CYTOPLASMIC DNA SYNTHESIS IN *AMOEBA PROTEUS*

II. On the Behavior and Possible Nature of the DNA-Containing Elements

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ABSTRACT

Nucleic acid–containing particles in the cytoplasm of *Amoeba proteus* (*cf.* reference 1) were counted after acridine orange staining. The number of particles per ameba was found to be correlated with cell age and size. Fresh daughters had a mean particle number of 5400, whereas predivision amebae contained around 11,000 particles. Amebae from two other strains contained similar particles. The particles were found to be clustered in fasted cells and redispersed after feeding. A marked increase in the particle population was noted in anucleate fragments. These results, together with those previously presented, suggest that the particles multiply intracellularly. Their nature and their relationship to previous work on nucleic acid labeling in *Amoeba* are discussed.

We have previously shown (1) that cytoplasmic incorporation of tritiated thymidine in *Amoeba proteus* occurs in association with particulate elements detectable with the light microscope. The staining behavior of the particles, together with the results of appropriate enzyme digestions, suggested that they contain DNA and RNA.

The occurrence of structures containing nucleic acids outside the nucleus of any cell raises the question of their significance with respect to the normal function of the cell within which they are seen. One approach to this question can be made by inquiring into the origin of the particles and the degree to which they are integrated with the cell's activities. The simple possibility of extracellular origin and limited retention is particularly likely in *Amoeba*, since this organism derives the bulk of its nutrients from the ingestion of other living systems.

The evidence to be presented here suggests that this simple explanation of continuous intake of the particles from the culture medium cannot be supported; our data make it likely that the particles are capable of self duplication within the cell and are sufficiently integrated with the ameba's metabolism to keep pace with the cell's development from one division to the next. It will also be shown that the particles respond to the absence of the ameba nucleus.

MATERIALS AND METHODS

In addition to this laboratory's strain of *Amoeba proteus*, cells of the same species were obtained through the courtesy of Dr. L. E. Roth from the Argonne National Laboratories and also from the General Laboratory Supply House, Chicago. Culture methods and conditions were those previously described (1).

Cells in known stages of the life cycle were obtained by collecting division figures from recently fed cultures brought to room temperature 0.5 to 1 hour previously. After division the fresh "daughters" were transferred to 35 × 10 mm plastic dishes and fixed at various periods thereafter; if fed, these cells divided after about 45 hours at 18°C.

In the enucleation experiments, amebae were cut

free hand by means of glass needles after elongation of the cells under a beam of light.

In some of the fasting experiments fresh daughters were used, one of each pair being fasted and the other fed; after different periods of starvation some cells were refed and then fixed at selected time intervals.

Particle counts were performed on acridine orange-stained cells, previously fixed and treated with ribonuclease as described in the previous paper (1), to which we refer also for details on the optical equipment used. Final magnification was 850 times, and areas 1×10^3 to 3×10^3 μ^2 were scanned for each ameba. Cells were then drawn over ruled paper and their areas measured by planimetry.

RESULTS

Over 130 amebae in known stages of the life cycle were subjected to particle counts. Fig. 1 shows a plot of the ameba sizes, as obtained from the drawings, against age. It can be seen that cells of the same age vary rather widely in size. However, the regression of cell size against age is significant below the 0.1 per cent level, and it can be seen that the areas measured approximately double between newly divided cells and predivision ones.

Fig. 2 shows the number of particles per ameba as correlated with cell age. A significant regression ($P < 0.1$ per cent) was obtained. Fresh daughters had a mean particle number of 5400, whereas predivision amebae contained about 11,000 particles on the average. Again the variability is large. When the number of particles per ameba is plotted against cell size (Fig. 3), a significant correlation (0.84 in this particular sample) is found. At least some of the variation in particle numbers within a given age could therefore be attributed to variation in cell size. The regression coefficient is highly significant ($P <$ 0.1 per cent).

In the course of the quantitative analysis of the

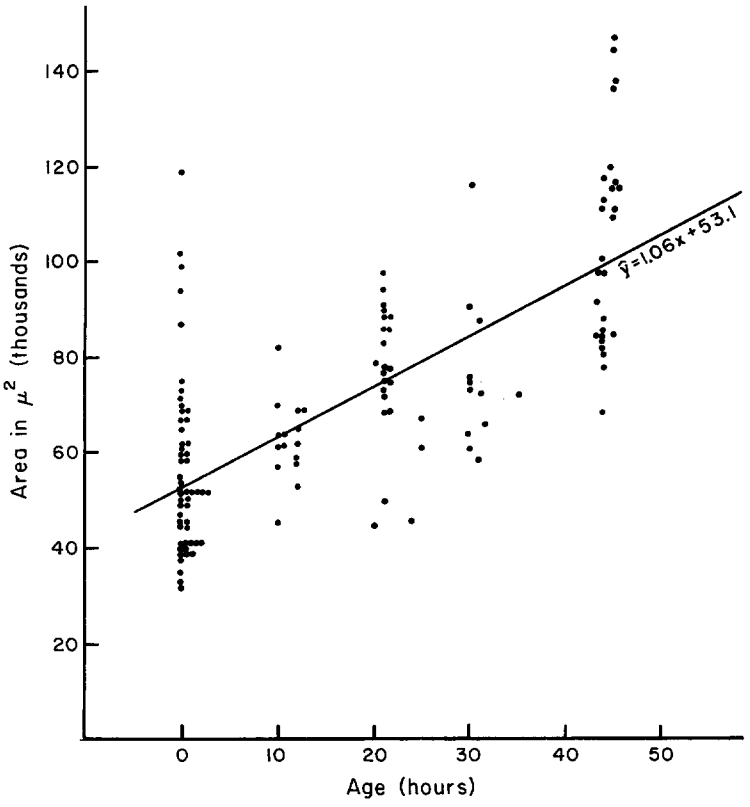

FIGURE 1

Plot of size of amebae against age. Standard error of the regression coefficient, $s_b = 0.112$; $t = 9.46$; $n = 132$.

particles, it became apparent that although their distribution was more or less uniform throughout the ameba cytoplasm, they exhibited a strong tendency to occur in pairs. Roughly one-third of the total number of particles were associated with a second particle in closer proximity than would be expected from a random distribution. Since this pattern was evident in centrifuged as well as in uncentrifuged cells, it may be indicative of a mode of replication by a process akin to binary fission.

Both the Argonne and the General Laboratory Supply House strains were checked for the presence of particles. Particles with the same staining characteristics and apparent morphology as described for the Madison strain were found in all cells examined. Although the particles were not subjected to precise quantitative analysis, it was obvious that cells of the Argonne strain contained several times as many particles as the others.

Effect of Fasting and Refeeding

Cells were stained with acridine orange after 24 hours, 2, 4, 8, and 15 days of fasting. At least 10 cells were studied for each time period. Already in the first group, but more clearly in the suc-

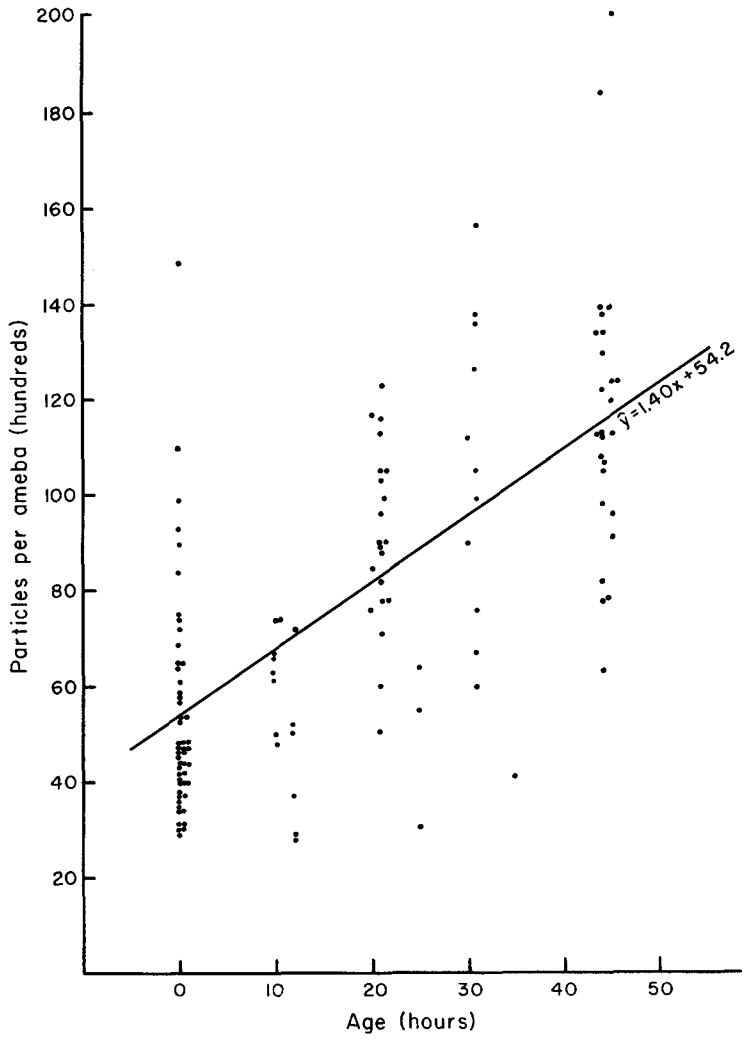

FIGURE 2

Plot of particle number per amoeba against age. $s_b = 0.135$; $t = 10.34$; $n = 128$.

ceeding ones, particles were found to be closely clustered in groups of 10 to 20; this precluded accurate counting, but the visual impression was that their numbers were similar to those in unfasted cells; in a small minority, however, the particles seemed to be markedly increased. Cells fasted for 8 days were refed and fixed at 4, 14, and 24 hours thereafter. In the first group, particle clusters were less numerous; at 14 and 24 hours, amebae presented as uniform a distribution of particles as that found in normally grown cells. The results were confirmed with paired cells (see under "Materials and Methods").

Enucleation Experiments

Cells were cut and the fasted nucleate and anucleate halves were fixed 2 and 7 days afterward. Nucleated amebae behaved like the fasted cells described above. In anucleates, however, a marked increase in the number of particles per unit area was found; no counts were performed, but this increase seemed to be a several-fold one, obliterating the clustering present in nucleate halves.

DISCUSSION

We have shown that the particles in the ameba cytoplasm increase in number from one division

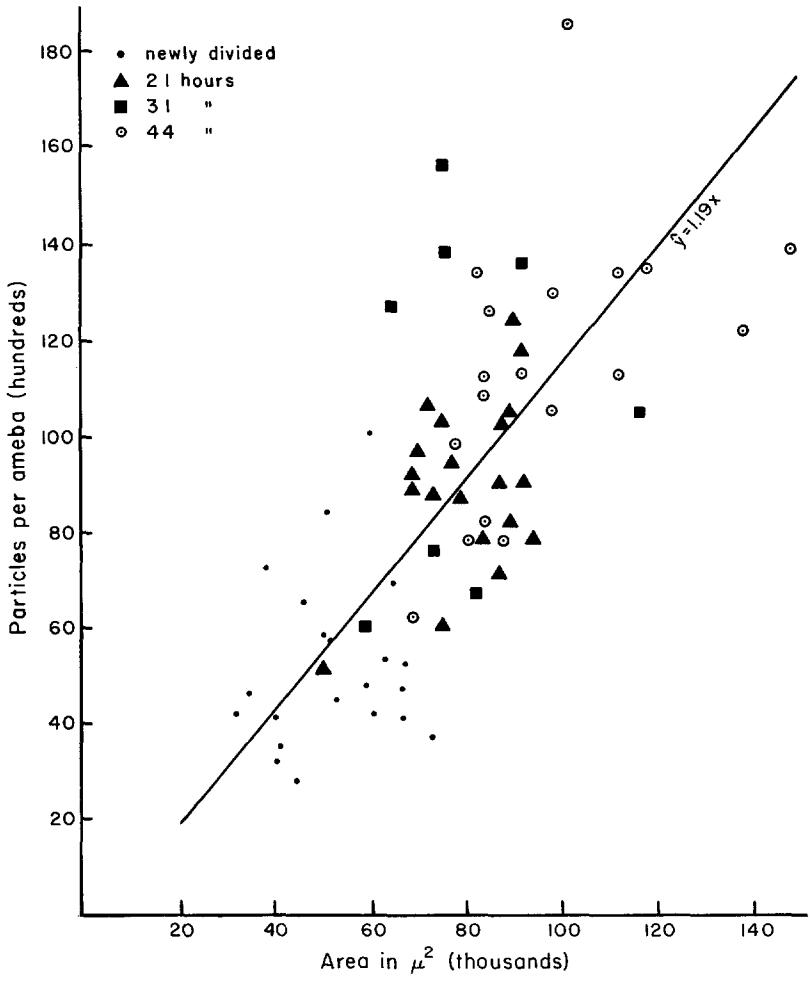

FIGURE 3

Plot of number of particles per ameba against size. All cells from one experiment. $s_b = 0.092$; $t = 13.0$; $n = 71$.

to the next, with an approximate doubling between freshly divided and predivision amebae. Moreover, statistical analysis indicates that the particle number is more strongly correlated with size than with age of the amebae. This numerical regularity suggests that the particles are in some way integrated with the cellular economy and argues against their being constantly taken up by the amebae from the medium. The lack of detectable association of the particles with the feeding process and with food vacuoles is a further argument against their uptake from the outside.[1]

The elimination of continuous ingestion from the medium as the origin of the particles leads us to consider various modes of intracellular derivation. The possibility of the particles' being associated with a normal cellular organelle (2, 3) seems to be contradicted by their already mentioned appearance in pairs, together with the increase in numbers in anucleate cells. We are left with the possibility that the particles occur independently of normal cell components and are themselves organized structures capable of intracellular multiplication.

Our data do not permit further elucidation of the structural and biochemical characteristics of the particles at the present time. Their nucleic acid content, size, and mode of reproduction by what may be binary fission suggest that they may be thought of as bacteria or rickettsia-like in nature. This suggestion should be regarded as highly tentative pending further information on the particle ultrastructure, enzymatic machinery, energy metabolism, and synthetic capabilities (4). Only with the availability of this information can the nature of the association between particles and ameba be described in precise terms. Some degree of control over particle activity by the ameba's nucleus is evidenced by the behavior of the particles in anucleate half cells. Both particle number and thymidine incorporation (5) are increased in the absence of the nucleus; in this connection it may be of interest to note that cytoplasmic thymidine incorporation in synchronized *Amoeba proteus* cells, although extending throughout the interphase, is maximal during that period in which nuclear incorporation occurs (6).

The suggestion of the "organismal" nature of the cytoplasmic particles warrants a short discussion of some previous pertinent work. Roth and Daniels (7) described with the phase and electron microscope "infective organisms" in *Amoeba proteus*, and suggested their origin by uptake from the medium. Although the strain of ameba used by these investigators has been shown above to contain large numbers of the cytoplasmic particles described here, their identification with the particles pictured by Roth and Daniels will not be attempted at present. It should be noted that in an earlier electron microscope study of the same organism (8), bodies identified as "alpha" particles were described and attention was called to their similarity to viral inclusions. Further ultrastructural work is obviously necessary to establish the identity of these variously described bodies. Prescott *et al.* (9) have recently suggested that cytoplasmic H^3-thymidine incorporation in *Amoeba proteus* could be due to the activity of cytoplasmic DNA polymerase in conjunction with DNA primer molecules originating from the degradation of food organisms or taken up by pinocytosis. The now demonstrated association of cytoplasmic thymidine incorporation with structures more complex than free DNA molecules does not help in substantiating their interpretation.

Finally, the demonstration of presumably self-duplicating DNA- and RNA-containing particles in the ameba cytoplasm raises the question whether or not these particles are responsible for the incorporation of various RNA precursors by anucleate cell halves (10). It may be pointed out in this connection that the incorporation of RNA precursors into such cells decreases rapidly after removal of the nucleus, whereas thymidine incorporation is markedly enhanced. Experiments now in progress may be expected to establish the degree to which the particles contribute to anucleate RNA synthesis.

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[1] A preliminary attempt was made to demonstrate similar particles in medium where amebae had been grown; about 100 ml were centrifuged at about 1000 *g* for 5 minutes and then at 35,000 *g* for 30 minutes. Smears of the sediment were fixed and stained with acridine orange. Although many bacteria were found, no bodies of size and staining comparable to the intracellular particles were seen. This negative evidence fits with the hypothesis that the particles replicate within the amebae and are not taken up from the medium.

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