MORPHOLOGICAL CHANGES AND THE REQUIREMENTS FOR MACROMOLECULE SYNTHESIS DURING EXCYSTMENT OF *ACANTHAMOEBA CASTELLANII*

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ABSTRACT

Light and phase-contrast microscopic observations of excystment in *Acanthamoeba castellanii* have been used to classify cells in excysting populations as free trophozoites, or mature, activated, or preemergent cysts. These categories have been used to describe the kinetics of excystment. A pH of 7 and a temperature of 30°C have been found to be optimal for the activation of mature cysts. Both activation and emergence are inhibited by cycloheximide and actinomycin D, but neither process is much affected by hydroxyurea. Cell-free extracts of high molecular weight components of cyst cytoplasm can support protein synthesis in vitro, although less efficiently than similar extracts from trophozoites. Evidence indicates that some of the functional RNA in the cyst extracts is synthesized before excystment.

INTRODUCTION

In 1964 Neff et al. reported on a system that they had developed for inducing synchronous encystment in axenic cultures of the small ameba *Acanthamoeba* sp., and Neff and Neff (1969) have recently reviewed subsequent studies on the encystment of this organism, now called *Acanthamoeba castellanii* (Neff strain) (Page, 1967). Although a considerable amount of information is available on encystment, there is a lack of studies on the morphology and biochemistry of excystment. Work in this laboratory has demonstrated that marked physiological changes occur during aging of unagitated cultures of *Acanthamoeba* (Byers et al., 1969) and that several of these changes can be considered preparations for encystment (V. Rudick, 1971). Among other things, these workers have shown that encystment is associated with a depression of RNA and DNA syntheses which occurs as cultures near termination of logarithmic growth. In the present study, the kinetics of encystment are described and, then, in an effort to further explore the nature of the suppression of biosynthesis during cyst formation, an examination has been begun of the synthetic capabilities of cysts and the biosynthetic requirements for reinitiating vegetative growth by excystment.

MATERIALS AND METHODS

Cell Growth

Acanthamoeba (Neff's clone I-12) was grown in optimal growth medium (OGM)[1] prepared as pre-

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[1] *Abbreviations used in this paper:* ATP, adenosine triphosphate; CTP, cytidine triphosphate; EM, encystment medium; GTP, guanosine triphosphate; OGM, optimal growth medium; PCA, perchloric acid; POPOP, 1,4-bis-2-(5-phenyloxazolyl)- benzene; PPO, 2,5-diphenyloxazole; TCA, trichloroacetic acid.

viously described (Byers et al., 1969). Trophozoites were grown in unagitated 150-ml cultures in Roux bottles or in 300-ml cultures in 2-liter low form Erlenmeyer flasks and were incubated at 30°C in the dark.

Cyst Preparation

Amebas were induced to encyst in Neff's nutrient-free encystment medium (EM) that gives a progressive increase in pH (Neff et al., 1964). Cells from older cultures were suspended at a final concentration of 10^6 amebas per milliliter in 1500 ml of encystment medium in 2-liter aspirator flasks. They were then incubated at 30°C with aeration at 4 ft^3/hr.

After 36–48 hr in EM, cysts were aseptically harvested and washed in 0.15 M KCl. Remaining trophic forms were lysed by overnight incubation of the cells at 5°C in sterile 2.5% deoxycholate, or in the latter plus 37 units/ml Nystatin (3000 units/mg powder) and 100 μg/ml Neomycin sulfate. Although this treatment does not affect the viability of cysts (Culbertson, 1961; Culbertson et al., 1965; Ensminger and Culbertson, 1966), it was eliminated when cysts were to be used for the preparation of cell-free extracts. In this case, trophozoites and thin-walled cysts were broken by homogenization in a suitable buffer medium, and thick-walled cysts were collected from the pellet after centrifugation at 700 g for 10 min.

Morphology and Kinetics of Excystment

Excystment has been followed in 3-ml test tube cultures containing OGM, inoculated with thick-walled cysts at about 1–2 × 10^4 cysts per milliliter, and incubated on a 5° slant at 30°C. For each observation, at least 1000 organisms were examined by phase-contrast microscopy or after staining. In the latter procedure, cells were harvested by centrifugation from an unfixed excysting culture and then were resuspended in 0.5 ml 0.1 M Na–acetate buffer at pH 5.15. Next, 0.1 ml of 2.5% iodine–5% KI was mixed with the suspension and, then, 0.1 ml of 1% eosine Y (Matheson Co., Inc., Rutherford, N.J.) in 0.1 M acetate buffer at pH 5.6 was added. The final volume was adjusted to 1 ml with acetate buffer of the lower pH. This pH gave the best eosine staining without precipitating its acid form.

Some observations were conducted with time-lapse cinematography and phase-contrast microscopy. In this case, cysts were suspended on a slide in micro-cultures 0.5 mm deep.

The effects of temperature and pH on excystment were studied with stained samples from replicate 50-ml cultures containing 7 × 10^5 cysts per milliliter in 250-ml Erlenmeyer flasks. For pH studies, un-filtered OGM (to avoid loss of compounds precipi-

tating at different pH's) was adjusted with KOH or HCl to the proper pH.

In Vivo Incorporation of Radioisotopes

Isotopic labeling of cells was achieved in replicate 3-ml cultures in screw-cap tubes. The isotopes used were: 5 μCi uridine-^{3}H (20 Ci/mmole)/3 ml for RNA; 3 μCi thymidine methyl-^{3}H (15 Ci/mmole)/3 ml for DNA; and 0.6 μCi proline-^{14}C (220 mCi/mmole)/3 ml for protein synthesis. Labeling was terminated by the addition of an equal volume of 10% trichloroacetic acid (TCA). After 24 hr in the cold, the cells were lysed with 1 ml 0.2 M Tris buffer, pH 8, and 25 ml 9 N NaOH, and the macromolecules were reprecipitated by adding 2 ml 10% TCA. The TCA-insoluble material was then collected on 0.45 μ pore membrane filters, dried, and the radioactivity was assayed by liquid scintillation spectrometry in a scintillation mixture of 0.5% 2,5-diphenyloxazole (PPO) and 0.01% 1,4-bis-2-(5-phenyloxazolyl)- benzene (POPOP) in toluene.

Cell-Free Protein Synthesis

High molecular weight cytoplasmic extracts for studies of cell-free protein synthesis were prepared from trophozoites or from mature cysts. Extracts were prepared at 0°–4°C in buffer A (50 mM Tris, pH 7.4; 5 mM $MgCl_2$; and 10 mM 2-mercaptoethanol) according to a modification of the method of Chesters (1968). Cysts or trophozoites were washed twice in buffer A, resuspended at about 10^7 cells per milliliter in the same buffer, and then homogenized with a ground-glass homogenizer with the pestle turning at 250 rpm. About 90–95% of the trophozoites were disrupted in 2.5 min (100 up and down strokes), while 25 min were required to achieve a similar level of breakage with cysts. Although these homogenizing times are very different, it was found that equalizing the times did not change the results obtained in the cell-free synthesis studies. The homogenates were centrifuged at 5000 g for 10 min, and the top lipid layer and the pellet were discarded. The turbid supernatant was filtered through 8–10 volumes of Bio-Gel P-4 (Calbiochem, Los Angeles, Calif.) equilibrated with buffer A and contained in a column of 1.3 cm inside diameter. Fractions equal in volume to the sample were collected and the two fractions containing the highest absorbance at OD_{260} were combined and used as the high molecular weight (mol wt greater than 3600) cytoplasmic extract.

Protein synthesis was assayed by the incorporation of leucine-^{3}H into hot TCA-insoluble material. The assay was performed in a reaction volume of 1 ml in a 10 ml Erlenmeyer flask which was shaken in a water bath at 32°C. All reagents were dissolved in buffer A and added to the reaction flask in the following order

and to give the following final concentrations: 1 mM each of 17 amino acids (glutamic acid, aspartic acid, tryptophan, cysteine, methionine, phenylalanine, glycine, tyrosine, valine, serine, lysine, arginine, threonine, histidine, isoleucine, proline, and alanine); 1 mM adenosine triphosphate (ATP); 0.25 mM guanosine triphosphate (GTP); 0.25 mM cytidine triphosphate (CTP); 5 mM creatine phosphate; 60 μg creatine phosphokinase; 10 μCi (50 μM) D,L-leucine-4,5-^{3}H; and to begin the experiment, 0.5 ml cytoplasmic extract containing 0.8–1.6 mg total RNA was added. Samples of 0.1 ml were taken and added to 2 ml of cold 10% TCA to stop the reaction. Precipitation was allowed to proceed for 30 min and then, in order to release any nonprotein-bound amino acids, the precipitate was heated at 90°C for 30 min and, finally, was cooled and kept in the cold TCA overnight. The resulting precipitate was collected by filtration, the filters were washed, dried, and assayed for radioactivity as described above.

The amounts of RNA in the cytoplasmic extracts were determined by the method of Fleck and Begg (1965). The TCA-insoluble material in 1 ml of cytoplasmic extract was collected by centrifugation at 5000 g for 15 min. The pellet was washed twice with 2 ml of 0.2 N perchloric acid (PCA), the excess acid was drained by blotting, and the RNA was hydrolyzed with 1 ml of 0.3 N KOH for 1 hr at 37°C. The reaction mixture was cooled for 10 min in an ice bath and then the unhydrolyzed material was precipitated with 0.5 ml 1.2 N PCA. The acid-soluble RNA hydrolysate was collected after centrifugation. The pellet was washed three times with 0.2 N PCA, the washings were combined with the original supernatant, and the OD was read at 260 nm. Yeast RNA treated in the same way was used as a standard.

RESULTS

Morphology and Kinetics of Excystment

The mature (resting) cyst (Fig. 1 a) has been described previously (Volkansky, 1931; Neff, 1957; Page, 1967; Bowers and Korn, 1969). In brief, it is spherical with a double wall, an outer exocyst that is continuous over the entire surface of the cyst, and an inner endocyst that is discontinuous in an area referred to as the ostiole. A layer of refractile granules is embedded in the outer margin of the cytoplasm.

In time-lapse, phase-contrast photographic studies of excystment, it has been observed that cytoplasmic movement is enhanced soon after the transfer of cysts from EM to OGM. In less than 7 min, dark refractile globules appear in the middle of the ameba (Fig. 1 b), move toward the plasma membrane, apparently coalesce with the smaller lipid granules there, and move back toward the center. After half an hour, cytoplasmic movement becomes more active and the peripheral layer of lipid granules completely disappears (Fig. 1 c). Then, a contractile vacuole becomes prominent in the center of the ameba (Fig. 1 d) and increases in size. The large vacuole moves toward the cyst wall (Fig. 1 e, f) where it seems to discharge its contents between the endocyst and the plasma membrane. The ameba then begins pulling away from the endocyst (Fig. 1 f, g). After 12–36 hr, the organism moves freely within the cyst wall and the emergence of a cytoplasmic bud is observed (Fig. 1 g). The emerging ameba attaches to any available surface and passes completely out of the cyst wall in 1–3 min (Fig. 1 h, i, j, k).

On the basis of the observed morphological changes, the process of excystment has been divided into four stages (Fig. 2): (a) the mature cyst stage, a stable form that only begins to undergo changes resulting in excystment after it has been exposed to optimal growth conditions; (b) the activated cyst stage, a form first recognized by the disruption of the peripheral lipid granules and ending with the free movement of the ameba inside the cyst wall; (c) the preemergence stage, characterized by complete detachment of the ameba from the cyst wall and ending when a cytoplasmic bud penetrates the wall; (d) complete emergence, begun when the ameba passes out through the cyst wall and resulting in a free trophozoite plus an empty cyst wall.

Excystment was also studied by light microscopy of cysts stained with iodine-eosine. The eosine stains the cyst wall pink while the iodine stains the ameba yellowish brown in the mature cysts and reddish brown in the activated cysts. The color change from yellowish to reddish brown is easily recognized and, thus, provides a relatively simple way of distinguishing the activated and mature cyst stages. The preemergent ameba stains like the activated cyst, but has a space between its plasma membrane and the inner cyst wall as in Fig. 1 g. The pink empty cyst wall can easily be identified by the absence of the ameba.

The kinetics of excystment were studied with the aid of the iodine-eosine staining procedure. In a typical experiment, it was observed that the total number of cyst walls remained constant for up to 75 hr after the cysts were placed in OGM (Fig. 3). The same constancy was observed in five other cases in which it was even more convincing than in the example illustrated. This

indicates that the empty cyst walls do not disintegrate during that period. Thus, the rate at which amebas emerge from cysts can be represented by the increase in numbers of empty cyst walls (Fig. 4 c). In fact, this is the only way to score emergence, since the amebas readily multiply after they leave the cysts walls (Fig. 3) and, thus, cannot themselves be used to quantitate emergence. In a typical experiment, there is a small initial burst of emergence during the first 10–15 hr after the cysts are placed in OGM and then a linear rate with a slope of about 5% per 10 hr. In Fig. 4 the activation of encysted amebas is illustrated by the disappearance of mature cysts (4 a), the subsequent appearance of activated cysts, and the latter's conversion to preemergent forms (4 b). Although only about 40% of the encysted amebas emerge within 70 hr (Fig. 4 c), it appears that at least 90% of the mature cysts become activated (Fig. 4 a). The disappearance of mature cysts is accompanied by the appearance of a peak in the activated cyst population (Fig. 4 b). The level of the latter begins to drop and a peak is then seen in the population of preemergent cysts. Finally, these forms begin to disappear as trophozoites continue to emerge.

The kinetics of excystment are affected by pH and temperature. The optimal pH for activation of excystment is 7 (Fig. 5) and the optimal temperature is 30°C (Fig. 6). In both cases, maximal initial rates and final extents of activation were achieved under the optimal conditions.

Excystment Activity in the Presence of Inhibitors of RNA, DNA, and Protein Syntheses

Since V. Rudick (1971) has demonstrated that both DNA and RNA syntheses are repressed in aging cultures of *Acanthamoeba* and, in confirming

the work of Neff and Neff (1969), that extensive encystment can occur when nucleic acid synthesis is inhibited, an attempt has been made in these studies to determine whether the synthesis of any of these macromolecules is necessary for excystment.

As illustrated in Fig. 7 a, cycloheximide effectively eliminates all incorporation of proline-^{14}C into protein when an attempt is made to induce excystment in OGM. The incorporation observed in the control cultures is probably mostly due to the presence of actively multiplying trophozoites, since, as already noted (legend, Fig. 3), the cyst preparations were always initially contaminated with 1–5% trophozoites, and the latter, as well as newly emerged amebas, actively multiply in the OGM (Fig. 3). However, analysis of the cysts revealed that the inhibitor severely repressed activation (Fig. 7 b) and eliminated emergence (Fig. 7 c). Likewise, there was no multiplication of the trophozoites that contaminated the preparation (Fig. 10 a). At 48 hr, samples of cysts were washed free of the inhibitor and were found to be still capable of excystment in plain OGM.

Although hydroxyurea inhibits DNA synthesis during the induction of excystment (Fig. 8 a), the pattern of activation in the presence of the inhibitor is indistinguisable from that in the control (Fig. 8 b). Emergence (Fig. 8 c) is only slightly reduced. As illustrated in Fig. 10 a, b, the number of trophozoites produced in the culture exceeds the number expected on the basis of the empty cyst walls produced and, therefore, there appears to be some multiplication in the presence of inhibitor. Thymidine incorporation in the control cultures (Fig. 8 a) characteristically shows a lag phase in contrast to the uptake patterns observed for proline (Fig. 7 a) and uridine (Fig. 9 a).

Actinomycin D effectively blocks RNA synthesis (Fig. 9 a) and seems to affect excystment in a manner similar to cycloheximide. That is, it

Figure 1   Photographs of excysting amebas. Photomicrographs a, b, and c were taken with a still camera, and pictures d to k are reprints from time-lapse film. (a) Mature cyst; arrow indicates intact layer of refractile granules located in the peripheral region of the ameba. The cyst wall is observed external to this layer. (b and c) Newly activated cysts; arrows indicate large internal refractile granules. Note the disruption (b) and subsequent disappearance (c) of the peripheral refractile granule layer. (d to f) A sequence showing a cyst in the process of terminating the activation stage and entering the preemergence stage; arrows indicate places where the cytoplasm is beginning to pull away from the cyst wall. A large vacuole (v) is seen to move from the center of the cell to a position adjacent to the cyst wall. (g) Cyst in preemergence stage; arrow indicates cytoplasmic bud beginning to penetrate the cyst wall. (h to k) A sequence showing the process of emergence. × 1100.
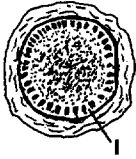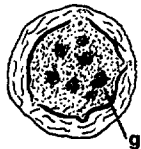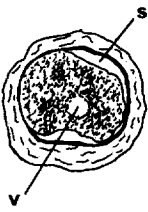
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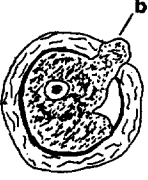
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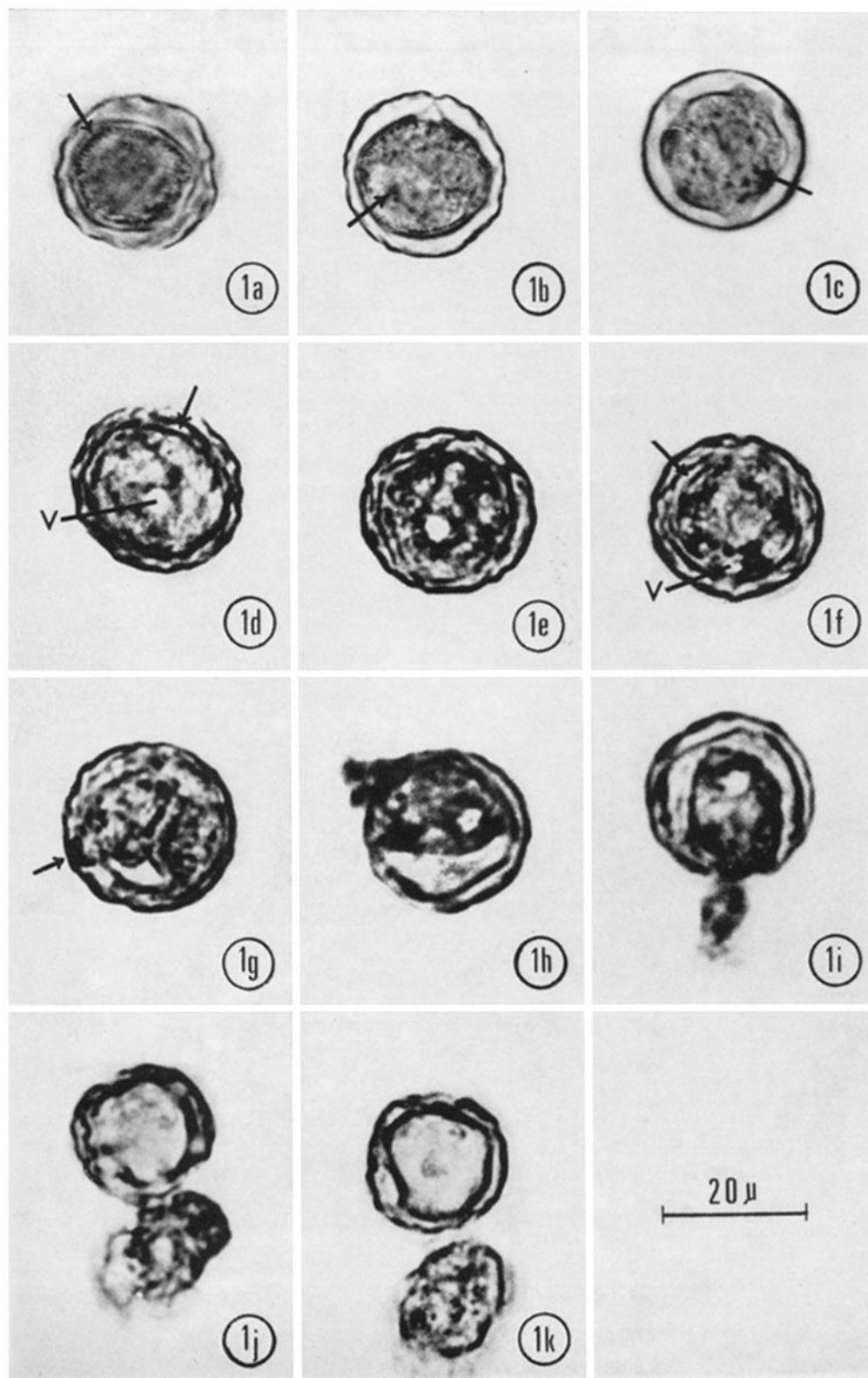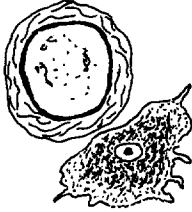
Cyst stage	Morphological description	Staining reaction	Duration of stage
Mature cyst stage	Spherical with a layer of lipid granules around the outer margin of the ameba (l)	Pink cyst wall and yellowish brown ameba	Indefinite under appropriate conditions in EM
Activated cyst stage	The granular layer disappears and larger globules (g) appear in the center of the ameba	Pink cyst wall and reddish brown ameba	0.5–4 hr after transfer of cysts to OGM
Early pre-emergence (P.E.) stage	The ameba detaches from the inner cyst wall forming a space (s) and a large central vacuole (v)	As above	4–8 hr
Late P.E. stage	The detachment of the ameba is more evident and a cytoplasmic bud (b) starts to penetrate the cyst wall	As above	8–24 hr
Complete emergence	A trophozoite emerges leaving behind an empty cyst wall	As above	3 min for emergence

FIGURE 2 The stages of excystment.

partially represses activation (Fig. 9 b), but completely inhibits emergence (Fig. 9 c). There is some increase observed in the trophozoite numbers in the absence of emergence (Fig. 10 a).

In Vitro Protein Synthesis

The inhibition of excystment by cycloheximide indicates that protein-synthesizing machinery is available in cysts even though its activity is prob- ably repressed. The inhibitory effects of actinomycin further suggest that some of this machinery is made by the cyst itself. In order to further examine the nature of this synthetic capability, a cell-free protein-synthesizing system has been used. With this system, the biosynthetic activities of cyst and trophozoite cytoplasmic extracts have been compared.

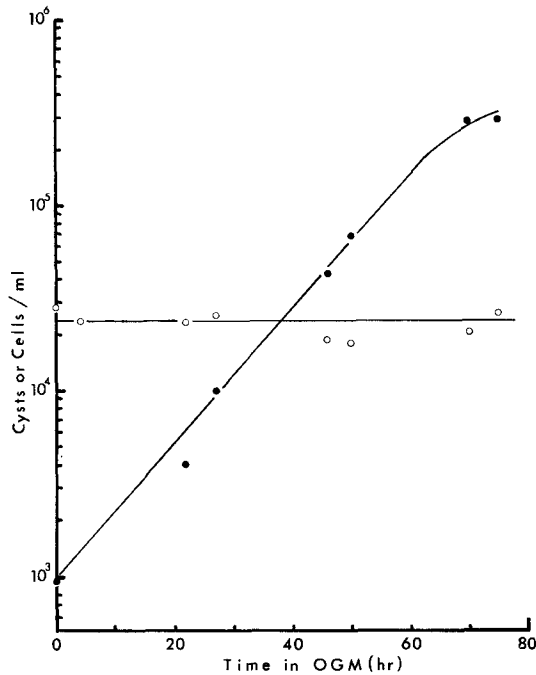The system was first studied with the more readily obtained extracts from free amebas. When

FIGURE 3 Concentrations of free trophozoites and of total cysts plus empty walls in a typical excystment experiment. A cyst preparation was used to inoculate OGM which was then distributed in 3-ml amounts to screw-cap tubes, incubated at 30°C, and periodically sampled. Each point on the graph represents an average count for three tubes. The initial preparation contained about 96% cysts and 4% trophozoites. The cysts in this initial population were catagorized as: 72% mature, 14.1% activated, 1.4% preemergent, and 11.5% empty walls. Open circles, cysts; closed circles, trophozoites.

the complete system of Chesters (1968) was compared with one lacking nucleotide triphosphates (ATP, CTP, and GTP) and an ATP-generating system (creatine phosphate plus creatine phosphokinase), the former system was found to be only slightly better (Fig. 11). Although the amount of leucine incorporated seems to be a linear function of concentration up to the maximum level used (Fig. 12), the upper limit used was chosen to avoid solubility problems with the amino acids in stock solutions.

A comparison of the incorporation of leucine-^{3}H into hot TCA-insoluble protein by cyst and trophozoite cytoplasmic extracts is illustrated in Fig. 13. It appears that cyst cytoplasm is only 7–10% as effective as trophozoite cytoplasm in promoting protein synthesis.
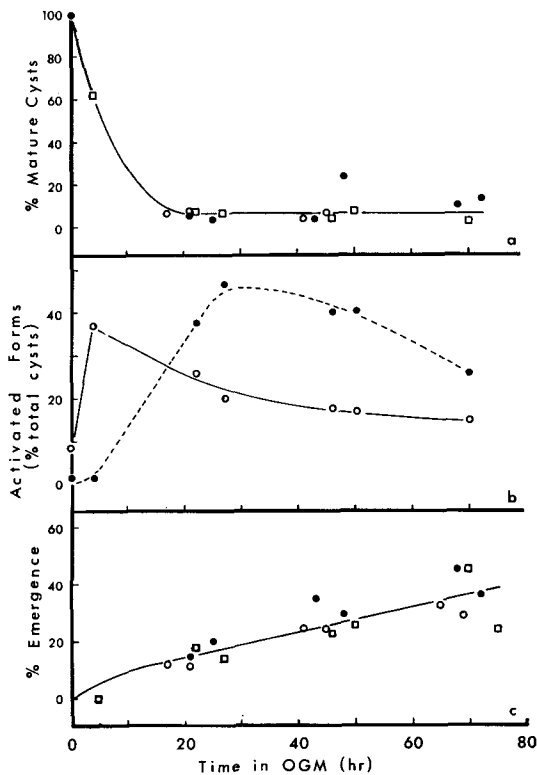
An effort was made to determine whether the

FIGURE 4 Kinetics of excystment. The data are from experiments performed as in Fig. 3 with similar initial cyst preparations. (a) Per cent of mature cysts: based on number of mature forms in initial population. Three experiments are represented. (b) Activated forms: a typical experiment illustrating the per cents of the total number of cyst walls (mature cysts [M] + activated cysts [A] + preemergent cysts [P] + empty walls [EW]) that were categorized as activated (open circles) or preemergent (closed circles) cysts. (c) Per cent of emergence: data from three experiments in which emergence is determined from the appearance of newly formed empty cyst walls. Per cent of emergence equals $100(EW_t - EW_0)/(M_0 + A_0 + P_0)$ where 0 is zero time and t is any subsequent time.

RNA active in supporting protein synthesis in cyst cytoplasm is synthesized by the cyst or before cyst formation. Post–log phase amebas were induced to encyst in EM with or without actinomycin. When encystment was complete, cytoplasmic extracts from both control and treated cysts were tested for their ability to support in vitro protein synthesis. Fig. 13 shows that extracts from treated cysts are capable of sustaining protein synthesis and, thus, the responsible RNA must be synthesized before the induction of encystment.
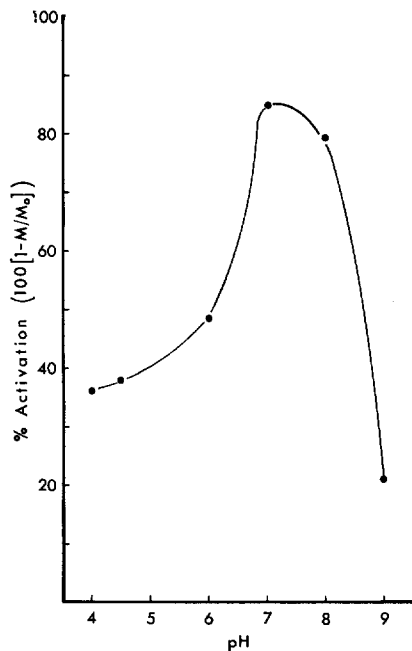
FIGURE 5 Optimum pH for activation of excystment. Cysts were incubated as described in text and per cent of activation was determined from the fractions of the initial mature cyst populations that were converted to activated forms in 24 hr. The initial cyst populations contained an average of 89.6% mature forms. The same cyst preparation was used in all cases, but on different days. M_0 equals the number of mature cysts at time zero and M equals the number after 24 hr.
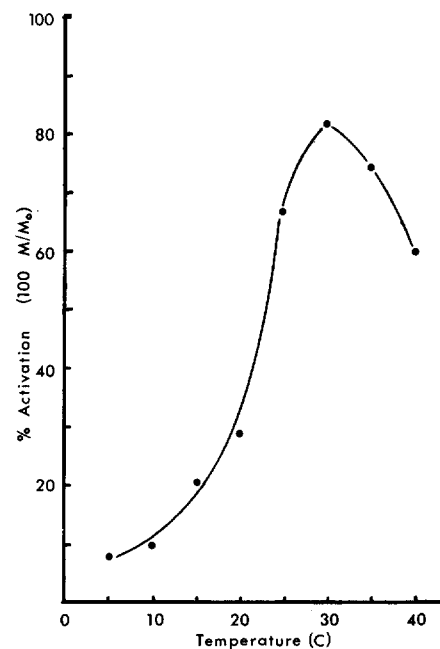

FIGURE 6 Optimum temperature for activation of excystment. Cysts were incubated as described in text and per cent of activation determined as in Fig. 5. The initial cyst populations averaged 77.7% mature forms.

DISCUSSION

Most methods for quantitating encystment and excystment in *Acanthamoeba* depend on microscopic methods and are tedious. In an effort to avoid this problem, Griffiths and Hughes (1968) have introduced an optical assay for cellulose as an indicator of encystment. Unfortunately, such an assay could not be used in these experiments because empty cyst walls remained in the cultures long after excystment had occurred. Consequently, the latter process still has to be followed microscopically. The iodine-eosine technique used in these studies is similar to the method introduced by Kessel (1925) for *Hartmannella hyalina* and has the advantage that it permits a distinction to be made between mature (resting) cysts and activated cysts that have initiated the events leading to emergence of the ameba. The basis of the color change observed upon activation is unknown, but the kinetic data (Fig. 4) indicate that, once the

change has occurred, the cysts progress toward completion of excystment. Further evidence to support this point is found in the observation that cycloheximide inhibits both the color change and excystment. The main disadvantage of the stain is that it seems to work best on fresh unfixed material, and, therefore, quantitation must occur at the same time that samples are taken.

The activation of mature cysts is optimum at 30°C. Unlike the situation with many bacterial and fungal spores, for which the temperature optima for germination are very different from those of vegetative growth (Sussman and Halvor son, 1966), the temperature optimum for activation of excystment is similar to that for growth of trophic forms of *Acanthamoeba*. Thus, a temperature change is not likely to be a primary trigger for excystment of this organism.

The optimum pH for excystment is 7 and lies within the range optimal for cell multiplication in OGM. It is often observed that encystment is accompanied by or induced at high pH's (Neff et al., 1964; Byers, 1970, unpublished results) and, therefore, although there is no real evidence
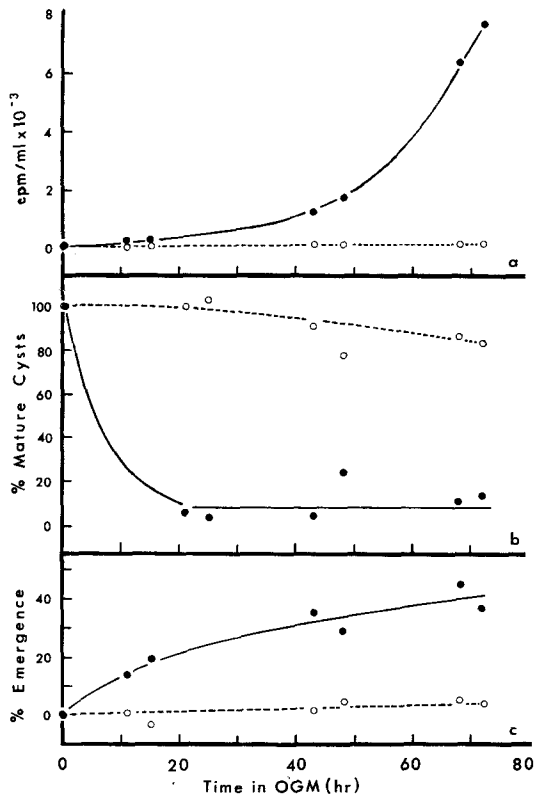
FIGURE 7 The influence of cycloheximide on excystment. Cysts were incubated and sampled as in Fig. 3. Control cultures (closed circles) contained plain OGM. Experimental cultures (open circles) contained OGM plus 50 μg/ml cycloheximide. (a) cpm proline-^{14}C incorporated into TCA-insoluble material per milliliter of culture. The label was continuously present. (b) Per cent of mature cysts remaining in the population. (c) Per cent of emergence.
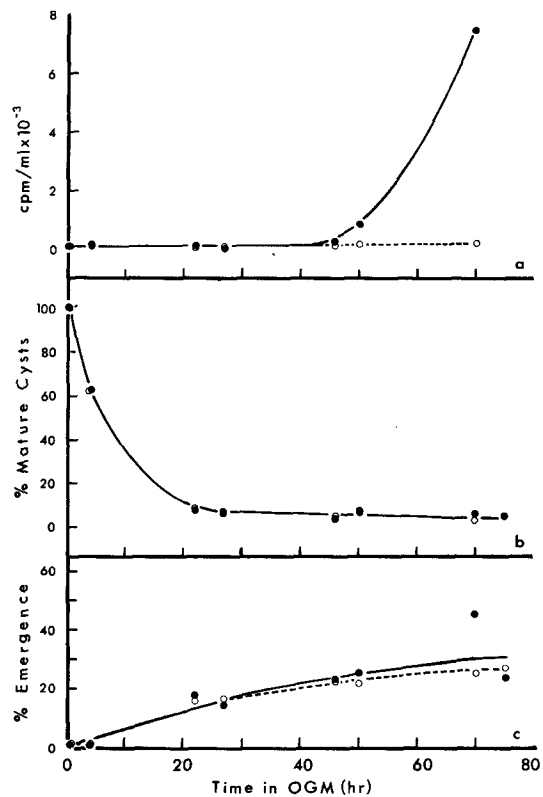
FIGURE 8 The influence of hydroxyurea on excystment. Cysts were incubated and sampled as in Fig. 3. Closed circles, control cultures; open circles, experimental cultures containing 30 mm hydroxyurea. (a) cpm thymidine-^{3}H incorporated into TCA-insoluble material per milliliter of culture. The label was continuously present. (b) Per cent of mature cysts remaining in the population. (c) Per cent of emergence.

for this point, it is possible that a pH drop is part of the stimulus for activation.

The fact that activation is induced by transferring cysts from a nonnutrient medium to a nutrient-rich medium suggests that nutrients are a major component of the inducing mechanism. In support of this, we have found that some degree of excystment can be achieved in EM simply by the addition of amino acids. However, we are now able to induce high levels of encystment in fresh OGM where nutrients are plentiful, and, therefore, it seems unlikely that nutrient deficiencies are the only factors that can maintain the resting cyst state.

As already pointed out, there is evidence that RNA and DNA syntheses are inhibited during

late logarithmic growth in cultures of trophozoites (Mattar, 1970; V. Rudick, 1971). The resultant depression in synthetic activity occurs before the time at which an increase in the proportion of cysts is typically observed in aging cultures (Byers et al., 1969). Furthermore, as also mentioned previously, Neff and Neff (1969) and V. Rudick (1971) have established that cysts can be induced to form in the presence of inhibitors of nucleic acid synthesis, although the latter author has shown that this is only true for RNA after this macromolecule has undergone an accumulation in late log- and early postlog population growth. Neff et al. (1958) have observed that respiration rates are minimal in cysts. Thus, there are indications that the mature cyst is metabolically relatively
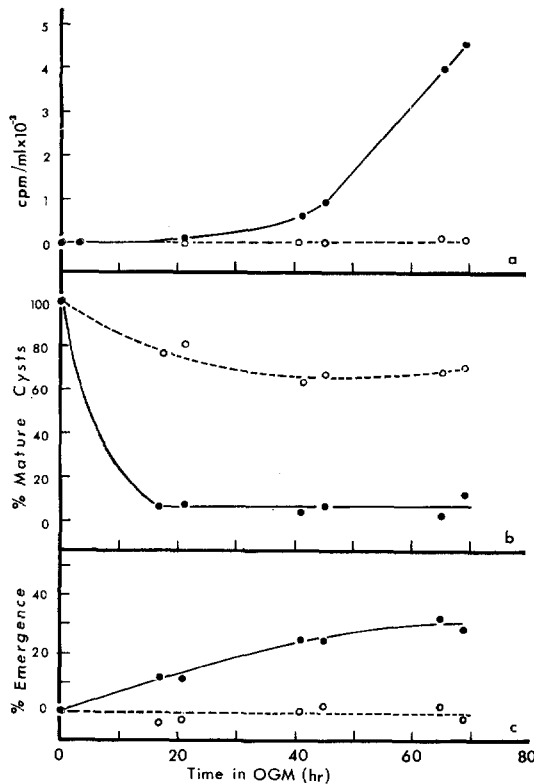
FIGURE 9 The influence of actinomycin D on excystment. Cysts were incubated and sampled as in Fig. 3. Closed circles, control cultures; open circles, experimental cultures containing 15 µg/ml actinomycin D. (a) cpm uridine-³H incorporated into TCA-insoluble material per milliliter of culture. The label was continuously present. (b) Per cent of mature cysts remaining in the population. (c) Per cent of emergence.
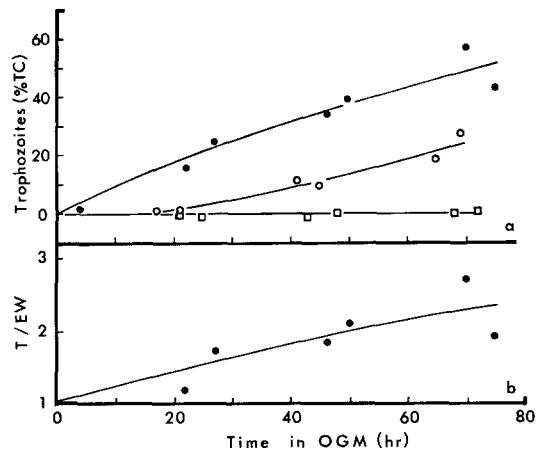

FIGURE 10 The influence of inhibitors on the increase in numbers of trophozoites in cultures of excysting amebas. (a) Increases in the numbers of trophozoites, expressed as per cent of total cyst walls in the population, in the presence of cycloheximide (open squares), actinomycin D (closed circles), and hydroxyurea (open circles). Data are from the same experiments as in Figs. 7-9. On the same scale, control cultures show an increase on the order of 1000% (see Fig. 3). (b) Ratio of trophozoites to newly formed empty cyst walls in the presence of hydroxyurea. T equals the number of trophozoites and EW equals empty cyst walls.

inactive. Our experiments with cell-free protein synthesis are consistent with this, but point out that the cyst does retain the capability for some protein synthesis and that the required RNA must be made before encystment (Fig. 13) since proline incorporation is still promoted by RNA from cysts formed in the presence of actinomycin D. The inefficiency of the cyst cell-free protein-synthesizing system compared to the trophozoite system suggests the possibility that encystment results in a defect or deficiency in one of the enzyme or RNA components, and we are currently investigating this.

The greater efficiency of the RNA extracted from cysts prepared in the presence of actinomycin D may be explained in several ways (Fig. 13). One of the more appealing is that the stable

RNA fraction becomes enriched during encystment in the presence of the drug because the bulk of the RNA is being degraded (see Neff and Neff, 1969) and the reutilization of the degradation products for turnover synthesis of the unstable fraction is prevented. Mattar (1970) has previously reported evidence for turnover.

Although the evidence mentioned above indicates that some cyst RNA may be made before encystment, it is clear that actinomycin D inhibits complete excystment. Only about 35-40% of the control level of activation can occur in the presence of the drug, and the emergence of amebas is prevented (Fig. 9 c). Since the cyst populations at the start of the inhibition experiments contained cells in all stages of excystment (legend, Fig. 3), it appears that both early and late stages are actinomycin sensitive and probably depend on RNA synthesis. Inhibition with cycloheximide produces similar results and, thus, it appears that protein synthesis is also required for early and late stages of excystment. As might be expected, this drug completely prevents the multiplication of the trophozoites that initially contaminate the cyst
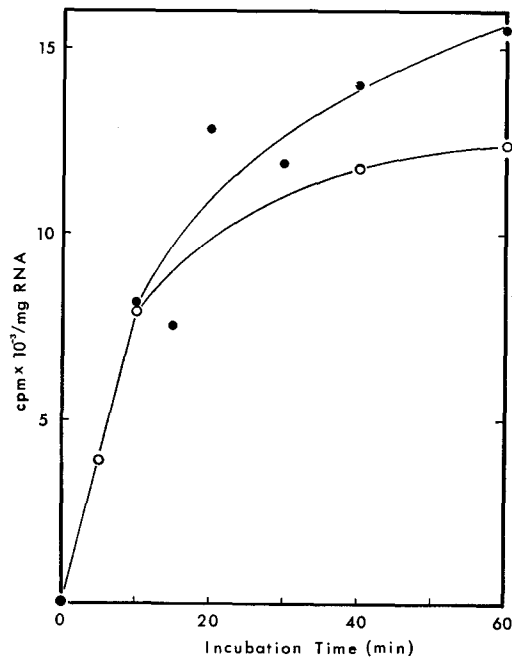
FIGURE 11 Effect of GTP, ATP, CTP, plus an ATP-generating system on protein synthesis by a high molecular weight trophozoite cytoplasmic extract. Leucine-^{3}H incorporation was studied in a complete reaction mixture (closed circles) containing the following components in a total volume of 1 ml: soluble trophozoite cytoplasmic components above mol wt 3600, 1 μmole each of 17 amino acids (see Materials and Methods), 1 μmole (10 μCi) leucine-^{3}H, 1 μmole ATP, 0.25 μmole GTP, 0.25 μmole CTP, 5 μmoles creatine phosphate, and 60 μg creatine phosphokinase. Incorporation was also studied in an incomplete system (open circles) lacking nucleotide triphosphates, creatine phosphate, and creatine phosphokinase. In both cases, all reagents were in buffer A (see text) and the incubation temperature was 32°C. Each point represents the counts in 0.1 ml of reaction mixture. Counts are corrected for background due to nonspecific adherence of the label to filters.
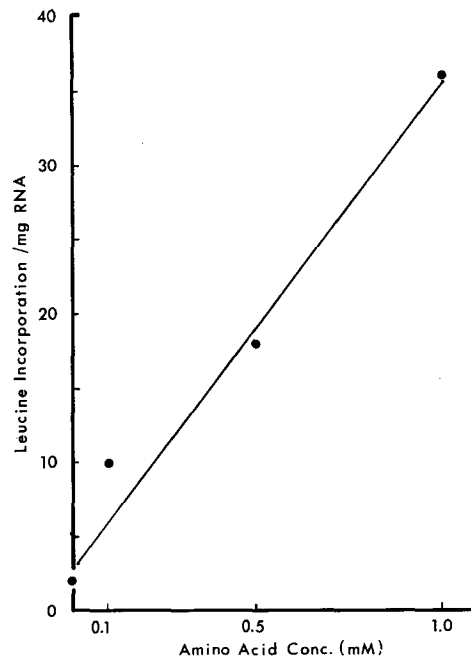
FIGURE 12 The relationship between amino acid concentration and leucine incorporation in a trophozoite cell-free protein-synthesizing system. The incorporation of leucine-^{3}H into protein in a complete system as described in Fig. 11 was studied in mixtures in which each amino acid had a concentration of 0, 0.1, 0.5, or 1.0 mM. Actually the leucine concentration in the absence of other amino acids was 2 μM. Leucine incorporation is expressed as cpm $\times$ 10^{-3} for a constant specific activity of 0.2 Ci/mmole and an incubation time of 60 min.

preparation (Fig. 10 a), but, unexpectedly, actinomycin does not seem to have this effect (Fig. 10 a). In fact, if the increase in trophozoites is plotted in the same manner as the data in Fig. 3, it can be concluded that all the trophozoites present at the end of the experiment arose from those present initially and that the latter multiplied with a generation time of 7–8 hr for about 40 hr before multiplication was inhibited by the actinomycin. The reason for the insensitivity of the trophozoites to the drug while the cysts were sensitive is obscure,

but the fact that the former survived the deoxycholate treatment used in preparing the cysts suggests that they represent an unusually resistant fraction of a typical trophozoite population. Possibly this resistance is mediated by the plasma membrane.

V. Rudick (1971) has presented evidence that DNA synthesis is inhibited in G_1, the prereplication phase, during preparation for encystment. If this is true, then any amebas emerging from cysts in the presence of an inhibitor of DNA synthesis should be unable to multiply. In the present experiments, hydroxyurea was used as the inhibitor and was found to have no influence on cyst activation and little if any influence on emergence (Fig. 8 b, c). However, approximately twice as many amebas were found in the cultures at the end of the experiment as would be expected on the basis of the number of empty cyst walls that appeared
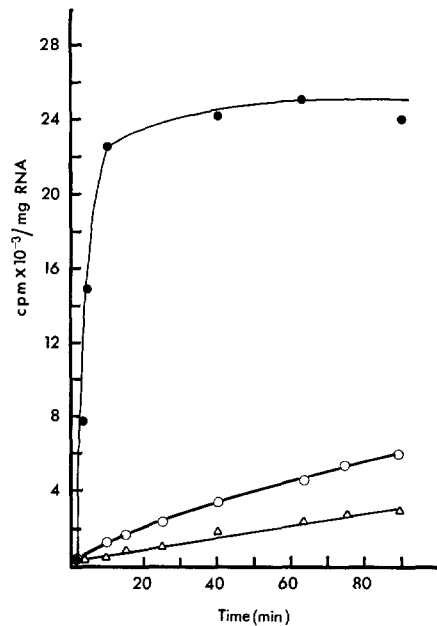
FIGURE 13 Leucine-^{3}H incorporation by cell-free extracts of cysts and trophozoites. The assay system is the same as the complete system described in Fig. 11, except that the final concentration of leucine-^{3}H was 0.05 μM. Closed circles, trophozoite cytoplasmic extract; open circles, cytoplasmic extract from standard cyst preparation (see Materials and Methods); open triangles, cytoplasmic extract from cysts induced to form in the presence of 10 μg/ml actinomycin D.

(Fig. 10 b). Although this observation might be explained as due to typical cell multiplication, there is a more likely explanation. The trophozites that were originally used to induce encystment were grown in an aspirator flask under conditions of agitation that would ordinarily result in a population with an average of 1.7–1.8 nuclei per ameba (James and Byers, 1967; M. Rudick, 1970). Our experience indicates that the multinucleates encyst as such, and, in confirmation of this, binucleated amebas were seen in some of the cysts in the present experiment. In contrast, all of the trophozoites seen after excystment in hydroxyurea were mononucleates. Therefore, it is probable that the doubling of trophozoite number was due to plasmotomy (James and Byers, 1967) either in the cyst or after emergence, and the data are still consistent with the proposal that encysted cells are blocked in G_1 or at the G_1-S transition point.

The lag observed in the incorporation of thymidine-^{3}H during excystment in control cultures

(Fig. 8 a) is especially interesting because it is reproducible and because it continues after amebas have emerged and undergone up to five replications. Thus, either the newly emerged amebas cannot utilize exogenous thymidine for DNA synthesis during the lag, or they contain enough DNA to permit several cell divisions in the absence of DNA synthesis. The evidence that hydroxyurea blocks cell multiplication, except for plasmotomy, during excystment implies that DNA synthesis is required for cell multiplication during the period when the lag in thymidine uptake is observed in the control cultures. Thus, the most likely explanation of the lag is that there is a delay in the ability of the trophozoites in these cultures to utilize exogenous thymidine.

In summary, the evidence suggests that RNA and protein syntheses are both needed for activation and emergence, although activation is more dependent on protein synthesis. The cell-free studies give evidence for a stable messenger RNA that is synthesized before encystment and retained by the cysts. Although the cyst can synthesize protein, its machinery for doing this is inefficient compared to that of the trophozoite. Our data are consistent with the hypothesis that amebas are blocked in G_1 during encystment, and indicate that DNA synthesis does not have to be resumed during excystment until after emergence. The lag in thymidine incorporation during excystment raises the interesting possibility that enzymes required for the utilization of exogenous thymidine are destroyed during encystment and that a considerable period of time is required for their reappearance. The fact that cyst walls are not completely degraded during excystment lends support to the idea that emergence is through a specialized region of the exocyst, the ostiole, and suggests that this region may be sensitive to an enzyme that cannot attack the rest of the wall.

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