encysted in Roux bottles, they were collected aseptically by centrifugation at 700–1000 $\times g$, and the pellet was washed once in 150 ml of 0.15 M NaCl, which is isotonic to the cells (Neff, Ray, et al., 1964). By taking into account an expected cell loss of 20%, the amoebae were then resuspended in 0.15 M NaCl to a concentration thought to be somewhat more than $2.2 \times 10^6$ cells/ml. A 1.0 ml sample of this suspension was diluted for counting on the Celloscope to determine the actual cell density. The number of additional ml of 0.15 M NaCl to add to bring the cells/ml to $2.2 \times 10^6$ was calculated, and this dilution performed. By using a 10 ml Mini-pet, 7.5 ml of inoculum was dispensed into 142.5 ml of EM in Roux bottles, making a final concentration of about $1 \times 10^5$ amoebae/ml. The bottles were capped with aluminum foil and placed in a horizontal position in a dark incubator at 30°C without aeration or agitation.

When 7-day PGD cells were induced to encyst in aerated cultures of EM, a slight modification of the device used by M. Rudick (1970) for cultivating trophozoites in suspension cultures of OGM was employed. In allowing for a 20% loss of cells during aseptic harvesting, enough amoebae were collected to obtain a final concentration of about $5 \times 10^5$ cells/ml in the 1.5 liters of EM used in the incubation vessel. The pellet of amoebae was first washed once in 300 ml of 0.15 M NaCl before the cells were resuspended in EM.

The encystment-sampling apparatus consisted of a 2 liter Pyrex aspirator bottle which was connected to (1) an aeration train, and (2) a sampling port. The assembled apparatus has been diagrammed by M. Rudick (1970). It was kept in a dark 30°C culture room, and was gassed at 4 $ft^2/hr$ with air.
from the laboratory compressed air supply. The only modification of M. Rudick's device made by this author was to insert into the line after the Millipore filter an additional bacterial and fungal filter consisting of a CaCl₂ drying tube packed with nonabsorbent cotton. This precaution was taken since the Millipore filter membrane was sometimes ruptured during autoclaving.

**Inducing Encystment in Optimal Growth Medium**

Encystment was also induced in this study by adding the drugs, ethidium bromide (Eb) or erythromycin (Ery) to OGM containing amoebae in the mid-LM (about 3 x 10⁴ cells/ml) phase of growth. Starvation was not the cause for encystment in this case, since amoebae were induced to differentiate in the presence of exogenous nutrients. Ethidium bromide is known to be an inhibitor of mitochondrial transcription, whereas erythromycin is an inhibitor of mitochondrial translation (Knight, 1969; Zylber, et al., 1969; Kroon & Arendzen, 1972).

In searching for mutant amoebae incapable of completing encystment, it was accidentally discovered by Kuhns & Byers (1972) that Eb induces differentiation when added to cultures of *A. castellanii* growing in OGM. Subsequent work by them, and also by Byers (1973), led to the conclusions that the effectiveness of Eb in inducing encystment in growth medium was dependent on (1) physiological "age," or state, of the cells, mid-LM amoebae being especially susceptible to the drug whereas older PGD cells were resistant to cyst induction; (2) cell concentration; (3) depth of the culture medium; and (4) concentration of the inhibitor, 25 ug/ml being most effective. Moreover,
for induction of encystment in OGM to work well, ethanol, at a final concentration of 0.7 %, must be added concurrently with Eb. Possibly, ethanol acts to increase permeability of the cellular membranes to the drug. By employing these conditions, 30–70 % formation of cysts is obtained in OGM after 4 days of treatment with Eb (Kuhns & Byers, 1972; Byers, 1973). Byers (1973) also demonstrated that addition to OGM of a final concentration of 1 mg/ml of Ery induces high levels of encystment in A. castellanii.

To induce encystment by using the mitochondrial inhibitors, amoebae were first cultured in horizontally-positioned Roux bottles containing 50 ml of OGM. This growth condition approximated the shallow cultures employed by Byers (1973) in his work. He grew the amoebae in 1.0 ml of OGM in vials routinely used for liquid scintillation counting. When the amoebae in the Roux bottles reached a density of about $3 \times 10^4$ cells/ml, Eb at a final concentration of 25 µg/ml, and Ery at a final level of 1 mg/ml, were added to separate sets of cells. A final concentration of 0.7 % ethanol was added in conjunction with the inhibitors, and was also added to control cultures. The density of $3 \times 10^4$ amoebae/ml at the time of inhibitor addition mimicked the concentration of cells that Byers (1973) found most susceptible to Eb-treatment in his vial cultures.

Both Eb and Ery were added in 0.5 ml quantities of stock solutions. Eb was dissolved in sterile distilled water with vigorous mixing. The stock of Ery was made by dissolving in 70 % ethanol. Both inhibitor solutions were prepared fresh the day of use. Eb-treated cultures received 0.5 ml of stock Eb and 0.5 ml of 70 % EtOH; Ery-treated cultures, 0.5 ml of stock Ery and 0.5 ml H₂O; and control cells, 0.5 ml of 70 % EtOH and 0.5 ml of H₂O.
Haemocytometer Counts of Encysting Cells

When encysting amoebae were harvested at various times to prepare cell-free homogenates, two 0.1 ml samples were removed with an Eppendorf pipet and fixed with 5 ml of CM in a centrifuge tube. The fixed cells were stored at 4 C until they could be counted on a haemocytometer to determine (1) the number of amoebae homogenized, and (2) the % cysts. Since fixed trophozoites tend to round up, no attempt was made to differentiate normal trophozoites from round forms of encysting cells. Amoebae were scored as cysts when a prominent cyst wall or wrinkled appearance to the cellular contour could be observed.

At the time of counting, the fixed amoebae were first pelleted by a 700 X g, 3 min centrifugation, and the supernatant was aspirated off to 0.5 ml or less, without disturbing the pellet. The cells were resuspended to a density which would give 400-800 amoebae per haemocytometer grid in a staining solution described by Mattar & Byers (1971). The stained cells were differentially counted under a light microscope equipped with a blue filter. Four grid counts were made for each original 0.1 ml sample. A total of 1500-2500 amoebae were counted for each set of cells harvested and homogenized. Neff (1964) reports that when 2000 total amoebae are counted in a blood cell counting chamber, the maximum variation from the averaged mean for the cell concentration of the sample is slightly less than ± 2.5 %.

In some cases, 12 ml samples of differentiating cells were removed from cultures to study the kinetics of encystment. In these instances, the amoebae were concentrated to yield conditions similar to the above, and the stained amoebae differentially counted.
**Harvesting and Homogenizing Cells**

Normally, 2-4 x 10^7 total amoebae were harvested for preparation of cell-free extracts at various times during population growth and encystment. An average cell loss of roughly 20% was routinely obtained. The cells were collected at 4°C by centrifugation at 700-1000 X g in a Sorvall RC2-B refrigerated centrifuge. Encysting cells were generally collected by use of a Sorvall KSB-R continuous flow system, employing a flow rate of 60 ml/min and the centrifugation speed of 1000 X g. Amoebic pellets were usually washed twice in 20 ml of 0.15 M KCl, a solution isotonic to trophozoites (Neff, Ray, et al., 1964), but if the cells were collected by continuous flow, the first wash was with 1.0 liter of 0.15 M KCl, which was used to flush OGM out of the centrifuge system.

In preparing axenically-grown trophozoites for homogenization, the washed cell pellet was resuspended in 3.0 ml of cold (4°C) homogenizing buffer (HB). HB consisted of a mixture of 0.05 M Tris-HCl (pH 8.0)–0.01 M mercaptoethanol–0.25 M sucrose. The sucrose was the RNase-free grade (Schwartz/Mann) generally used for making density gradients. Since the pH of Tris buffer varies markedly with temperature (Campbell & Sargent, 1967), HB was made with cold distilled water so its pH would be 8.0 during cellular homogenization at 4°C. This pH was above the optimum for the hydrolases assayed in this study (Fig. 4), and this precaution, plus the addition to HB of mercaptoethanol, a sulphydryl-protection agent, were undertaken to maintain the integrity of the enzymes. Sucrose at 0.25 M was added to HB to preserve lysosomes intact in order to perform fractionation studies on cellular extracts. This concentration of sucrose is routinely used by investigators...
to maintain the integrity of organelles in cell-free homogenates (Vaes, 1966; de Duve, 1971). However, sucrose was always included in HB even if the homogenates were not fractionated, so that enzyme data between various experiments could be compared.

After resuspending the trophozoites in 3.0 ml of HB, three 0.1 ml samples were removed with an Eppendorf pipet, and each fixed with 5 ml of CM. These samples were diluted and counted on the Celloscope to determine the number of cells homogenized. The remaining 2.7 ml of suspension was poured into a glass Potter-Elvehjem homogenizer. The sample tube was rinsed twice with 0.5 ml of HB, and these rinses also added to the homogenizer. Using a motor-driven Teflon pestle, the cells were homogenized at 4 C for a total of 10 min, 65-75 strokes/2 min, with 30 sec stops in between to keep down foaming which might denature proteins. The Teflon pestle was then rinsed with 1.6 ml of HB, which was combined with the cellular homogenate. The actual volume of final homogenate was measured by use of blow-out pipets.

Three 0.05 ml samples were removed and quick-frozen in a dry ice-methanol bath for storage at -70 C until they could be assayed to determine total cellular protein. Then 2.3 ml of the crude homogenate was centrifuged at 20,000 X g for 30 min to pellet the lysosomes. Of course, other things, such as mitochondria, peroxisomes, unbroken nuclei, cysts, and large cellular debris, would also be pelleted under these conditions. The supernatant was quickly poured off, and its volume measured by pipet. The pellet was resuspended in HB to the original volume of 2.3 ml. Triton X-100 (Packard Instrument Company), a nonionic detergent which ruptures lysosomal membranes (Wattiaux & de Duve, 1956), was added to the crude, pellet, and supernatant
samples at a final concentration of 0.1%. All three samples were quick-frozen in a dry ice-methanol bath and stored at -70 C until enzyme activities could be assayed. Lowry & Passonneau (1972) have stated that storage of cell-free extracts at -50 C, rather than at the -20 C typically used by investigators, is more preservative of enzyme activities. This author found that cellular extracts of *A. castellanii* which were stored at -70 C, if un-thawed only once (at the time of assay), maintained stable activities of APase, DNase, and RNase for as long as 4 months.

To prepare encysting cells for homogenization, the collected amoebae were washed twice, and then resuspended in an appropriate volume of HB. If the cells were mostly trophozoites, they were resuspended in 3.2 ml of HB; if the number of cysts was greater than 10%, the amoebae were resuspended in 2.7 ml. Two 0.1 ml samples were removed by Eppendorf pipet, and each fixed with 5 ml of CM until it could be differentially counted later on a haemocytometer. The remaining suspension was poured into a special bottle used for breaking cells in a B. Braun "MSK" mechanical cell homogenizer. The sample tube was rinsed either twice or thrice with 0.5 ml of HB, and these rinses added to the homogenizing bottle. Three rinses were used if the number of cysts was greater than 10%, because clumps of cysts tended to adhere to the glass walls of the sample tube.

The total volume of amoebic suspension homogenized was 4.0 ml. To this amount was added 8.0 "ml" of cold, acid-cleaned glass beads (0.45-0.50 mm diameter). The encysting cells were broken by grinding with the glass beads for 30 sec in the B. Braun "MSK" homogenizer. The agitated bottle was kept cool by blowing gaseous CO₂ over the homogenizing chamber.
In a 4 C cold room, the mixture of amoebic homogenate and glass beads was scraped into a 30 ml glass syringe which lacked a needle and was fitted on the bottom with a small piece of cheesecloth for holding back the beads; the syringe emptied into a collecting tube. The homogenate was blown off the beads into this tube by using a gentle pressure of filtered N$_2$. About 75% of the original sample was recovered by this technique. Duplicate or triplicate samples of 0.04 ml were removed, quick-frozen in a dry ice-methanol bath, and stored at -70 C for subsequent protein assays. To 2.0 ml of the remaining homogenate, Triton X-100 was added at a final concentration of 0.1%, and the extract frozen and stored at -70 C for later enzyme assays.

**Lowry Protein Assay**

The protein assay used in this study was an adaptation of the method originally described by Lowry, et. al. (1951) and modified by Oyama & Eagle (1956). The general principle is as follows: In an alkaline solution, a complex is formed between protein and copper. This protein-copper complex then reduces a Folin-Ciocalteau phenol reagent to form a bluish compound that is measured colorimetrically at 660 nm.

The reagents for this protein assay are listed in Table 5, and the preparation of bovine serum albumin (BSA) standards is shown in Table 6. Data obtained from six different standard curves of BSA were used to calculate this linear regression equation with a Hewlett-Packard Model 9100B desk computer: $y = 0.0022x + 0.0134$. The standard deviations for the slope were ± 0.0001, and for the intercept, ± 0.0018. This regression equation was used to calculate the protein content in samples of *A. castellani* extracts.
Table 5. Reagents for Lowry protein assay

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2.0% Na$_2$CO$_3$ and 0.002% KNa.tartrate in 0.1 N NaOH</td>
</tr>
<tr>
<td>B</td>
<td>0.5% cupric sulfate</td>
</tr>
<tr>
<td>C</td>
<td>50 parts of A and 1 part of B mixed fresh on day of use</td>
</tr>
<tr>
<td>D</td>
<td>5 parts of Folin-Ciocalteau phenol reagent mixed with 7 parts H$_2$O</td>
</tr>
<tr>
<td>E</td>
<td>Standard solution of bovine serum albumin, 200 ug/ml</td>
</tr>
</tbody>
</table>

Table 6. Preparation of bovine serum albumin (BSA) standards

<table>
<thead>
<tr>
<th>Tube No.</th>
<th>Stock BSA (ml)</th>
<th>H$_2$O (ml)</th>
<th>Amt of BSA (ug)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (BLANK)</td>
<td>0.00</td>
<td>1.00</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0.05</td>
<td>0.95</td>
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</tr>
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<td>0.70</td>
<td>60</td>
</tr>
<tr>
<td>8</td>
<td>0.35</td>
<td>0.65</td>
<td>70</td>
</tr>
<tr>
<td>9</td>
<td>0.40</td>
<td>0.60</td>
<td>80</td>
</tr>
<tr>
<td>10</td>
<td>0.45</td>
<td>0.55</td>
<td>90</td>
</tr>
<tr>
<td>11</td>
<td>0.50</td>
<td>0.50</td>
<td>100</td>
</tr>
<tr>
<td>12</td>
<td>0.55</td>
<td>0.45</td>
<td>110</td>
</tr>
<tr>
<td>13</td>
<td>0.60</td>
<td>0.40</td>
<td>120</td>
</tr>
<tr>
<td>14</td>
<td>0.65</td>
<td>0.35</td>
<td>130</td>
</tr>
<tr>
<td>15</td>
<td>0.70</td>
<td>0.30</td>
<td>140</td>
</tr>
<tr>
<td>16</td>
<td>0.75</td>
<td>0.25</td>
<td>150</td>
</tr>
<tr>
<td>Samples</td>
<td>0.00</td>
<td>1.00</td>
<td>-</td>
</tr>
</tbody>
</table>
Protein in 0.04 or 0.05 ml samples of _A. castellanii_ extracts was prepared for the Lowry assay by an overnight precipitation at 4°C with an equal volume of 10% TCA plus an additional amount of either 1.0 or 2.0 ml of 5% TCA. Precipitating the protein was necessary because the Tris, mercaptoethanol, and sucrose present in HB all interfere with the Lowry assay (Gerhardt & Beevers, 1968; Hinton, et al., 1969; Geiger & Besman, 1972; Ji, 1973). Sometimes, Triton X-100 was present in extracts assayed for protein, and this compound also interferes with the Lowry procedure (Holloway, 1973).

The precipitate was centrifuged at 4°C in a Sorvall RC2-B refrigerated centrifuge at 12,000 X g for 7-10 min, using a swinging bucket. The supernatant was gently aspirated off, and the pellet was either (1) washed twice in 1.0 ml of 5% TCA, or (2) washed once in 2.0 ml of 5% TCA. Prior to washing, the precipitate was scraped up by using a mini-stirring rod made by flaming closed the tip of a Pasteur pipet.

After washing, the pellet was resuspended and dissolved in 2.0 ml of Reagent C (Table 5), and this mixture poured into a sample assay tube (Table 6). The original tube was rinsed with 3.0 ml of Reagent C, and this material also added to the assay tube. Five ml of Lowry C was added to BSA standards when constructing a standard curve. After an incubation of 10 min or longer at room temperature (23-25°C) to dissolve the protein, 0.5 ml of Reagent D (Table 5) was rapidly squirted into the assay tubes, and the contents vigorously mixed. After 1 hr of color development, the absorbance was measured at 660 nm. If the amoebic samples contained a large amount of broken cyst walls, a 5000 X g, 5 min centrifugation was first performed to sediment these cyst walls along the edge of the tube. Absorbance readings were made.
with a Gilford Model 2400 spectrophotometer equipped with a rapid sampling
system, a digital absorbance meter, and a data lister.

Assays of Lysosomal Enzymes

The ingredients for assaying APase, DNase, and RNase are listed in Ta­
bles 7, 8, and 9, respectively. APase and DNase assays were conducted for
12 min, whereas RNase was assayed for 60 min. Enzyme assays were normally
begun by adding substrate, and were performed either in duplicate or tripli­
cate. Sample absorbances were measured against blanks, which contained all
the ingredients for a particular assay, but to which substrate was added
after that ingredient which normally stops the enzyme reaction. Absorbances
were measured with the Gilford Model 2400 spectrophotometer previously
mentioned.

For convenience, all enzyme assays were performed at pH 5.0, which ap­
ppeared to be the pH optimum for APase (Fig. 4). However, the pH optima for
DNase and RNase were about 4.8 and 5.2, respectively (Fig. 4). Muller
(1969b) obtained a pH optimum of 5.0 for APase, and of 5.5 for RNase, in cel­
lar extracts of Acanthamoeba sp.; he did not assay DNase.

Enzyme assays were verified by this author to exhibit a linear corres­
pondence with both extract concentration and time within the assay conditions
employed in this study. Zero order kinetics were observed for all three hy­
drolases. No deviant curves to suggest the action of inhibitors or activa­
tors, or of competitive enzymes, were noticed. A possible acid hydrolysis
of substrates by the pH 5.0 acetate buffer was also checked, and found to be
either negligible or nonexistent.
All enzyme assays were performed in a water bath at 30°C, the temperature optimal for growth of *A. castellanii* (Neff, et al., 1958), on cell-free extracts that had been frozen and thawed once in the presence of 0.1% Triton X-100. A homogenate volume of 0.05 ml that contained 40-150 μg of total protein was added to assay tubes containing 1.0 ml of 0.1 M acetate buffer (pH 5.0), and allowed to incubate 30-45 min at 30°C before adding substrate to start the reactions. This preliminary incubation ensured that all latent enzyme activity was released. In preliminary experiments, this author demonstrated that latency of acid hydrolases in *A. castellanii* extracts was effected by (1) freezing and thawing, and (2) incubation with different concentrations of Triton X-100. These results were in agreement with findings originally reported by Griffiths & Bowen (1969). Furthermore, incubation of cellular extracts at pH 5.0 effects latency of acid hydrolases in rat liver (Appelmans & de Duve, 1955; Gianetto & de Duve, 1955), and lysosomes from the ciliate *Tetrahymena* are unstable on exposure for 30 min to temperatures greater than 10-15°C (Lee, 1971).

The assay used in this study for acid phosphatase (E.C. 3.1.3.2: orthophosphoric monoester phosphohydrolase) was a modification of procedures used by Holter & Lowy (1959) and Lazarus & Scherbaum (1967b, 1968). The substrate was disodium p-nitrophenyl phosphate (#104 Phosphatase Substrate, Sigma Chemical Company), or NPP. Hydrolysis of this compound yields a yellow-colored product, p-nitrophenol, in alkaline solution. The enzyme reaction was terminated by adding cold (4°C) 1.6 N NaOH, whose high basicity prevents alkaline phosphatase activity, and also develops the yellow color of p-nitrophenol. According to Muller (1969b), the molar absorptivity of
p-nitrophenol at 410 nm, the absorbance at which the yellow color was measured in this study, was $18 \times 10^3 \text{M}^{-1}\text{cm}^{-1}$. This $E_{410}$ was found by this author to have a similar value for the assay conditions of APase employed here, and so it was used to calculate units of APase activity. The formation of 1 umole of p-nitrophenol/min was defined as being due to one unit of enzyme activity. APase activity/amoeba was expressed as fmoles of p-nitrophenol formed per min. Specific activity of APase was calculated as nmoles of p-nitrophenol formed per min per mg protein.

Some objection has been raised that in various tissues, acid p-nitrophenyl phosphatase activity is not confined entirely to lysosomes (Beaufay, 1972). Presumably, if APase is assayed with b-glycerophosphate as substrate, and the enzyme activity determined by measuring the amount of free phosphate formed as product, then a true index of lysosomal APase can be obtained (Beaufay, 1972). However, Triton-treated extracts were used in this study, and this detergent interferes with the Fisk-Subbarow determination of free phosphate (See & Pitt, 1972). Furthermore, in extracts of *Tetrahymena* subjected to sucrose-density gradient centrifugation, APase had the same distribution whether assayed with p-nitrophenyl phosphate or b-glycerophosphate as substrate (Brightwell, et. al., 1968). Moreover, the same sedimentation profile obtained in this study for APase, DNase, and RNase (Fig. 6) suggests that all three enzymes are found in the same type of organelle.

The assay for acid deoxyribonuclease (E.C. 3.1.4.5: deoxyribonucleate oligonucleotido-hydrolase) was a modification of procedures used by de Duve, et. al. (1955) and Holm (1966). The substrate was a sodium salt of salmon sperm DNA (Calbiochem). A stock solution (1 mg/ml) of it was made in 0.15 M
NaCl by gently stirring overnight at 4 C with a magnetic stirrer. This stock was stored, unfrozen, in the cold, also. The enzyme reaction was terminated by adding cold (4 C) 1.2 N PCA, which precipitated unreacted substrate after 30 min at 4 C. This precipitate was removed by centrifugation for 5 min at 5000 X g in a swinging bucket of a Sorvall RC2-B centrifuge. The A_{260} of the supernatant was measured with the Gilford Model 2400 spectrophotometer. One unit of enzyme activity was taken as being equivalent to that amount of acid-soluble oligonucleotide which caused an increase in absorbancy at 260 nm of 1.0/hr. DNase/amoeba was expressed in terms of uUnits of activity per cell. Specific activity of DNase was calculated simply as Units/mg total protein.

The assay for acid ribonuclease (E.C. 2.7.7.16: polyribonucleotide 2-oligonucleotido-transferase, cyclizing) was a modification of the method employed by Lazarus & Scherbaum (1967a). The substrate used was yeast soluble RNA (Type III, Sigma Chemical Company). The reaction was terminated by adding a cold (4 C) mixture of 10 % HCl-95 % ethanol-0.01 M MgCl$_2$ (AEM), which precipitated unreacted RNA after 30 min at 4 C. The precipitate was removed as described in the assay for DNase. The obtained supernatant of both sample and blank tubes was diluted with water to yield an A_{260} of less than 2.0. One unit of enzyme activity was defined as being equivalent to that amount of acid-soluble oligonucleotide which effected an increase in absorbancy at 260 nm of 1.0/hr. RNase/amoeba was expressed as uUnits of activity per cell. Specific activity of RNase was calculated as Units/mg total protein.
### Table 7. Ingredients for acid phosphatase (APase) assay

<table>
<thead>
<tr>
<th>No.</th>
<th>Description</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>0.1 M acetate buffer, pH 5.0</td>
<td>1.00 ml</td>
</tr>
<tr>
<td>2.</td>
<td>Cell extract (Triton-treated)</td>
<td>0.05 ml</td>
</tr>
<tr>
<td>3.</td>
<td>0.02 M NPP (10 umoles/0.5 ml)</td>
<td>0.50 ml</td>
</tr>
<tr>
<td>4.</td>
<td>1.6 N NaOH</td>
<td>1.50 ml</td>
</tr>
<tr>
<td></td>
<td><strong>TOTAL</strong></td>
<td>3.05 ml</td>
</tr>
</tbody>
</table>

### Table 8. Ingredients for acid deoxyribonuclease (DNase) assay

<table>
<thead>
<tr>
<th>No.</th>
<th>Description</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>0.1 M acetate buffer, pH 5.0</td>
<td>1.00 ml</td>
</tr>
<tr>
<td>2.</td>
<td>Cell extract (Triton-treated)</td>
<td>0.05 ml</td>
</tr>
<tr>
<td>3.</td>
<td>0.1% DNA (0.5 mg/0.5 ml)</td>
<td>0.50 ml</td>
</tr>
<tr>
<td>4.</td>
<td>1.2 N PCA</td>
<td>0.90 ml</td>
</tr>
<tr>
<td></td>
<td><strong>TOTAL</strong></td>
<td>2.45 ml</td>
</tr>
</tbody>
</table>

### Table 9. Ingredients for acid ribonuclease (RNase) assay

<table>
<thead>
<tr>
<th>No.</th>
<th>Description</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>0.1 M acetate buffer, pH 5.0</td>
<td>1.00 ml</td>
</tr>
<tr>
<td>2.</td>
<td>0.09 M EDTA</td>
<td>0.10 ml</td>
</tr>
<tr>
<td>3.</td>
<td>Cell extract (Triton-treated)</td>
<td>0.05 ml</td>
</tr>
<tr>
<td>4.</td>
<td>2.0% RNA (2 mg/0.1 ml)</td>
<td>0.10 ml</td>
</tr>
<tr>
<td>5.</td>
<td>AEM</td>
<td>1.20 ml</td>
</tr>
<tr>
<td></td>
<td><strong>TOTAL</strong></td>
<td>2.45 ml</td>
</tr>
</tbody>
</table>
RESULTS

Population Growth of Trophozoites

A normalized growth curve for trophozoites of *A. castellanii* axenically cultured in optimal growth medium (OGM) is depicted in Fig. 1. With the sampling procedure employed, no lag phase was observed. The growth curve can be divided into two phases: (1) a period of logarithmic multiplication (LM), which lasts about 40 hr, and is arbitrarily taken to end when the concentration of amoebae is $8 \times 10^4$ cells/ml; and (2) population growth deceleration (PGD), which constitutes the rest of the curve. The mean generation time (GT), or population doubling time, for LM trophozoites was $6.50 \pm 0.40$ hr, while the mean growth rate constant during this period was $0.1074 \pm 0.0050$ hr$^{-1}$.

Breakage Curves

Fig. 2 shows a breakage curve for trophozoites of *A. castellanii* obtained by using a motor-driven glass-Teflon Potter-Elvehjem homogenizer. A breakage curve for cysts obtained by using a B. Braun "MSK" mechanical cell homogenizer is depicted in Fig. 3.

Biochemical Evidence for Lysosomes in Trophozoites

Fig. 4 attests to the acid pH optima of all three enzymes assayed in this study. Sedimentation of these enzymes at 20,000 X g in 0.25 M sucrose
Figure 1. Average population growth curve for trophozoite cultures of *A. castellanii*. Cells were grown axenically in OGM as described in the Methods. Data have been normalized (see Methods) from 20 separate experiments. The growth points shown here for LM were calculated by using the mean growth rate constant of 0.1074 hr$^{-1}$. The points during PGD represent average values for selected growth periods. The arrows indicate those cell densities at which amoebae were induced to encyst in sundry experiments.
Figure 2. Breakage curve for trophozoites obtained with a glass-Teflon Potter-Elvehjem homogenizer. Two sets of about 3.3 x 10^7 cells each, and containing 0.0% cysts, were collected and homogenized as described in the Methods. At different times, 0.1 ml was removed for making breakage counts on a haemocytometer. A total of 9 such samples was removed for each set. Even after 10 or 15 sec, most of the trophozoites were shredded and not actually whole cells. Therefore, this figure is primarily a representation of the complete homogenization of these large chunks of cellular debris. Breaking up these large pieces of debris was essential for accurate study of the sedimentation at 20,000 x g in 0.25 M sucrose of acid hydrolases within isolated, yet intact, lysosomes in cell-free homogenates. The arrow depicts the point at which homogenized extracts were taken for use in sundry experiments.

Figure 3. Breakage curve for cysts obtained with a B. Braun "MSK" mechanical cell homogenizer. Four sets of about 3.0 x 10^7 cells each, 98.8% of which were cysts, were broken with the "MSK" device (see Methods) to construct this curve. At different times, 0.1 ml was removed for making breakage counts on a haemocytometer. A total of 5 such samples was removed from each set. The arrow depicts the point at which homogenized extracts were taken for subsequent assays.
Fig. 2

% Intact Trophozoites vs. Breakage Time (min)

Fig. 3

% Intact Cysts vs. Breakage Time (sec)
Figure 4. Lysosomal enzyme activities as a function of pH. All enzymes were assayed as described in the Methods in a crude homogenate prepared from 3-day PGD cells. The different pH's were made with 0.1 M acetate buffers. The ordinate for APase is the absorbance at 410 nm for the assay tubes after terminating enzyme reactions. The ordinates for DNase and RNase represent the absorbances at 260 nm for the supernatants obtained after centrifuging precipitated, unreacted substrate out of the reaction tubes.
is shown by the data in Table 10, as well as by the sedimentation profile of the enzymes as a function of culture aging (Fig. 6). Three different kinds of experiments demonstrated latency of the enzymes assayed in this study. These experiments are described in the legends of Tables 11 and 12, and in the caption under Fig. 5. For the data in Table 11 and Fig. 5, latency is defined as that percentage of the total hydrolase activity that is not detectable in the absence of treatment with Triton X-100. The data in Table 12 were acquired primarily to check for any noticeable discrepancies in either enzyme or protein data when amoebae were homogenized by the two different procedures used in this study. No appreciable differences in the parameters measured were obtained, however, except for one case: % sedimentation of acid hydrolases at 20,000 X g in 0.25 M sucrose, which was markedly reduced by using the B. Braun "MSK" device (Table 12). Only about 8 % of the total hydrolase activity sedimented in the 20,000 X g pellet, although slightly more than 50 % of the total protein was found in this fraction. These data suggest a latent release of lysosomal enzymes effected by this more rigorous homogenizing procedure.

Lysosomal Activity during Population Growth

Figs. 6-9 depict changes in lysosomal enzyme activities and cellular protein content during population growth in A. castellanii. The % sedimentation at 20,000 X g in 0.25 M sucrose for all three acid hydrolases increases from about 40 % in mid-LM to a plateau of about 80 % in PGD (Fig. 6). In younger cells, which represent both LM and early PGD ages, the pg protein per amoeba is about 30 % less than that found in older organisms, in which
Table 10. Sedimentation of lysosomal enzymes at 20,000 X g as a function of sucrose inclusion in the homogenizing buffer (HB)

<table>
<thead>
<tr>
<th>Culture Age (hr)</th>
<th>Cells/ml (x 10^5)</th>
<th>Sucrose Conc in HB</th>
<th>% APase in Pellet</th>
<th>% DNase in Pellet</th>
<th>% RNase in Pellet</th>
</tr>
</thead>
<tbody>
<tr>
<td>48.0</td>
<td>1.48</td>
<td>0.00 M</td>
<td>26.5</td>
<td>18.9</td>
<td>10.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.25 M</td>
<td>54.3</td>
<td>53.9</td>
<td>33.3</td>
</tr>
<tr>
<td>168.0</td>
<td>5.81</td>
<td>0.00 M</td>
<td>52.4</td>
<td>61.7</td>
<td>48.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.25 M</td>
<td>83.2</td>
<td>79.3</td>
<td>82.1</td>
</tr>
</tbody>
</table>

Amoebae of each age were collected by centrifugation (see Methods) and then 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, while the other set was homogenized in a similar buffer which contained 0.25 M sucrose, as well. Cell breakage was effected in a Potter-Elvehjem homogenizer (see Methods). The homogenates were centrifuged at 20,000 X g for 30 min, and enzyme activities were determined in the pellet and supernatant fractions to calculate the % of activity in the pellet.
Table 11. Percent latency of lysosomal enzymes in homogenates of PGD trophozoites

<table>
<thead>
<tr>
<th>Culture Age (hr)</th>
<th>Cells/ml (x 10^5)</th>
<th>Percent Latency</th>
<th>APase</th>
<th>DNase</th>
</tr>
</thead>
<tbody>
<tr>
<td>98.2</td>
<td>3.28</td>
<td>46.8</td>
<td>37.8</td>
<td></td>
</tr>
<tr>
<td>101.8</td>
<td>2.67</td>
<td>43.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>149.5</td>
<td>4.79</td>
<td>43.1</td>
<td>40.1</td>
<td></td>
</tr>
<tr>
<td>170.5</td>
<td>5.55</td>
<td>41.0</td>
<td>41.3</td>
<td></td>
</tr>
<tr>
<td>186.6</td>
<td>9.32</td>
<td>35.7</td>
<td>32.3</td>
<td></td>
</tr>
<tr>
<td>261.3</td>
<td>10.70</td>
<td></td>
<td>42.0</td>
<td></td>
</tr>
</tbody>
</table>

Extracts of amoebae were prepared with a Potter-Elvehjem homogenizer (see Methods), and then divided into two portions. Release of latent activities was effected in one of these portions by adding a final concentration of 0.1% Triton X-100, with subsequent incubation at 4°C for 60 min prior to performing enzyme assays. Non-latent activities were determined on the remaining portion of freshly-prepared homogenate by assaying enzymes as quickly as possible. Latency was calculated as that % of the total hydrolase activity (which was considered as being assayed in the Triton-treated homogenate) that was not detectable in the absence of Triton-treatment.
Table 12. Lysosomal enzyme and cellular protein data for 7-day PGD trophozoites as a function of homogenizing procedure

<table>
<thead>
<tr>
<th>Description</th>
<th>Homogenizing Procedure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Potter-Elvehjem</td>
</tr>
<tr>
<td><strong>APase</strong></td>
<td></td>
</tr>
<tr>
<td>% Recovery</td>
<td>108.3</td>
</tr>
<tr>
<td>% Sedimented</td>
<td>83.2</td>
</tr>
<tr>
<td>Activity/Amoeba</td>
<td>74.0</td>
</tr>
<tr>
<td><strong>DNase</strong></td>
<td></td>
</tr>
<tr>
<td>% Recovery</td>
<td>107.0</td>
</tr>
<tr>
<td>% Sedimented</td>
<td>79.3</td>
</tr>
<tr>
<td>Activity/Amoeba</td>
<td>78.0</td>
</tr>
<tr>
<td><strong>RNase</strong></td>
<td></td>
</tr>
<tr>
<td>% Recovery</td>
<td>103.4</td>
</tr>
<tr>
<td>% Sedimented</td>
<td>82.1</td>
</tr>
<tr>
<td>Activity/Amoeba</td>
<td>89.0</td>
</tr>
<tr>
<td><strong>Protein</strong></td>
<td></td>
</tr>
<tr>
<td>% Recovery</td>
<td>102.2</td>
</tr>
<tr>
<td>% Sedimented</td>
<td>43.8</td>
</tr>
<tr>
<td>pg/Amoeba</td>
<td>488.0</td>
</tr>
</tbody>
</table>

About $6 \times 10^7$ total cells were harvested and divided into two equal groups. One group was homogenized with a glass-Teflon Potter-Elvehjem homogenizer, and the other with a mechanical B. Braun "MSK" device, as described in the Methods. The homogenates were centrifuged at $20,000 \times g$ for 30 min, and enzyme activities and protein amts were determined in the pellet and supernatant fractions to obtain the % sedimented data, as well as to calculate enzyme activities/cell. The sums of the data in the fractions were compared against values obtained in crude extracts to determine % recoveries. Cellular protein content was calculated by using the sample value of the crude homogenate. Enzyme activities/amoeba are as defined in the Methods.
Figure 5. Percent latency of APase in a 20,000 X g homogenate pellet. An extract of 7-day PGD amoebae was prepared with a Potter-Elvehjem homogenizer (see Methods), and then subjected to a 30 min, 20,000 X g centrifugation. The pellet was resuspended in homogenizing buffer to the original volume centrifuged. Portions of this resuspended pellet were then incubated at 4 C with a final concentration of 0.1 % Triton X-100. Latency was calculated in a fashion similar to that described in the legend of Table II.
Figure 6. Percent sedimentation of lysosomal enzymes as a function of culture age. The data are from 7 separate experiments, and have been normalized (see Methods). Amoebic homogenates were prepared with a Potter-Elvehjem homogenizer and fractionated at 20,000 \( \times g \) in 0.25 M sucrose as described in the Methods. Enzyme activities were assayed in the pellet and supernatant fractions to obtain the % sedimented data. Shaded circles, APase. Unshaded circles, DNase. Stars, RNase.
Figure 7. Changes in cellular protein content as a function of culture age. The normalized data (see Methods) are from 7 separate experiments.
Figure 8. Lysosomal enzyme activities per cell as a function of culture age. The normalized data (see Methods) are from 7 separate experiments. Amoebic homogenates were prepared with a Potter-Elvehjem homogenizer and fractionated at 20,000 X g as described in the Methods. Enzymes were assayed in the pellet and supernatant fractions, and the sums used to calculate activities/cell. When compared to crude homogenates, essentially 100% recovery of the enzymes was obtained in the fractions. Enzyme activities/cell are as defined in the Methods.
Fig. 8

- LM PGD
- Rnase/Amoeba
- APase/Amoeba
- DNase/Amoeba

Normalized Culture Age (hr)
Figure 9. Specific activities of lysosomal enzymes as a function of culture age. The normalized data (see Methods) are from 7 separate experiments. Amoebic homogenates were prepared with a Potter-Elvehjem homogenizer, and a portion was fractionated at 20,000 X g as described in the Methods. Total enzyme activity was determined by using the sums of the enzyme values found in the pellet and supernatant fractions. Total protein was computed by using the sample value of the crude homogenate. Activities/mg protein were subsequently calculated. Enzyme units are as defined in the Methods.
Fig. 9

- LM
- PGD

Normalized Culture Age (hr)

- APase/mg Protein
- DNase/mg Protein
- RNase/mg Protein
protein remains at a rather constant value of 350 pg/cell (Fig. 7). The activity/amoeba of both APase and DNase remains basically unchanged as a function of culture age (Fig. 8). RNase activity, on the other hand, is about twofold higher in LM and early PGD cells than it is in older trophozoitites; its activity decreases about 50% by day 5 of growth (Fig. 8). As shown in Fig. 9, the activity/mg protein of all three enzymes is highest during the first 70 hr of population growth, when the total protein/amoeba is about 30% less than at later times (Fig. 7). This result is not surprising for the RNase, which exhibits a higher activity per cell during this period. However, these data represent an enrichment of both APase and DNase in younger amoebae.

**Differentiation of 7-day PGD Cells in Unagitated EM**

The % cell loss and encystment kinetics for 7-day PGD cells differentiating in Roux bottles containing 150 ml of unagitated EM are depicted in Figs. 10 and 11. A synchronous encystment commences between the 15th and 18th hr, and by 24 hr, nearly 70% of the cells have differentiated. By 48 hr, over 90% of the amoebae are cysts. As shown in Fig. 10, a cell loss in the culture of about 20% occurs during incubation, so that only 70% of the inoculum cells actually form cysts. Clumps of cysts, sometimes including 25 cells or more, are formed in these encysting cultures of 7-day PGD cells. A qualitative analysis of encystment by this author suggests that the degree of clumping is a complex phenomenon dependent on (1) age, or physiological state, of the cells; (2) cell concentration; (3) whether or not the ambient medium is agitated, either by mechanical shaking or bubbling with air; and
Figure 10. Percent loss of 7-day PGD cells during encystment in unagitated EM. The points represent average values for selected periods of time.

Figure 11. Encystment kinetics of 7-day PGD cells in unagitated EM. The data are from 5 separate experiments.
method of cyst induction: i.e., whether by starvation in EM or by adding drugs to OGM.

During the first 10 hr of encystment by 7-day amoebae in unagitated EM, the % sedimentation of both APase and DNase is maintained at the initial value of about 80 %, whereas pelleted RNase activity gradually decreases to 60 % (Fig. 12). There is a selective decrease in the cellular levels of lysosomal hydrolases during incubation in EM, and this decline in enzyme activity commences even before the appearance of thick-walled cysts (Figs. 13, 11). The decrease in RNase is especially pronounced; by 6 hr in EM, the cells have lost about 60 % of their initial activity (Fig. 13). The changes in cellular protein content of encysting amoebae are shown in Fig. 14. By 72 hr, about 60 % of the initial protein content has been lost.

The specific activities of APase, DNase, and RNase as a function of incubation in EM are depicted in Fig. 15. RNase/mg protein quickly decreases in a fashion similar to that for activity/cell (Figs. 15, 13). DNase/mg protein diminishes at a slower rate. There is, however, an enrichment of APase in encysting amoebae, even though its total cellular level is gradually decreasing (Figs. 15, 13). An increase of about 40 % in APase/mg protein is obtained after 48 hr of encystment (Fig. 15). This specific activity of APase subsequently drops back to the value at 0-time.

In view of the possibility that the decrease in lysosomal enzyme activity and total protein was due to the synthesis of proteases during the induction of encystment, differentiation in A. castellanii was examined in the presence of 0, 5, 10, 25, and 50 ug of cycloheximide per ml of EM. Cycloheximide is a known inhibitor of cytoplasmic protein synthesis (Sisler & Siegel,
Figure 12. Percent sedimentation of lysosomal enzymes for 7-day PGD cells encysting in unagitated EM. The data are from 2 separate experiments. Amoebic homogenates were prepared; fractionated, and assayed for enzyme activities as described in the legend of Fig. 6. Shaded circles, APase. Unshaded circles, DNase. Stars, RNase.
Figure 13. Changes in lysosomal enzyme activities of 7-day PCD cells encysting in unagitated EM. The data are from 2 separate experiments. Enzymes were assayed in crude amoebic extracts prepared with a B. Braun "MK" homogenizer (see Methods). Enzyme activities/amoeba are as defined in the Methods. Shaded circles, APase. Unshaded circles, DNase. Stars, RNase.

Figure 14. Changes in protein content of 7-day PCD cells encysting in unagitated EM. The data are from 2 separate experiments.
Fig. 13

Fig. 14

Activity/Amoeba

pg Protein/Amoeba

Incubation Time (hr)
Figure 15. Specific activities of lysosomal enzymes for 7-day PGD cells encysting in unagitated EM. The data are from 2 separate experiments. Enzymes were assayed in crude amoebic extracts prepared with a B. Braun "MSK" homogenizer (see Methods). Enzyme units are as defined in the Methods. Shaded circles, APase. Unshaded circles, DNase. Stars, RNase.
1967; Kroon & de Vries, 1970; Kroon & Arendzen, 1972). Byers (1973) has demonstrated that 10 ug/ml of this compound reduces encystment by nearly 90% when LM amoebae are induced to differentiate in growth medium with the chemical agent, ethidium bromide. M. Rudick (1970) showed that 50 ug/ml of cycloheximide reversibly blocks incorporation of radioactively labeled amino acids into protein of A. castellanii trophozoites; he did not test lower concentrations. Table 13 illustrates that after 56 hr, encystment of 7-day PGD cells in unagitated EM has not been significantly reduced by any of the cycloheximide concentrations employed. It, therefore, appears that new protein synthesis is not required for differentiation in cells of this age. Consequently, even if new proteases participate in hydrolase degradation, their synthesis is not a necessary event for encystment.

The conservation and enrichment of APase during encystment (Fig. 15) might be related to a requirement for free phosphate in encysting cells. Addition of phosphate to EM might prevent this conservation of APase activity, since amoebae would be able to obtain free phosphate from the ambient medium. To test this hypothesis, KH$_2$PO$_4$ was added to EM at final concentrations of 0.00, 0.25, 0.50, and 0.75 mM, and the degree of encystment, and cellular levels of protein and APase activity, were determined after 75 hr. The data are shown in Table 14. No significant differences between control and experimental cultures can be observed.

**Differentiation of 3-day PGD Cells in Unagitated EM**

The % cell loss and encystment kinetics for 3-day PGD cells differentiating in Roux bottles containing 150 ml of unagitated EM are depicted in
Table 13. Percent encystment\textsuperscript{a} after 56 hr of 7-day PGD cells as a function of cycloheximide addition to encystment medium

<table>
<thead>
<tr>
<th>Cycloheximide Concentration (ug/ml)</th>
<th>% Cysts</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>94.8</td>
</tr>
<tr>
<td>5</td>
<td>86.2</td>
</tr>
<tr>
<td>10</td>
<td>88.3</td>
</tr>
<tr>
<td>25</td>
<td>90.2</td>
</tr>
<tr>
<td>50</td>
<td>91.5</td>
</tr>
</tbody>
</table>

\textsuperscript{a}The data have been averaged from two separate experiments.

Table 14. Encystment data\textsuperscript{a} after 75 hr for 7-day PGD cells as a function of phosphate addition to encystment medium

<table>
<thead>
<tr>
<th>KH\textsubscript{2}PO\textsubscript{4} Concen (\textmu M)</th>
<th>% Cysts</th>
<th>Pg Protein Per Cell</th>
<th>APase Per Cell</th>
<th>APase/mg Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>97.9</td>
<td>218</td>
<td>33</td>
<td>150</td>
</tr>
<tr>
<td>0.25</td>
<td>98.6</td>
<td>265</td>
<td>36</td>
<td>136</td>
</tr>
<tr>
<td>0.50</td>
<td>98.4</td>
<td>256</td>
<td>30</td>
<td>117</td>
</tr>
<tr>
<td>0.75</td>
<td>95.7</td>
<td>257</td>
<td>35</td>
<td>138</td>
</tr>
</tbody>
</table>

\textsuperscript{a}The data have been averaged from two separate experiments.
Figs. 16 and 17. RNase activity/amoeba was about twofold higher in these 3-day cells, as compared to 7-day amoebae (Figs. 8, 13, 18). Synchronous encystment of the 3-day cells is shown in Fig. 17 to commence sometime between the 8th and 15th hr in EM. By 24 hr, over 90% of the amoebae have formed cysts, as compared to about 70% with the 7-day PGD cells (Figs. 17, 11). At later time periods, nearly 100% of the 3-day amoebae are cysts. However, a cell loss of about 30% occurs during incubation in EM (Fig. 16), so only 70% of the inoculum cells actually differentiate. Actually, this figure is probably even higher, for great difficulty was encountered in recovering encysting amoebae of the 3-day PGD type from their culture vessels. This difficulty was due to the appearance of especially large clumps of cysts which readily adhered to the walls of the Roux bottles as amoebic suspension was poured out. Therefore, the data shown in Fig. 16 are likely not all due to cell lysis. Sometimes these clumps contained over 100 cells. They were much larger than clumps obtained during encystment of 7-day PGD cells in unagitated EM.

Changes in lysosomal enzyme activities and pg protein/amoeba during encystment of the 3-day amoebae are shown in Figs. 18 and 19. The data are roughly comparable to those obtained for the 7-day cells (Figs. 13, 14). However, because of the twofold higher level of RNase in the 3-day amoebae, the absolute values per cell of this enzyme differ from those for 7-day organisms (Figs. 18, 13). The initial pg protein/cell for 3-day amoebae are slightly less than in 7-day cells (Figs. 19, 14). A glance at Fig. 7 shows that this is a difference which might be expected, however. The specific activities of APase, DNase, and RNase shown in Fig. 20 exhibit the same
Figure 16. Percent loss of 3-day PGD cells during encystment in unagitated EM. The points represent average values for selected periods of time.

Figure 17. Encystment kinetics of 3-day PGD cells in unagitated EM. The data are from 3 separate experiments.
Figure 18. Changes in lysosomal enzyme activities of 3-day PGD cells encysting in unagitated EM. The data are from 3 separate experiments. Enzymes were assayed in crude amoebic extracts prepared with a B. Braun "MSK" homogenizer (see Methods). Enzyme activities/amoeba are as defined in the Methods. Shaded circles, APase. Unshaded circles, DNase. Stars, RNase.

Figure 19. Changes in protein content of 3-day PGD cells encysting in unagitated EM. The data are from 3 separate experiments.
Figure 20. Specific activities of lysosomal enzymes for 3-day PCD cells encysting in unagitated EM. The data are from 3 separate experiments. Enzymes were assayed in crude amoebic extracts prepared with a B. Braun "MSK" homogenizer (see Methods). Enzyme units are as defined in the Methods. Shaded circles, APase; Unshaded circles, DNase; Stars, RNase.
general patterns as seen for 7-day PGD cells (Fig. 15). There is a decrease in activity/mg protein of both RNase and DNase, and an enrichment of APase activity.

**Differentiation of 7-day PGD Cells Suspended in Aerated EM**

The encystment experiments with 7-day and 3-day PGD cells thusfar described were conducted in unagitated, "monolayer" cultures of EM. It was of interest to learn if the alterations in cellular levels of protein content and lysosomal enzyme activities were similar if starvation-induced encystment were effected by aerating suspension cultures of 7-day PGD cells in EM. This is the technique generally used for inducing differentiation in *A. castellanii* (Neff, Ray, et. al., 1964), and there was reason to believe that "monolayer" and suspension cultures respond differently to a number of treatments (Byers, et. al., 1969; M. Rudick, 1970; V. Rudick, 1971; Byers, 1973).

The encystment kinetics for these aerated, suspension cultures are shown in Fig. 21. Higher levels of cysts were obtained in a shorter period of time with these aerated EM cultures of 7-day PGD cells, as compared to un-aerated ones (Figs. 21, 11). This result was somewhat surprising since the initial concentration of amoebae in suspension cultures was $5 \times 10^5$ cells/ml, as compared to $1 \times 10^5$ cells/ml in "monolayer" cultures. Synchronous encystment in aerated EM appears to commence by the 10th hr, and by 24 hr, over 90% of the cells have differentiated. Furthermore, in spite of the higher concentration of amoebae employed, smaller and less numerous clumps of cysts were obtained in these aerated cultures, as compared to cells in unagitated
Figure 21. Encystment kinetics of 7-day PGD cells suspended in aerated EM. The data are from 2 separate experiments.
Clumps from aerated EM generally contained no more than 10-15 cells.

For some reason, the initial protein contents for 7-day PGD amoebae aerated in EM was about 30% higher than might be expected for cells of this age (Figs. 23, 7, 14). This higher protein content was reflected in initial enzyme activities that also were about 30% higher than expected (Figs. 22, 8, 13).

The data for changing cellular levels of protein and acid hydrolase activities, as well as for specific activities of the lysosomal enzymes, for 7-day amoebae encysting in aerated EM are shown in Figs. 22-24. Again, the patterns roughly paralleled those obtained for either 7-day or 3-day PGD cells encysting in unagitated EM (Figs. 13-15, 18-20). Especially apparent are (1) the decrease in pg protein/amoeba, (2) the sharp decline in RNase activity, even before the appearance of thick-walled cysts, and (3) the enrichment of APase activity.

**Encystment of Mid-LM Amoebae in Optimal Growth Medium**

Do these same alterations in cellular levels of protein and acid hydrolase activities occur if encystment is induced in OGM, where the amoebae have access to exogenous nutrients? The experiments described thusfar have been with PGD cells differentiating under starvation conditions. To answer the question posed, encystment of mid-LM amoebae was induced in OGM by adding either ethidium bromide (Eb) or erythromycin (Ery) to the cultures. The subsequent effect of these drugs on growth kinetics of *A. castellanii* is depicted in Fig. 25. The arrow indicates that point at which the inhibitors were added to experimental cultures.
Figure 22. Changes in lysosomal enzyme activities of 7-day PGD cells encysting in aerated EM. The data are from 2 separate experiments. Enzymes were assayed in crude amoebic extracts prepared with a B. Braun "MK" homogenizer (see Methods). Enzyme activities/amoeba are as defined in the Methods. Shaded circles, APase. Unshaded circles, DNase. Stars, RNase.

Figure 23. Changes in protein content of 7-day PGD cells encysting in aerated EM. The data are from 2 separate experiments.
Figure 24. Specific activities of lysosomal enzymes for 7-day PGD cells encysting in aerated EM. The data are from 2 separate experiments. Enzymes were assayed in crude amoebic extracts prepared with a B. Braun “MSK” homogenizer (see Methods). Enzyme units are as defined in the Methods. Shaded circles, APase. Unshaded circles, DNase. Stars, RNase.
Control cultures increased to a concentration of $2.5 \times 10^6$ amoebae/ml in 6 days, and LM for these cells appeared to terminate at about 50 hr of growth, when the density was $2 \times 10^5$ cells/ml. Eb-treated amoebae were substantially inhibited in their growth. Growth was halted in Ery-treated cells, but death of amoebae did not occur since these cells were capable of encystment. The influence of mitochondrial inhibitors on growth is seen better in Fig. 26, in which the number of population doublings is plotted against time of drug exposure. By 120 hr, control populations had doubled 6 times, and Eb-treated cells nearly 3 times, whereas Ery-treated amoebae remained at the same level as at 0-time.

The encystment kinetics obtained after treatment with Eb and Ery are shown in Fig. 27. Ery is obviously a more potent inducer of encystment under the conditions employed. By 40 hr in Ery, nearly 60% of the amoebae have encysted, as compared to only 10% for Eb-treated cells. The peak values for encystment are obtained by 80 hr, when 92% of the Ery-exposed cells have differentiated, and 36% of the Eb-treated amoebae. A slight appearance of cysts commences by 80 hr in control cultures, but only increases to 10% after 120 hr (Fig. 27).

Changing levels of cellular protein as a function of drug exposure are shown in Fig. 28. In control cells, protein gradually increases to attain a value of 350 pg/amoeba, which might be expected for aging trophozoites (Fig. 7). Protein content in drug-exposed cells exhibits an increase by 20 hr, and then in the Ery-treated amoebae, in which over 90% of the cells form cysts (Fig. 27), a pronounced decline in cellular protein of about 60% later occurs (Fig. 28). Protein content in Eb-treated amoebae appears to drop
slightly, but only back to the level found at 0-time.

The activities/cell of the two nucleases are shown in Fig. 29. In Ery-treated amoebae, pronounced decreases in cellular levels of both DNase and RNase occur, which compare roughly with similar declines found for PCD cells differentiating in EM (Figs. 13, 18, 22). The enzyme data for Eb-treated cells, in which only 36% encystment is obtained as a peak value (Fig. 27), are rather midway between those for Ery-treated amoebae and controls (Fig. 29). Also as might be expected, RNase activity/amoeba drops about 50% during aging in control cells (Figs. 29, 8).

The specific activities for all three lysosomal enzymes are depicted in Fig. 30. Especially evident are (1) the reduction in nuclease activities in drug-exposed amoebae, and (2) the enrichment of APase in Ery-treated cells.

In conclusion, it is seen that in Ery-treated amoebae, in which over 90% encystment is induced in OGM, the obtained data closely resemble those acquired for PCD cells encysting in starvation medium. These similarities include (1) decrease in cellular protein, (2) selective declines in DNase and RNase activities, and (3) enrichment of APase. The protein and enzyme data for Eb-treated cells, which only reached a maximal encystment of 36%, are rather midway between those for Ery-exposed amoebae and controls. This result might be expected for a population in which only one-third of the cells are encysting, while two-thirds of them are still slowly growing.
Figure 25. Kinetics of growth for control, Eb-treated, and Ery-treated cells of *A. castellanii*. The data are from 2 separate experiments, and have not been normalized. Cultures were prepared and inoculated with the drugs as described in the Methods. The arrow indicates that point at which either Eb or Ery was added to growth medium. Shaded circles, controls. Stars, Eb-treated. Unshaded circles, Ery-treated.
Figure 26. Number of population doublings for control, Eb-treated, and Ery-treated cells. The data are from 2 separate experiments. The abscissa indicates the time of exposure of amoebae to either Eb or Ery (which would not apply for controls). 0-time on this graph corresponds to that point indicated by the arrow in Fig. 25. Shaded circles, controls. Stars, Eb-treated. Unshaded circles, Ery-treated.

Figure 27. Encystment kinetics for control, Eb-treated, and Ery-treated cells. The data are from 2 separate experiments. The abscissa has been described in the legend of Fig. 26. Shaded circles, controls. Stars, Eb-treated. Unshaded circles, Ery-treated.
Figure 28. Changes in protein content of control, Eb-treated, and Ery-treated cells. The data are from 2 separate experiments. The abscissa has been described in the legend of Fig. 26. Shaded circles, controls. Stars, Eb-treated. Unshaded circles, Ery-treated.
Figure 29. Changes in lysosomal nuclease activities in control, Eb-treated, and Ery-treated cells. The data are from 2 separate experiments. The abscissa has been described in the legend of Fig. 26. Enzymes were assayed in crude amoebic extracts prepared with a B. Braun "MSK" homogenizer (see Methods). Enzyme activities/amoeba are as defined in the Methods. Shaded circles, controls. Stars, Eb-treated. Unshaded circles, Ery-treated.
Figure 30. Specific activities of lysosomal enzymes for control, Eb-treated, and Ery-treated cells. The data are from 2 separate experiments. The abscissa has been described in the legend of Fig. 26. Enzymes were assayed in crude amoebic extracts prepared with a B. Braun "MSK" homogenizer (see Methods). Enzyme units are as defined in the Methods. Shaded circles, controls. Stars, Eb-treated. Unshaded circles, Ery-treated.
DISCUSSION AND CONCLUSIONS

Population Growth of Trophozoites

The 20 growth curves for axenically cultured *A. castellanii* had kinetic profiles similar to growth curves obtained by others in this laboratory. There were only two, distinct phases: (1) logarithmic multiplication (LM), followed by (2) population growth deceleration (PGD). No noticeable lag, maximum stationary phase, or death phase were observed during 12 days of growth (Fig. 1).

In this study, the average GT for LM trophozoites was $6.50 \pm 0.40$ hr. Byers, *et al.* (1969) reported a GT of $7.48 \pm 0.90$ hr, whereas M. Rudick (1970) obtained a doubling time of $7.35 \pm 0.37$ hr. There are four possible reasons for the faster GT's in this study: (1) adaptation of cells to OGM due to subculturing for several years; (2) always inoculating growth cultures with actively dividing LM amoebae; (3) acclimation of OGM to 30 C, the temperature optimal for growth of *A. castellanii* (Neff, *et al.*, 1958), prior to cellular inoculation; and (4) maintenance of stock media in the dark until ready for use, a precaution taken since Dolphin (1968, 1970) learned that OGM was partly photodegradable.

Biochemical Evidence for Lysosomes in Trophozoites

Figs. 4, 5, and 6, and Tables 10, 11, and 12 all provide biochemical evidence for the existence of lysosomes in cell-free homogenates of
**A. castellanii.** All three of the hydrolases assayed possessed acid pH optima (Fig. 4). The inclusion of 0.25 M sucrose in the homogenizing medium, with the consequent sedimentation of sizable amounts of enzyme activity (Table 10, Fig. 6) by centrifugation at 20,000 X g, attests to a particle-bound nature of the enzymes. Some preservative osmotic effect on lysosomes is also provided by homogenizing buffer (HB) itself, or else all the hydrolase activity shown in Table 10 would appear in the 20,000 X g supernatant when sucrose was excluded from HB. Finally, treatment of extracts by procedures which rupture lysosomal membranes, such as incubation at 4 °C with 0.1 % Triton X-100 or grinding trophozoites with glass beads, was found to promote latency of acid hydrolases (Fig. 5, Tables 11, 12).

A pH curve for DNase activity in *A. castellanii* (Fig. 4) has not appeared in the literature. The only reference to assay for this enzyme in a hartmannellid is that reported by Morgan & Griffiths (1972).

**Lysosomal Activity during Population Growth**

Fig. 6 shows that APase, DNase, and RNase all exhibit a similar sedimentation profile as a function of culture aging. The % sedimentation for the three enzymes increases from about 40 % in mid-LM amebae to attain a plateau of about 80 % in PGD cells. These data do not necessarily mean that all three enzymes are contained in a common organelle, however, for heterogeneous populations of lysosomes have been observed in rat liver cells (Rahman, *et al.*, 1967; Rahman & Cerny, 1969), in the ciliate Tetrahymena (Müller, 1970, 1972; Lloyd, *et al.*, 1971), and in rabbit leukocytes (Baggiolini, *et al.*, 1969, 1970).
The acid hydrolase sedimentation of 80% is a figure commonly reported for other cell types, and the 20% "free" activity is generally attributed to rupture of lysosomes during homogenization (de Reuck & Cameron, 1963; Muller, et al., 1966; Dingle & Fell, 1969). However, it also is possible that free activity is the result of (1) naturally occurring soluble enzyme, or (2) is due to enzymes being synthesized in the endoplasmic reticulum, or stored in the Golgi, that have either become solubilized during breakage, or else become contained within microsomes that do not sediment at 20,000 X g.

There are at least four explanations for the lower sedimentation of acid hydrolases in younger amoebae: (1) more free activity in rapidly dividing, younger cells; (2) more fragile lysosomes in the young amoebae, so that the sedimentation profile as a function of culture age is merely an artifact of the breakage procedure; (3) smaller lysosomes, not all of which sediment at 20,000 X g, in the younger cells; and (4) a higher synthesis of acid hydrolases in the endoplasmic reticulum, or greater storage of these enzymes in the Golgi, of young cells, so that upon cellular homogenization, these enzymes appear either in the supernatant or else are contained in non-sedimenting microsomes.

By an extrapolation of latency data he obtained in cell-free homogenates of Tetrahymena, Lee (1970) calculated that about 85% of the APase activity in stationary phase cells was contained within lysosomes, whereas only 35% was lysosome-bound in log phase organisms. These data are strikingly similar to those obtained in this study for A. castellanii (Fig. 6). Lee (1970) postulated that free lysosomal enzymes participated in regulating mitosis in log phase Tetrahymena; he cited references to work on other types
of animal cells to support this view. Allison (1969) has likewise reviewed evidence for lysosomal involvement during mitosis in animal cells. Furthermore, by cytochemical staining, Jurand & Saxena (1972) observed APase in macronuclear fragments of Paramecium aurelia following both conjugation and autogamy. They suggested that this enzyme functions to elicit disintegration of the old macronucleus.

During axenic culture of A. castellanii, total cellular protein increases about 30% from mid-LM and early PCD phases of growth to reach a rather constant value of 350 pg/cell in older amoebae (Fig. 7). This value is less than the 450 pg/amoeba reported by V. Rudick (1969). Some of her data suggest a lower content of protein in younger cells, but were not expressed in this fashion. The discrepancy in cellular levels of protein between this study and that of V. Rudick (1969) could be due to the differences in mean GT's between the amoebae of the two studies. In this work, amoebae had an average GT of 6.50 hr, whereas the cells used by V. Rudick (1969) doubled every 7.48 hr. Certain metabolic differences in the two groups of amoebae might therefore be expected. In this study, the 30% increase in pg protein per amoeba might be caused by a slight unbalanced growth effected by aging, and an accumulation in the cultures of non-mitosing, predivison cells.

The activity/cell of both APase and DNase remains rather constant as a function of culture age (Fig. 8), which suggests that both enzymes are constitutive. These data need not imply, however, that the hydrolase activity is due to only one APase and one DNase. Indeed, by electrophoresis of cellular extracts and subsequent staining of acrylamide gels, this author has demonstrated the existence of at least three APases in A. castellanii.
Moreover, Holm (1971) has obtained evidence for the presence of two acid DNases in cells of *Tetrahymena*.

RNase activity/amoeba is twofold higher in younger cells, dropping 50% by day 5 of growth (Fig. 8). This decrease in RNase activity roughly parallels the cellular decline in RNA synthesis (V. Rudick, 1969). A functional role of RNase in regulating turnover of cellular RNA is suggested by these data, but would be difficult to prove.

There are several explanations for the alteration in RNase activity during aging: (1) the presence of activators in young cells, or of inhibitors in older amoebae, which could be checked by mixing homogenates from cells of different ages and assaying enzyme activity; (2) extracellular secretion of part of the RNase; and (3) the existence of multiple RNases, one or several of them being selectively degraded upon culture aging, or else being secreted into the ambient medium. Extracellular secretion could be verified by assaying for RNase in growth medium at sundry times during culture aging.

This author has assayed for APase and DNase in ultrafiltered OGM that initially contained LM amoebae, and was surprised to learn how much hydrolase activity seemed to be in this extracellular compartment; no detailed experiments were performed as a follow-through, however, nor was RNase activity measured. The presence of multiple RNases in extracts of *A. castellanii* could possibly be demonstrated by electrophoretic techniques. Or, the enzymes could be isolated from cellular homogenates by using standard biochemical techniques. Lazarus & Scherbaum (1967a) have isolated three acid RNases from extracts of *Tetrahymena*.

When enzyme data are expressed as activity/mg protein, an enrichment of
all three hydrolases is observed in younger amoebae (Fig. 9). This enrichment, coupled with the apparent increase in soluble hydrolases in young cells (Fig. 6), suggests that these enzymes might participate in regulating the mitotic process of rapidly dividing amoebae, such as by breaking up the nuclear envelope, the nucleolus, or the mitotic spindle. The enzymes might also help regulate the turnover of protein and nucleic acids which is possibly more enhanced in younger cells. Phosphatases have been associated with nucleotide metabolism in *Tetrahymena* (Conner & MacDonald, 1964). Furthermore, the rather high levels during growth of cellular activity of APase, DNase, and RNase is consistent with the notion that these enzymes function primarily as digestive hydrolases in heterophagic vacuoles. Certainly, an important role for acid hydrolases in Protozoa does seem to be a digestive one (Müller, et al., 1963; Müller, 1967a; Dembitzer, 1968; Esteve, 1970; Ricketts, 1971; Chapman-Andresen, 1973).

**Encystment Kinetics of PCD Cells**

The kinetics of starvation-induced encystment of PCD cells (Figs. 11, 17, 21) were observed to be a function of (1) inoculum culture age, and (2) whether or not amoebae were aerated in suspension cultures. The faster differentiation and higher levels of encystment obtained with 3-day amoebae in unagitated EM (Fig. 17), as contrasted with 7-day cells under similar conditions (Fig. 11), demonstrate a greater competence for encystment by the younger organisms. The reason for this difference in competency is difficult to explain, and is likely due to multiple factors. The higher % cysts obtained in 24 hr for 7-day amoebae aerated in suspension cultures
(Fig. 21), as compared to similar cells in unagitated EM (Fig. 11), possibly indicates that both aeration and a deplenishment of crowding by suspension of the cells enhance differentiation in *A. castellanii*.

The data for loss of cells during incubation in unagitated EM are due to (1) cell lysis, and (2) failure to recover all the encysting amoebae from their culture vessels. The latter was especially a problem with 3-day PCD cells encysting in unagitated EM. These amoebae tended to form some rather large clumps of over 100 cells, and these clumps adhered to the glass walls when amoebic suspension was poured out of the Roux bottles. No logical reason can be given for the differences in sizes of cyst clumps obtained in the several types of encystment experiments.

**Changes in Protein Content during Encystment of PCD Cells**

Regardless of inoculum age, or the method of inducing encystment in EM, a gradual decline in pg protein/amoeba to a final value 30-60% less than that at 0-time was observed in this study (Figs. 14, 19, 23). Neff & Neff (1969) and Griffiths (1970) have reviewed data by other investigators that also demonstrate a massive decrease in protein content of encysting amoebae. This cellular decline in protein is usually ascribed to endogenous degradation, but it could partly also be due to extracellular secretion. Neff, Neff, & Benton (1964) reported that a considerable portion of the nitrogen released from encysting *A. castellanii* in starvation medium was due to amino acids, which suggested an endogenous degradation of protein, followed by a subsequent excretion.
Lysosomal Activity during Encystment of PGD Cells

In spite of minor differences which are difficult to explain, it was observed in the several encystment experiments with PGD cells that there was (1) a selective decrease in the activity/cell of all three hydrolases, (2) a rather sharp decline in RNase activity of 60-80% even before the appearance of thick-walled cysts, and (3) an enrichment of APase activity. A decrease during encystment in both APase and RNase was observed by Tibbs & Marshall (1970) in the ciliate Colpoda steinii, but they offered no explanation for this decline. Neff & Neff (1969) stated that APase activity in A. castellanii decreased during encystment, but they gave no supportive data.

Since a massive decrease in cellular contents of DNA, RNA, protein, glycogen, and lipid occurs during starvation-induced encystment in A. castellanii (Neff & Neff, 1969; Griffiths, 1970), one might suspect that lysosomal hydrolases play a prominent role in this intracellular degradation. Lysosomal enzymes participate in a wide variety of catabolic processes in eukaryotes (Table 1). An increase in cellular activity of these enzymes, at least during initial stages of encystment, might even logically be expected during differentiation in A. castellanii, but on the contrary, activities/cell of APase, DNase, and RNase all decrease during encystment. This is unlike the case in Euglena gracilis, in which starvation results in autophagy and an increase in lysosomal enzyme activity (Brandes, et. al., 1964; Bertini, et. al., 1965).

The decrease in acid hydrolase/cell does not preclude that these enzymes play no prominent role in intracellular breakdown of macromolecules during encystment. The electron micrographs of Bowers & Korn (1969) show a
pronounced increase in autophagic vacuoles during differentiation in
*A. castellanii*, and these vacuoles stain positively for APase. The *in vivo*
substrate turnover rates for APase, DNase, and RNase in *A. castellanii* are
not known, but they might well be sufficient for these enzymes to partici­
pate significantly in autolysis of cellular components during encystment.

The decrease during encystment in activity/cell of the hydrolases could
be due to several factors: (1) the presence of inhibitors in encysting cells;
(2) the loss of activators from differentiating amoebae, (3) secretion into
the ambient medium; or (4) intracellular degradation of the hydrolases by
protease enzymes. The first two possibilities could be checked by mixing
cyst and trophozoite extracts and assaying the enzyme activities. The third
explanation could be investigated by assaying the medium for enzyme activi­
ties during encystment. If possibility 4 is the correct one, then the data
in this study suggest that there are differences in susceptibility to prote­
ase attack in the three hydrolases assayed.

A greater susceptibility of RNase to protease attack is suggested by
the data in Fig. 12, which indicates the % sedimentation of lysosomal en­
zymes for 7-day PGD cells differentiating in EM. Although the % sedimenta­
tion of both APase and DNase remains rather constant at 80 %, pelleted RNase
gradually decreases in 10 hr to 60 %. Particle-bound RNase might, therefore,
be more sensitive than the other two enzymes to degradation by proteases.
Huisman, *et. al.* (1973) have presented data suggesting that lysosomal prote­
asces might function to break down intracellular RNase.

It should also be mentioned that the % sedimented data shown in Fig. 12
can be used to argue for the presence of heterogenous populations of
lysosomes in A. castellanii.

If APase, DNase, and RNase all function primarily as digestive enzymes in heterophagic vacuoles, their decrease in cellular activity during encystment might merely represent a disposal of enzymes not needed in dormant cysts. The electron microscopic observations of Bowers & Korn (1969) demonstrate a depletishment of heterophagic vesicles during encystment in A. castellanii.

The sharp decline in RNase activity during encystment, which occurs even before the appearance of thick-walled cysts, might represent an intrinsic adaptation of the cells to protect against too extensive a degradation of RNA essential for encystment. It is known that new RNA synthesis occurs during the beginning stages of encystment, even though the total cellular levels of RNA are decreasing at this time (Neff & Neff, 1969; Stevens & Fachler, 1973).

The enrichment of APase activity during encystment could be interpreted as being due to synthesis of novel APases. However, such synthesis is not likely to be a requirement for encystment, since if it were, cells would be inhibited from differentiating in the presence of cycloheximide, and such is not supported by the data in Table 13. However, the maximal concentration of cycloheximide used in this study was 50 μg/ml. Rudick & Weisman (1975) report that a cycloheximide concentration of 500 μg/ml blocks encystment in A. castellanii without causing cell death for at least 48 hr.

The enrichment of APase during encystment suggests that this enzyme might function to maintain a pool of free phosphates for use (1) in an energy-generating system, and/or (2) as precursors for synthesizing the phosphoprotein and cellulose of the cyst wall.
If free phosphate is required for a functioning electron transport system, treatment of cells with an uncoupler of oxidative phosphorylation, such as 2,4-dinitrophenol, might inhibit encystment. However, this author found that when 7-day PGD amoebae were placed into EM containing a final concentration of this drug of 85 μg/ml, which inhibits Eb-induced encystment of LM amoebae in OGM (Byers, 1973), 88% of the cells formed cysts in 71 hr.

If encysting cells conserve APase to provide free phosphate for synthesizing the cyst wall, addition of phosphate to EM should promote a decline in enzyme activity, since amoebae could presumably acquire phosphate from the medium if no permeability barriers were established. Addition of free phosphate to growth medium of yeast (Schurr & Yagil, 1971) and Euglena gracilis (Price, 1962) were shown to effect a decrease in APase activity. However, in A. castellanii, APase activity per cell, as well as total cellular protein and % cysts, are unaffected by adding free phosphate to EM (Table 14). These data suggest that APase levels are not regulated by exogenous phosphate during this period.

Encystment Induced in Mid-LM Cells by Mitochondrial Inhibitors

A novel means of inducing encystment in LM trophozoites by adding inhibitors of mitochondrial function to OGM was used in this study. Both compounds, ethidium bromide (Eb) and erythromycin (Ery), retard normal growth of axenic cultures of A. castellanii (Fig. 25). Eb is also known to restrict axenic growth in the ciliate Tetrahymena (Meyer, et al., 1972; Rohatgi & Krawiec, 1973). The failure of Eb to suppress growth completely in A. castellanii suggests that (1) either a population of the amoebae was
resistant to the drug, or (2) inhibition by Eb of that cellular function which induces encystment is "leaky."

After 3 days of drug exposure, 36% of the Eb-treated cells had encysted; within the same time period, 92% of the Ery-treated amoebae formed cysts (Fig. 27). Levels of cellular protein and acid hydrolase activities for LM amoebae induced to encyst by these drugs in the presence of exogenous nutrients roughly paralleled data obtained for PGD cells differentiating in nutrient-free EM. These results were more apparent with the Ery-treated amoebae, nearly all of which formed cysts in OGM. The obvious similarities between Ery-exposed cells encysting in OGM and PGD cells differentiating in EM were (1) decrease in cellular protein, (2) selective declines in DNase and RNase activities, and (3) enrichment of APase.

Therefore, the intracellular changes observed to occur during starvation-induced encystment in PGD cells are not simply due to starvation itself, since they also occur when LM amoebae are induced to encyst in the presence of adequate nutrients. These data support the idea that the observed patterns of change are important for encystment. It cannot be ascertained, however, if the decline in cellular levels of lysosomal enzyme activities are a cause, or a consequence, of encystment. It does appear, though, that intracellular degradation during encystment may be essential even in the presence of exogenous nutrients, possibly because permeability barriers are established between differentiating amoebae and the ambient medium.
SUMMARY

There are three biochemical criteria for the presence of lysosomes in an organism: (1) acid pH optima of constituent hydrolases; (2) sedimentation of these hydrolases at 20,000 X g in 0.25 M sucrose, thereby attesting to their particle-bound nature; and (3) latency, or the enhancement of enzyme activity upon treatment of homogenates with procedures that rupture lysosomal membranes. By assaying three lysosomal marker enzymes, APase, DNase, and RNase, it was demonstrated that cell-free homogenates of *A. castellanii* trophozoites in PGD fulfill these three criteria.

During axenic culture of *A. castellanii*, total cellular protein increases about 30% from mid-LM and early PGD stages of growth to reach a rather constant value of 350 pg/cell in older amoebae. The sedimentation of lysosomal enzymes at 20,000 X g in 0.25 M sucrose gradually increases from about 40% in mid-LM to obtain a plateau of 80% throughout most of PGD. The activity/cell of both APase and DNase remains constant as a function of culture age, whereas that of RNase is twofold higher in younger amoebae, dropping 50% by 5 days of growth. When enzyme data are expressed as activity/mg protein, an enrichment of all three hydrolases is observed in younger amoebae. This enrichment, coupled with the apparent increase in soluble hydrolases in young cells, suggests that these enzymes might participate in regulating (1) the mitotic process of rapidly dividing amoebae,
and/or (2) the turnover of protein and nucleic acids which could be more enhanced in younger cells. Furthermore, the rather high levels during growth of cellular activity of APase, DNase, and RNase is consistent with the notion that these enzymes function primarily as digestive hydrolases in heterophagic vacuoles.

The kinetics of starvation-induced encystment of PGD cells were observed to be a function of (1) inoculum culture age, and (2) whether or not amoebae were aerated in suspension cultures. The faster differentiation and higher levels of encystment obtained with 3-day amoebae, as contrasted with 7-day cells, demonstrate a greater competence for encystment by the younger organisms. The higher % cysts obtained in 24 hr for 7-day amoebae aerated in suspension cultures, as compared to similar cells in unagitated EM, possibly indicates that both aeration and a depletion of crowding by suspension of the cells enhance differentiation in A. castellanii. The failure of cycloheximide and 2,4-dinitrophenol to block encystment of 7-day amoebae in unagitated EM suggests that cells of this age have sufficient protein and energy reserves to complete starvation-induced differentiation.

During the first 10 hr of encystment by 7-day amoebae in unagitated EM, the % sedimentation of both APase and DNase was maintained at the initial value of about 80 %, whereas pelleted RNase activity gradually decreased to 60 %. This result (1) argues for the heterogeneity of lysosomes in A. castellanii, or (2) suggests a greater susceptibility of particle-bound RNase to degradation by proteases.

During differentiation of PGD cells in EM, (1) total cellular protein dropped 50-60 % by 3 days of encystment, (2) a selective decrease in the
activity/cell of all three hydrolases was observed, (3) a rather sharp decline in RNase activity occurred even before the appearance of thick-walled cysts, and (4) although its activity/cell was gradually decreasing, there still was a relative enrichment of APase activity. Work by other investigators that is confirmed by the protein data of this study shows that during differentiation in *A. castellanii*, there is a rather massive decrease in the cellular contents of DNA, RNA, protein, lipid, and glycogen, which is generally ascribed to endogenous degradation. This degradation could be caused by activity of lysosomal enzymes, but the biochemical data in this study neither conclusively support, nor preclude, this possibility.

The sharp decline in RNase activity during encystment might represent an intrinsic adaptation of the cells to protect against too extensive a degradation of RNA essential for encystment. The enrichment of APase suggests that this enzyme might function to maintain a pool of free phosphates for use (1) in an energy-generating system, and/or (2) as precursors for synthesizing the phosphoprotein and cellulose of the cyst wall. APase activity per cell, as well as total cellular protein and % cysts, are unaffected by adding free phosphate to EM, however.

A novel means of inducing encystment in LM trophozoites by adding inhibitors of mitochondrial function to OGM was used in this study. One of these compounds, ethidium bromide, is an inhibitor of mitochondrial transcription, while the other, erythromycin, blocks mitochondrial translation. Both compounds markedly retard normal growth of axenic cultures of *A. castellanii*. After 3 days of drug exposure, 36 % of the Eb-treated cells had encysted, whereas 92 % of the Ery-treated amoebae were cysts. Levels of cellular
protein and acid hydrolase activities for LM amoebae induced to encyst by these drugs in the presence of exogenous nutrients roughly paralleled data obtained for PGD cells differentiating in nutrient-free EM.

The results suggest that in *A. castellanii*, the changing levels in lysosomal enzyme activities and cellular protein content are characteristic features of encystment, and are independent of environmental conditions or age of the cells.
PERSPECTIVES

This study has described and offered explanations for alterations in levels of lysosomal enzyme activities and cellular protein content during population growth in OGM, and for encystment induced under different conditions, for the soil amoeba, *A. castellanii*. Additional avenues of possible, subsequent investigation are now possible.

An obvious extension of the present study would be to ascertain the patterns of lysosomal enzyme activities when cells of different ages that have been induced to encyst by various methods are then allowed to excyst. Would an expected increase in lysosomal enzyme activities, and a rise in pg protein/amoeba, typify outgrowing cells? Also, what effect would inhibitors of nuclear transcription or cytoplasmic translation have on excystment in general, and on lysosomal enzyme activities in particular? Furthermore, no assays for cellular contents of DNA, RNA, glycogen, or lipid have thusfar been performed during outgrowth of dormant cysts of *A. castellanii*. Although it can be argued that performing such assays is trivial, these experiments still need to be performed if a complete understanding of the life cycle of *A. castellanii* is ever to be obtained. Moreover, some unexpected results of real significance might be uncovered.

The work conducted in this dissertation could be added to by (1) obtaining additional protein and acid hydrolase data for mid-LM trophozoites.
induced to encyst in OGM by Eb- or Ery-treatment, in order to clarify and verify the limited data presented in this study; (2) determining latency of acid hydrolases in different ages of LM cells, to confirm by another manner the apparent increase in free lysosomal enzyme activity found in this study for these cells; and (3) repeating experiments described in this dissertation with assays of other lysosomal enzymes, especially to ascertain if a decrease in all acid hydrolase activity/cell is a characteristic event of encystment. Other lysosomal enzymes previously reported to be in cells of *A. castellanii* are acid proteinase, b-N-acetylglucosaminidase, a-glucosidase, a- and b-galactosidase, and b-glucuronidase (Muller, 1969b; Morgan & Griffiths, 1972). Would some of these enzymes also be enriched during encystment as was observed in this study for APase?

The reason for the increase in sedimentation of acid hydrolase activity from 40% in mid-LM to a plateau of 80% in PGD cells needs to be ascertained. Several explanations for this phenomenon have been offered: (1) more free activity in rapidly dividing, younger cells; (2) more fragile lysosomes in the young amebae, so that the sedimentation profile as a function of culture age is merely an artifact of the breakage procedure; (3) smaller lysosomes, not all of which sediment at 20,000 X g, in the younger cells; and (4) a higher synthesis of acid hydrolases in the endoplasmic reticulum, or greater storage of these enzymes in the Golgi, of young cells, so that upon cellular homogenization, these enzymes appear either in the supernatant fluid or else are contained in non-sedimenting microsomes. Insight could be gained into which is correct of these alternate explanations by performing a differential centrifugation on cell-free homogenates to
obtain a 20,000 and 100,000 X g pellet, and a 100,000 X g supernatant. If interpretations 1 or 2 are correct, about 60% of the hydrolase activity in mid-LM amoebae should be in the 100,000 X g supernatant. The verity of possibilities 3 and 4 would be shown by the appearance of substantial enzyme activity in the 100,000 X g pellet. A distinction between these latter two explanations might possibly be made by examining the 100,000 X g pellet with the electron microscope.

An involvement of lysosomal hydrolases in regulating metabolism in younger, possibly more synthetically active, trophozoites of *A. castellanii* is suggested by the enrichment of APase, DNase, and RNase in extracts from the younger cells, and by the higher activity/cell of RNase in the young amoebae. Perhaps all three enzymes, or at least the RNase, participate in regulating the turnover of macromolecules during this point in the life cycle of *A. castellanii*. Some very difficult and intricately conceived experiments would have to be devised to assess the verity of this possibility.

A method for determining half-lives of acid hydrolase activities has been reported for mouse cancer cells (Rosmann, 1972). The cells are resuspended in a medium containing 100 µg/ml of cycloheximide, and the activities of the enzymes are determined after different times of exposure to the drug. The resultant decrease in the cellular levels of enzyme activities suggests that the hydrolases are being constantly degraded and resynthesized intracellularly. In this study, it was discovered that both APase and DNase remain at a rather constant level of cellular activity during population growth in *A. castellanii* (Fig. 8). Are these enzymes static, constitutive hydrolases, or are they in a constant state of degradation and resynthesis? The answer
to this question might be obtained by using the methods of Bosmann (1972).

The effect of erythromycin on inducing encystment in *A. castellanii* in OGM has not yet been accurately assessed as a function of (1) culture age, (2) cell concentration, and (3) depth of the culture medium. These experiments need to be performed to lay groundwork for more intensive encystment studies. Neff & Neff (1966, 1969, 1972) have reported that mitomycin C, an inhibitor of DNA synthesis and, also, an anti-tumor agent, is a potent inducer of encystment in *A. castellanii* when added to cells growing in OGM. This compound might also be used as another tool for inducing differentiation of *A. castellanii* in growth medium so that changes in macromolecule content and enzyme activities can be assayed for amoebae encysting in the presence of exogenous nutrients.

Except for kinetics studies (Neff & Neff, 1966, 1969, 1972; Kuhns & Byers, 1972; Byers, 1973), encystment of *A. castellanii* in growth medium has not yet received much attention. Many biochemical and physiological studies remain to be performed, and it is hoped that this dissertation will pave the way for such efforts. It was observed in this study that a dramatic decrease in the cellular content of protein occurs when encystment of mid-LM amoebae is effected by adding Ery to growth medium. Do decreases in the cellular levels of DNA, RNA, glycogen, and lipid also occur when encystment is induced in the presence of exogenous nutrients? Bowers & Korn (1969) showed that in starvation-induced encystment in *A. castellanii*, there was a depletion of the heterophagic vacuolar system and a pronounced appearance of autophagosomes. Do such intracellular changes also occur in cells encysting in growth medium? Electron microscopy would provide the answer to that
It was found in this study that concentrations of both cycloheximide and 2,4-dinitrophenol which inhibit Eβ-induced encystment of LM trophozoites growing in OGM do not block encystment of 7-day PGD cells placed in encystment medium. These data suggest a possible build-up and storage of protein and energy reserves in aging trophozoites in preparation for encystment; cytokodifferentiation subsequently ensues if environmental conditions become favorable. Experiments need to be conducted in which trophozoites of different ages are inoculated into EM containing various concentrations of either cycloheximide or 2,4-dinitrophenol, and the resultant level of encystment determined. The effect of other protein synthesis inhibitors, such as puromycin or emetine, might also be tested. This experimental protocol was used by V. Rudick (1969, 1971) to demonstrate how actinomycin D prohibited encystment of LM amoebae inoculated into EM, but not that of older PGD cells, which, apparently, had already synthesized and stored part of that RNA required for cyst wall formation.

The decrease in total cellular protein and in activity/cell of APase, DNase, and RNase reported in this study for amoebae induced to encyst under a variety of conditions was attributed to several possibilities, including (1) extracellular secretion, and (2) endogenous degradation. Such results are generally ascribed to endogenous degradation, although assaying enzyme activity in the ambient medium would verify if extracellular secretion also occurs. If intracellular degradation is characteristic of encystment, there might be an enhancement of protease activity in encysting amoebae. These proteases could be lysosomal enzymes, or they could also be enzymes
functioning at an alkaline pH. Regardless, assaying for protease activity
during encystment might yield information pertinent to understanding cytodi-
ferentiation in *A. castellanii*. This author has confirmed the report by
Muller (1969b) that there is present in extracts of *A. castellanii* trophozo-
ites an acid proteinase having a pH optimum of about 3.0. No assays for
this enzyme have been made during encystment, however.

If intracellular events occurring during the life cycle of
*A. castellanii* are ever to be fully understood, more assays of different en-
zymes must be performed on extracts from growing, encysting, and excysting
amoebae. This author has begun laying the groundwork for such an undertak-
ing. Spectrophotometric assays have been successfully tested for catalase,
hexokinase, alcohol dehydrogenase, glucose 6-phosphate dehydrogenase, succin-
ate dehydrogenase, phosphoenolpyruvate carboxylase, malate dehydrogenase,
b-hydroxybutyrate dehydrogenase, and acid proteinase. Moreover, electrophor-
etic techniques have been devised for analysing homogenates from
*A. castellanii* trophozoites and cysts. Staining procedures for specific en-
zymes have been successfully tried for APase, non-specific esterase, malate
dehydrogenase, b-hydroxybutyrate dehydrogenase, alcohol dehydrogenase, and
hexokinase.


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