

LOCALIZATION OF CYTOPLASMIC NUCLEIC ACID DURING GROWTH AND ENCYSTMENT OF *ENTAMOEBIA INVADENS*

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ABSTRACT

With a modified color-translating ultraviolet microscope, the distribution of material showing an absorption maximum at $265\text{ m}\mu$ was studied in samples from whole cultures of *Entamoeba invadens* at intervals during growth and from cysts allowed to mature under controlled conditions. Absorption by the cytoplasm in general gradually increased as trophozoites approached the period of maximum encystment. In late trophozoites and precystic forms, the absorbing material was concentrated into small bodies which coalesced to form large crystalloids of very high specific absorption. Maximum crystallization occurred in early cysts, where cytochemical tests have shown the large crystalloids to be ribonucleoprotein. Electron micrographs show that the crystalloids are composed of particles 200 to 300 A in diameter. During cyst maturation the amount of absorbing material per cyst is not visibly reduced, but the large bodies fragment into smaller units until finally there is only a very high diffuse absorption over the entire cyst. From these and other results the hypothesis is advanced that the large crystalloids ("chromatoid bodies") are a manifestation of a special parasite-host adaptive mechanism; ribonucleoprotein is synthesized under favorable conditions, crystallized in the resistant cyst stage, and dispersed in the newly excysted amebae thereby enabling them to establish themselves in a new host by a period of quick growth.

INTRODUCTION

Since the completion of a study of the cyclic changes in the chromatoid bodies of *Entamoeba invadens*, using classical cytochemical techniques, electron microscopy, and biochemical estimations of the total RNA in cells (3, 5), a number of reasons for reopening this investigation have materialized.

The recent work by Balamuth (2) on the factors affecting growth and encystment of *E. invadens* has now made possible cultures in which we can predict, with some certainty, the growth stage of the majority of cells in any 24-hour period.

In previous studies, our only control in the polyxenic cultures stemmed from MacConnachie's (25) correlation of mass encystment with the depletion of starch. It had been possible to obtain 84 per cent encystment in some cultures, but the response was varied and controlled conditions were never satisfactorily achieved. Balamuth (2) concluded that the concentration of rice starch in the medium was inversely related to the onset of encystation and that encystation also varied inversely with the bottom surface area of culture

tubes. The availability of these more reliable and standardized cultures referred to above made repetition of the previous experiments desirable.

Dissatisfaction with and criticism of the RNA estimations obtained in the previous studies, together with the uncertainty that events measured by the use of whole populations truly reflected the events in a single cell, led to a search for other methods of measuring changes in nucleic acid in living single cells.

The convincing demonstrations by Svihla and co-workers (26-28) that the ultraviolet microscope can be of considerable use in locating and following the fate of ultraviolet-absorbing substances in living cells provided impetus for the present study. The ability to measure ribonucleoprotein in the chromatoid bodies of living cells has enabled us to draw a much more precise picture of the changes in the concentration of RNA in the cells than has hitherto been possible.

MATERIALS AND METHODS

Three strains of *Entamoeba invadens* were used. Strain IP(B) grown monoexenically with *Clostridium perfringens* was kindly provided by Professor W. Balamuth, University of California, Berkeley. This strain was subcultured in: (a) aqueous egg-yolk infusion medium modified from Balamuth's medium (1); (b) Balamuth's liver-egg yolk medium; or (c) modified Jones' medium (22). IP-1 and PZ polyxenic cultures with undetermined bacterial flora were supplied by E. Meerovitch, MacDonald College, McGill University, Canada, and grown in Balamuth's egg-yolk infusion medium and Jones' medium.

Standardized, reproducible encystation responses were obtained with monoxenic cultures by adding 3 mg of rice starch to 15 ml of Balamuth's medium in 150 x 16 mm sterile plastic culture tubes. These conditions were essentially those specified by Balamuth (1, 2). Cultures were grown at $30^{\circ} \pm 1^{\circ}\text{C}$.

Random samples were taken from duplicate cultures at 24-hour intervals during the growth period. For studies of cyst maturation, cultures were treated with N/10 HCl for 1 hour to eliminate trophozoites, and the remaining cysts were placed in exhausted medium. Mature cysts were transferred to fresh medium for excystation studies. Viability of the late cysts was ascertained at the end of each experiment by subculturing. In certain cases the whole process of encystation and maturation of cysts was observed in moist chambers made by sealing Vycor covers to Vycor slides with paraffin oil or melted paraffin.

All samples were examined by phase contrast and by ultraviolet microscopy. In addition, early cysts

were fixed in buffered osmium tetroxide, embedded in methacrylate, Araldite, or Epon, sectioned, and examined by electron microscopy.

The instrument used for ultraviolet microscopy in these investigations was a modified color-translating ultraviolet microscope Model UV-91 built by Scientific Specialties Corporation of Boston (23, 24, 29). Its important characteristic is rapid access to projected images of photographic negatives obtained automatically at three selected wavelengths.

Choice of wavelengths was dictated by the specific absorptions of the material studied and the characteristic emissions (line and continuum) of the high pressure, water-cooled mercury vapor source. Since the UV-91 has means for controlling the source voltage and emitted energy, it was easy to adjust the voltage to secure maximum line emission (about 550 v). By setting both the exit and entrance slits of the grating monochromator to the same value, and keeping the exit slit smaller than the width of the selected spectral line, problems of spectral purity and calibration were minimized. Band width was less than $4\text{ m}\mu$.

A variety of optics was used, but the most useful combination was a 100 X, na 1.25, glycerin immersion Ultrafluar objective (Zeiss) used in conjunction with its companion condenser and projective. Our experience with ultraviolet optics has been similar to that reported by Zworykin and Berkley (31).

Observation and focusing had been carried out with green light (near $546\text{ m}\mu$) but it was found to be a great advantage to observe and focus by means of an RCA 7404 image-converter tube in a suitable housing, utilizing the weak $275\text{ m}\mu$ line. The absorption at $275\text{ m}\mu$ was sufficient for observations and less damaging to the specimen than $265\text{ m}\mu$, near the nucleic acid absorption peak. To avoid ultraviolet irradiation effects, short, overlapping periods of development were followed, with new fields being selected when necessary.

Automatically controlled exposures were made on 35 mm Kodak Spectrum Analysis Film No. 1, usually at 295, 275, and $265\text{ m}\mu$. The film was automatically processed in the instrument at 90°F in Kodak D-8 developer diluted 2:1 (plus paraformaldehyde as hardener) for 7 seconds, fixed with Kodak x-ray fixer, washed with distilled water, and dried with the aid of 95 per cent ethyl alcohol. Processing was complete in 30 seconds.

Exposures were adjusted to give a background optical density of about 2 units. While the quality of the processed image was not completely satisfactory, rapid access did permit corrections of ultraviolet focusing errors, which still occurred in spite of the image-converter tube, and tracking of a moving specimen. Since exposures at $265\text{ m}\mu$ were of about 5 seconds' duration, it was difficult to obtain pictures of some stages. The use of the image-converter tube per-

mitted visual observations of rapidly occurring events in the living organism.

RESULTS

The constant encystation responses obtained with the monobacterial combination of *E. invadens* and *C. perfringens* in liquid medium made it unnecessary to maintain diphasic cultures. Growth curves and

90 per cent cysts in 4 days, can be attributed to our use of all liquid medium.

The chromatoid bodies have been described by Dobell (12) as "of variable shape, but usually seen as rods or thick bars of substantial mass which stain deeply with the chromatin stains and are found in the cytoplasm of a variable proportion of the cysts of *Entamoeba histolytica* and certain other *Entamoeba*."

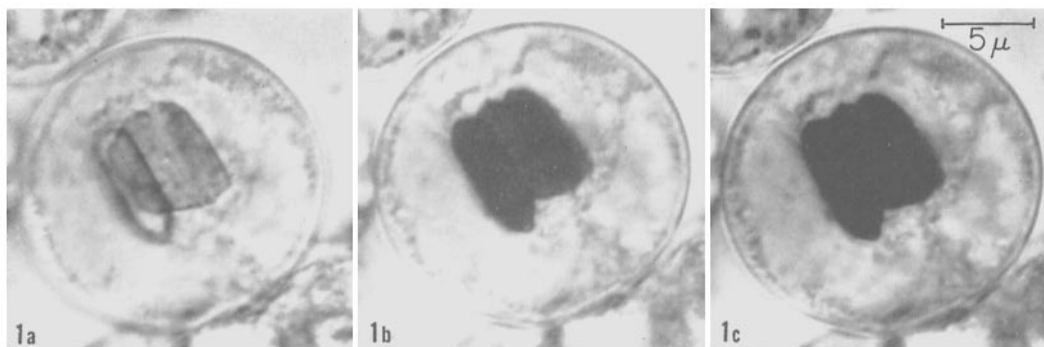


FIGURE 1 Early cyst stage of *E. invadens* photographed at wavelengths of 295, 275, and 265 $m\mu$ (a, b, and c, respectively), illustrating characteristic absorption by chromatoid body. $\times 2400$.

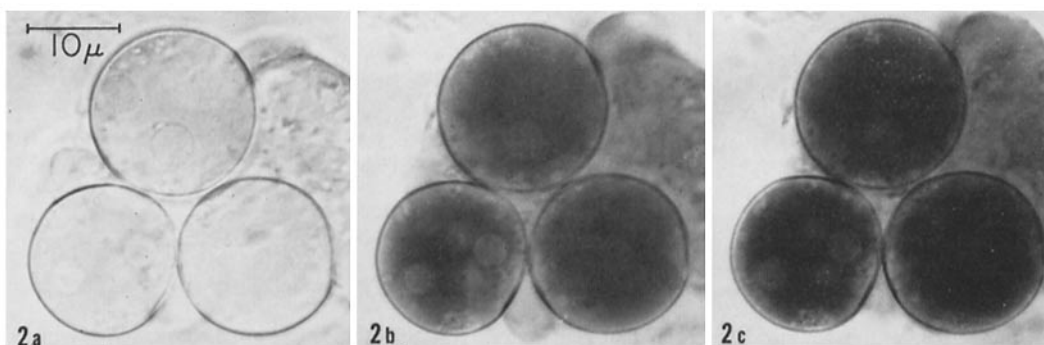


FIGURE 2 Late cysts and newly hatched trophozoite of *E. invadens* photographed at wavelengths of 295, 275, and 265 $m\mu$ (a, b, and c, respectively), showing cytoplasmic absorption. $\times 1200$.

percentage cyst measurements indicated that the maximum numbers of amebae were found on the 4th day and the percentage of cysts exceeded 90 per cent by the 6th day after inoculation into liquid medium. These results were obtained only with cultures at 30°C. Those grown at 25°C gave variable responses. The difference between our results in this respect and those of Balamuth (2), who obtained maximum numbers in 48 hours and

In the early cysts the ribonucleoprotein nature of the chromatoid body (3, 5, 7) gave it highly specific absorption characteristics. Fig. 1 shows absorption by the crystalloids at three wavelengths of ultraviolet light. Intense absorption by the bodies at 265 $m\mu$ and 275 $m\mu$ was apparent. At 295 $m\mu$ the bodies were almost non-absorbing. The cytoplasm of the early cysts absorbed very little at any of these wavelengths.

In contrast, the cytoplasm of late cysts and newly hatched trophozoites showed strong absorption at 265 $m\mu$ to 275 $m\mu$, but again there was very little absorption at 295 $m\mu$ (Fig. 2).

A comparison of the photographic densities (degrees of blackening) of the three images obtained at different wavelengths permits one to distinguish between specific and non-specific absorption. Similar densities of corresponding areas in each of the three images is a result of non-specific absorption or scatter. Dissimilar densities result from specific absorption plus a certain amount of scatter. In Fig. 1, the chromatoid body of the 265 $m\mu$ micrograph is black because of specific absorption, but note the edge effect of the crystal evident in the 295 $m\mu$ micrograph. It is likely that a small portion of the density in the 265 $m\mu$ micrograph in the corresponding area is also due to scatter. These factors must be considered in interpreting the images.

Although demarcations were not distinct, sometimes making it extremely difficult to classify a particular ameba, the life cycle of *E. invadens* was arbitrarily divided into 6 to 8 stages:

1. Newly-hatched amebae (premetacystic in Dobell's (13) terminology of *E. histolytica*).
2. Primary division amebae (metacystic), which were defined as all amebae resulting from the first divisions of the newly hatched amebae.
3. Young trophozoites, including the eight amebae which were the result of the final division of the primary division amebae.
4. Late trophozoites, which were found in cultures approaching mass encystment.
5. Precystic amebae, which were smaller than the late trophozoites.
6. Early cysts, which contained the classical rod- or bar-shaped chromatoid body, a single nucleus, and glycogen mass.
7. Maturing cysts, characterized by 2 to 4 nuclei and lesser amounts of glycogen.
8. Mature cysts, containing little or no glycogen and 4 nuclei.

Absorption Characteristics of the Life Cycle Stages

Young trophozoites were characterized by low specific absorption of the cytoplasm and were generally packed with starch (Fig. 3 *a*). They were very motile and consequently difficult to photograph. Late trophozoites revealed a general in-

crease in the total absorption of the cytoplasm but the absorption tended to be concentrated in a large number of small, intensely absorbing bodies (Fig. 3, *b* and *c*).

We have observed apparently different aspects of the late trophozoites and precystic amebae. In some it seemed that there was a differential division of the cytoplasm into two parts. One portion was clear, non-granular cytoplasm and contained large chromatoid bodies. The other was granular, vacuolated, and had the appearance of the cytoplasm of an actively feeding trophozoite. The clear area with chromatoid bodies (Fig. 3 *d*) separated from the trophozoite-like portion of the cytoplasm to become a cyst (Fig. 4 *d*). In other late trophozoites, as they passed through the precystic to early cyst stages, the smaller crystalloids apparently condensed or aggregated into large single or double crystalloids (Fig. 4, *a*, *b*, and *c*).

Certain forms were observed in which the chromatoid bodies appeared to be arranged in a ring, because of optical sectioning (Fig. 5). These forms were found at the same time as, but were nearly always slightly larger than, the early cysts.

Early cysts usually contained 1 to 3 chromatoid bodies which accounted for most of the specific absorption. The single nucleus contributed very little (Figs. 1 and 6). Electron microscopy, at this stage, revealed the bodies to be composed of particles 200 to 300 A in diameter (Fig. 7) arranged in a close cubic or hexagonal array. Each particle contained six or eight 50 to 70 A subunits (4-6).

In maturing cysts there was a gradual fragmentation or solution of the crystalloids until the cytoplasm was filled with intensely absorbing material (Fig. 4, *e* to *i*). There appeared to be no reduction in the gross amount of material per cyst although the amount of absorbing material varied according to the number and size of the crystalloids originally in the cyst (Fig. 8). In some cases the cyst appeared to enlarge as the solution of the crystalloid proceeded. Mature cysts were easily recognizable by the uniformly high absorption of the cytoplasm. This was frequently so strong that the four nuclei were completely obscured.

There may be more than one method of excystation, but the one that fits the majority of our observations is a reduction of the cyst wall over the greater part of the cyst (*cf.* reference 8) followed by the protuberance of several small pseudopods

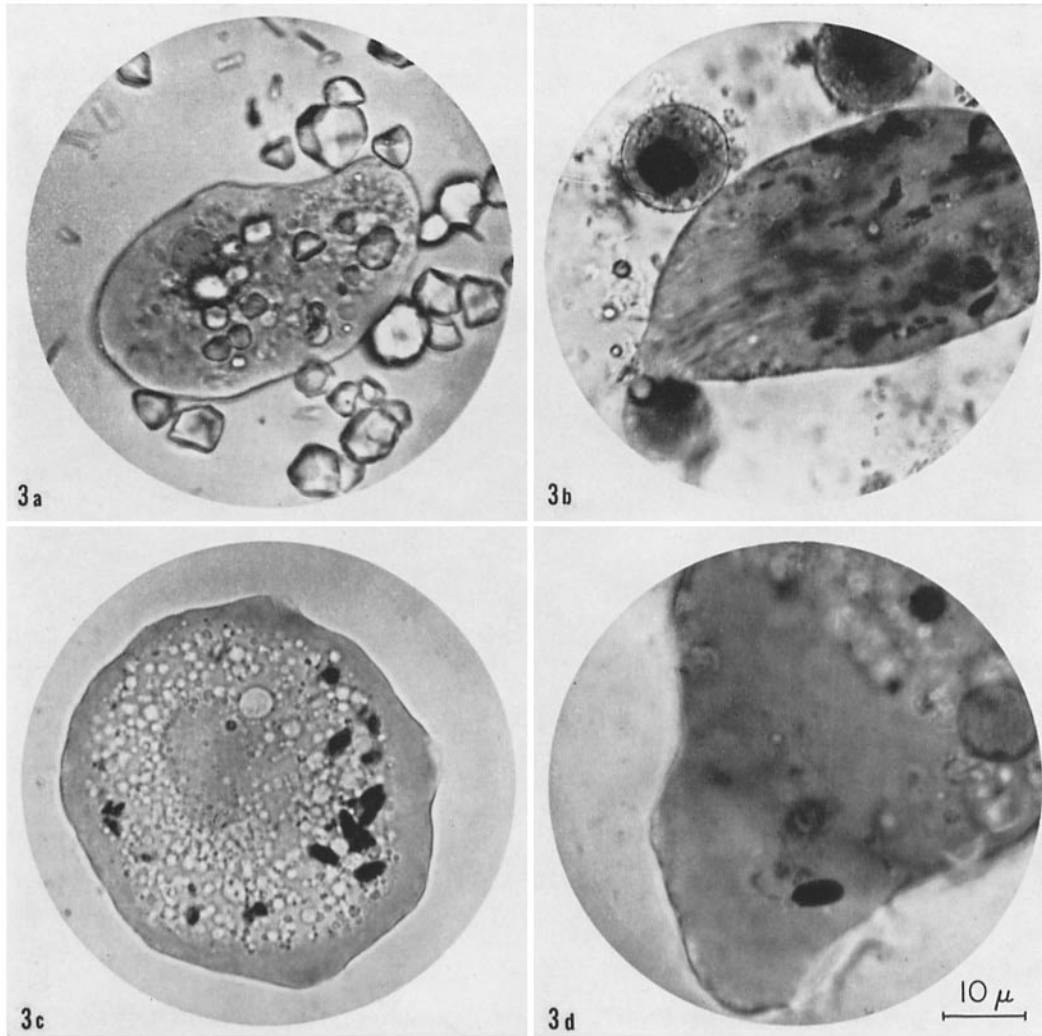


FIGURE 3 Trophozoites of *E. invadens* photographed at wavelength of 265μ . $\times 1100$.

(a) Early stage containing starch. Monoxenic culture.

(b) Late stage containing many chromatoid bodies. Polyxenic culture.

(c) Late stage containing chromatoid bodies. Axenic culture.

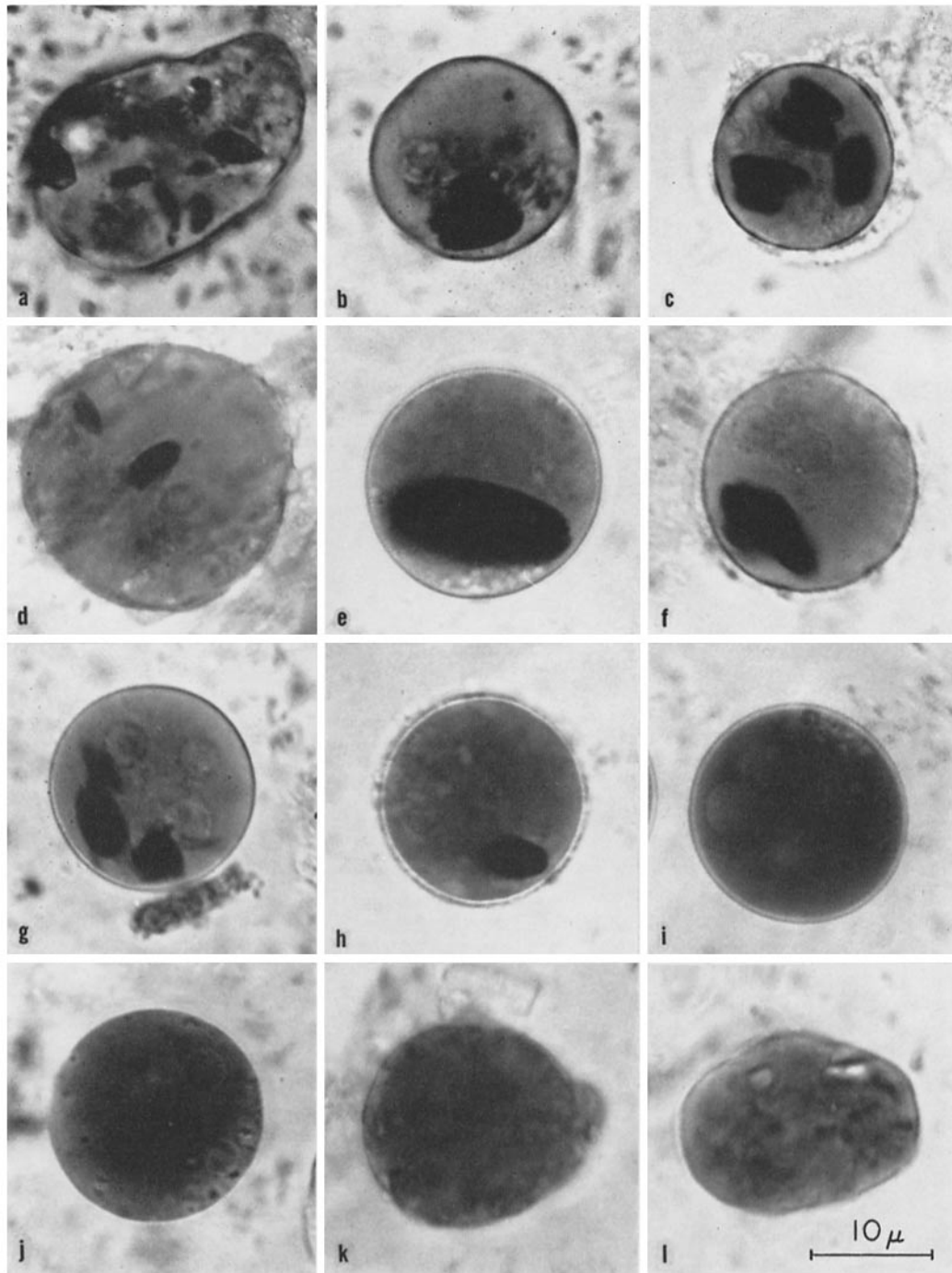
(d) Late stage with chromatoid body in clear cytoplasm about to be separated from main portion to produce precyst illustrated in Fig. 4 d.

over a very large surface area at irregular intervals (Fig. 4, *j* and *k*). Feeding begins at this time. Regardless of the method of excystation, the newly hatched amebae had uniform, intensely-absorbing cytoplasm like that of the mature cyst (Fig. 4 *l*). They progressed rather slowly at first, frequently rounding up. The metacystic or primary division amebae passed through a complicated division series leading to the formation of eight young

trophozoites from each cyst. During the divisions the absorbing material was diluted, by the general growth of the small amebae.

DISCUSSION

Since 1912, when Hartmann (18) first reported that the chromatoid bodies were consumed during development of the cysts, most authorities have accepted this as true (11, 13, 14). James (21), in a



study of the development of chromatoid bodies, came to the conclusion that they were derived from the cytoplasm by a process of condensation. The appearance of chromatoid bodies in the trophozoites has long been regarded as a rare or abnormal occurrence (12), although Geiman and Ratcliffe (16) described chromatoid bodies in the trophozoites of *E. invadens*. Hopkins and Warner

bodies increases with age in trophozoites and decreases with age in cysts. Deutsch and Zaman (15) showed, in electron micrographs of trophozoites, that a great number of small chromatoid bodies arise by a process of aggregation of small groups of 200 to 300 A units to form crystalline masses in the early cysts, and that in the maturing cysts these masses again fragment into separate units.

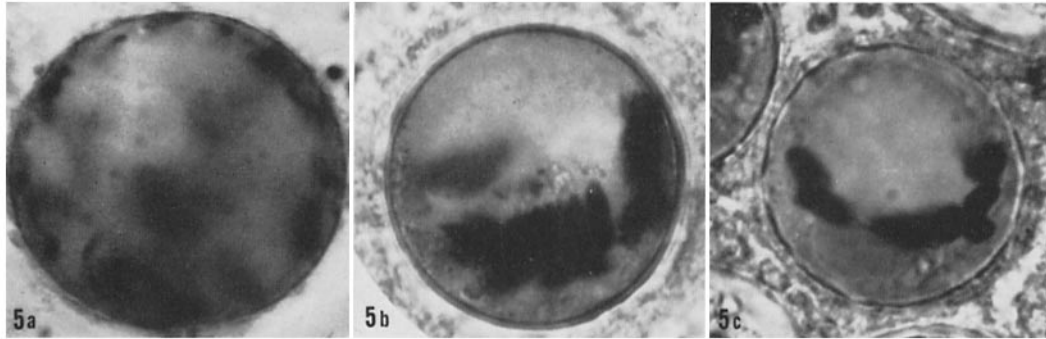


FIGURE 5 Stages in development of ring cyst of *E. invadens* photographed a wavelength of 265μ . $\times 1700$.
(a) Late precyst with numerous chromatoid bodies.
(b and c) Cysts in which consolidation of chromatoid material is occurring.

(20) found that they were present in *E. histolytica*, before the secretion of the cyst wall, being formed by a coalescence of clear vacuoles containing foodstuffs. Hakansson (17) was able to make dispersed or latent chromatoid material reappear by osmotic shock. He thought it was doubtful that the substance was actually consumed, preferring the view that the bodies merely fragmented into smaller units. Barker and Deutsch (7) gave an indication that the average size of the chromatoid

The specific ultraviolet-absorbing material is assumed to be ribonucleoprotein, because cytochemical tests have failed to identify any other substances in the chromatoid bodies (5). If this assumption is correct, then the results presented considerably amplify our knowledge of the distribution of ribonucleoprotein in the life cycle of *E. invadens*.

It now appears that there is very active synthesis of ribonucleoprotein in the trophozoites in all parts

FIGURE 4 Stages in encystation and excystation of *E. invadens* photographed at wavelength of $265 m\mu$. $\times 1750$.

- (a) Precyst. Small portion of a late trophozoite which had just separated from one of the type illustrated in Fig. 3 b. Still motile.
- (b) Similar to Fig. 4 a, but a later stage. Non-motile.
- (c) Early cyst with several chromatoid bodies derived from a precyst of the type shown in Fig. 4 a.
- (d) Precyst. Portion separated from a late trophozoite of the type illustrated in Fig. 3 d.
- (e) Cyst with typical single chromatoid body.
- (f) Maturing cyst with two nuclei.
- (g) Maturing cyst with four nuclei. Chromatoid body beginning to disperse.
- (h) Later stage of dispersion of chromatoid body.
- (i) Mature cyst. Chromatoid body completely dispersed.
- (j) Cyst wall disappearing.
- (k) Pseudopod formation. Feeding begins.
- (l) Newly hatched trophozoite. Motile.

of the cytoplasm during the period when the most favorable conditions for growth are present. Towards the end of the logarithmic growth phase,

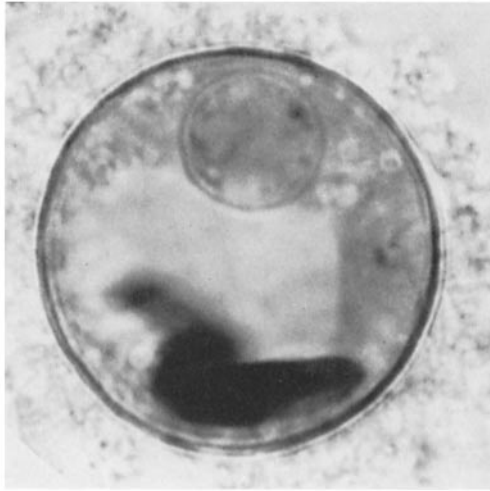


FIGURE 6 Early cyst of *E. invadens* photographed at wavelength of 265 m μ . Chromatoid body (dark), glycogen vacuole (light), and nucleus are evident. $\times 2650$.

when the majority of cells are in the late trophozoite or precystic stages, large amounts of newly synthesized ribonucleoprotein are concentrated in a number of small, highly specific, ultraviolet-absorbing crystalloids. With the onset of encystation, the ribonucleoprotein is further condensed into polycrystalline bodies.

We think that the observations we have made, of the kind illustrated by Figs. 3 *d* and 4 *d*, may represent a morphological manifestation of previously enacted nuclear events. Experiments are being designed to test the hypothesis that, during the mass encystation period, late trophozoites go through a nuclear division, which is followed by a differential division of the cytoplasm. The result is the formation of two unlike daughter cells. By this method, one late trophozoite will give rise to one cyst with large chromatoid bodies and a trophozoite. The trophozoite thus formed has two alternatives: either it goes on to another differential division, or it can form a cyst by the gradual condensation of the small crystalloids into large chromatoids. This is also consistent with our observations that a trophozoite can form a cyst without going through a differential division.

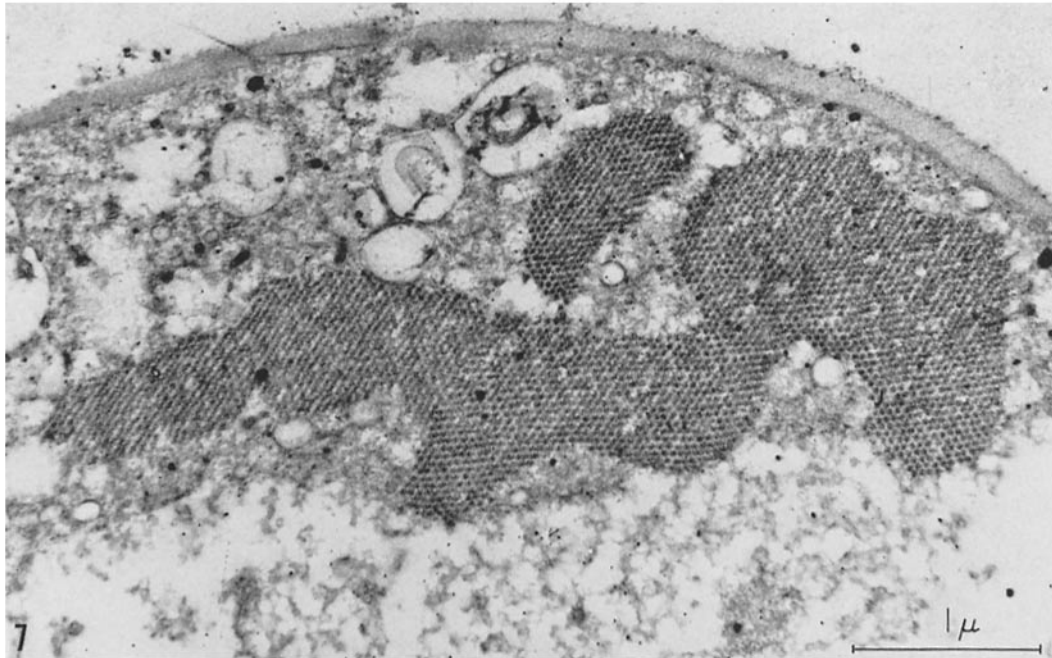


FIGURE 7 Electron micrograph of a portion of a cyst showing aggregation of 200 to 300 Å particles to form chromatoid bodies. $\times 2500$.

The type of differential division visualized is, of course, well known to embryologists in the differentiation of cells in metazoans, but we are unaware of any report of similar differential division in protozoans. However, Dobell (14) thought that characteristics of precystic *E. histolytica* indicated that they were the products of a recent nuclear division.

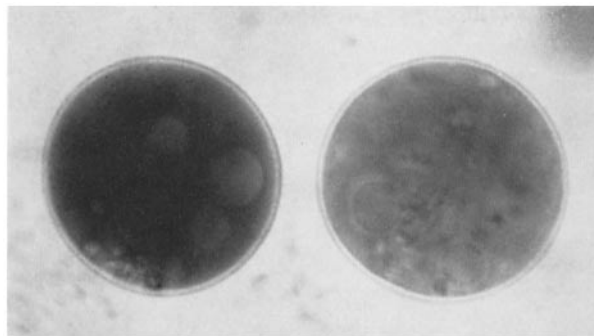
After the cyst wall is laid down, the intensely absorbing material is revealed by electron microscopy to be crystalline masses of 200 to 300 Å units packed in close cubic or hexagonal array. As the cysts mature there is no apparent decrease in the amount of absorbing material per cyst, but the crystalloids are isolated and dispersed until the entire cytoplasm of the mature cysts shows intense

found a few ring forms in all samples of cysts, including maturing and mature cysts.

The young excysted amebae also have highly absorbing cytoplasm, and this absorption does not become weaker until the division into eight small amebae is complete. The reduction in absorption per ameba is probably due to growth rather than to an actual reduction in the amount of absorbing material.

The use of improved culture conditions, resulting in predictable encystation responses, and the ability to study living cells with ultraviolet microscopy have given us a much more satisfactory picture of the distribution of ribonucleoprotein throughout the life cycle. On this and other evidence (3-7) the hypothesis is advanced that the

FIGURE 8 Mature cysts photographed together (wavelength 265 mμ) to illustrate the variation in amount of absorbing material that may be present. × 1750.



absorption. We have been unable to confirm or refute previous descriptions of excystation (9, 30, 14, 10, 19). The exact nature of cysts in which the chromatoid bodies are in a ring under the cyst wall is in doubt. In a previous study of the strain N of *E. invadens* (5), Barker described similar ring forms which apparently occurred in the maturing cysts. For technical reasons strain N has not been studied by ultraviolet microscopy. The previous observation may have been an artifact arising from the technique, since, in this study, the majority of ring cysts were found in association with precystic or early cysts.

On the other hand, some, if not all, ring cysts may be incomplete cysts, which never advanced beyond this stage to become mature cysts. We

large crystalloids, the chromatoid bodies, are a manifestation of a special parasite-host adaptive mechanism. Ribonucleoprotein is synthesized under favorable conditions, crystallized during the encystation period, stored as aggregates of ribosome-like particles in the cyst, dispersed in the mature cysts, and finally shared by the eight young amebae, which are thus supplied with previously synthesized "ribosomes" for a period of quick growth to establish themselves in a new host.

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