CYTOPLASMIC DNA SYNTHESIS IN *AMOEBA PROTEUS*

I. On the Particulate Nature of the DNA-Containing Elements

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ABSTRACT

The incorporation of tritiated thymidine in *Amoeba proteus* was reinvestigated in order to see if it could be associated with microscopically detectable structures. Staining experiments with basic dyes, including the fluorochrome acridine orange, revealed the presence of large numbers of 0.3 to 0.5 μ particles in the cytoplasm of all cells studied. The effect of nuclease digestion on the dye affinity of the particles suggests that they contain DNA as well as RNA. Centrifugation of living cells at 10,000 g leads to the sedimentation of the particles in the centrifugal third of the ameba near the nucleus. Analysis of centrifuged cells which had been incubated with H_3-thymidine showed a very high degree of correlation between the location of the nucleic acid–containing granules and that of acid-insoluble, deoxyribonuclease-sensitive labeled molecules and leads to the conclusion that cytoplasmic DNA synthesis in *Amoeba proteus* occurs in association with these particles.

Extranuclear DNA as a normal cell component has been reported in a variety of biological systems. In several instances the DNA is associated with presumably autonomous or semiautonomous cytoplasmic organelles. The DNA's of the kinetoplast of trypanosomes (1), of proplastids (2), and of chloroplasts (3, 4) fall into this category. In other cases, however, normal cytoplasmic location of DNA has not been related to a recognizable structure. These include the DNA in the cytoplasm of amphibian eggs (5, 6) as well as the presumptive DNA's reported in the cytoplasm of lily anthers (7), *Tetrahymena* (8), fern eggs (9), and *Amoeba proteus* (10, 11). This second group of cytoplasmic DNA's raises the question whether or not DNA or a DNA-like polymer can in fact be synthesized and maintained in a cell in the absence of structural organization beyond the molecule itself. The possibility of such synthesis is made plausible by the *in vitro* synthesis of deoxyribonucleotide polymers, with or without priming molecules, by means of the polymerase reaction (12, 13), as well as by the demonstration of polymerase activity in the cytoplasm of mammalian cells (14) and its speculatively inferred presence from less direct experiments in the cytoplasm of *Amoeba proteus* (15).

The present report deals with studies undertaken to learn whether or not cytoplasmic incorporation of tritiated thymidine into a deoxyribonuclease-sensitive molecule in *Amoeba proteus* is associated with a DNA-containing cell component with microscopically detectable structure. The application of cytochemical techniques, coupled with autoradiography and centrifugal stratification of living cells, has led to evidence which indicates that most if not all cytoplasmic thymidine incorporation into this organism occurs in or on discrete particles which can be resolved with light microscope optics but are below 1

micron in diameter when visualized with standard basic dyes or with the fluorochrome acridine orange.

MATERIALS AND METHODS

The cells used in this study were *Amoeba proteus* of the strain maintained in this laboratory for the past 6 years. They were grown at 18°C in a dilute salt solution (16) and fed with the ciliate *Tetrahymena pyriformis*.

Labeling

Tritiated thymidine (Schwarz BioResearch, 1900 or 3000 $\mu c/\mu M$) was administered to the amebae in food-free medium over a 24 hour period at a level of approximately 25 μc/ml; labeled cells were washed 4 or 5 times in normal medium prior to subsequent treatments.

Centrifugation

The cells, suspended in normal medium, were layered over a 15 per cent solution of Ficoll (Pharmacia, Uppsala, Sweden) in distilled water in 4 × 25 mm glass tubing, closed at one end with a rubber cap. After gentle rotation to produce a smooth gradient between the salt medium and the viscous polymer solution, the tubes were subjected to 10,000 *g* for 20 minutes in the SW39 swinging bucket head of the Spinco model L centrifuge under refrigeration. After completion of the centrifugal run the contents of the tubes were dumped into 10 to 20 ml of chilled (4°C) 45 per cent acetic acid; the cells were removed from this fixing bath within 10 minutes, placed on gelatinized slides, and flattened under a no. 2 coverglass. Subsequent processing of the slides was carried out as with uncentrifuged cells.

Preparation of Slides

Amebae were placed on gelatinized slides individually or in small groups with a minimal volume of medium. They were fixed and flattened by the application of a no. 2 coverslip bearing a small drop of 45 per cent acetic acid (except in the case of centrifuged cells, where the acid solution was already present on the slide). After freezing over dry ice and removal of the coverslip the slides were placed in acetic acid–ethanol (1:3) for approximately 5 minutes for postfixation of the cells, passed through absolute ethanol and 95 per cent ethanol, 2 changes of about 5 minutes each, and finally air dired.

Enzymatic Digestions

Ribonuclease digestion of fixed cells was carried out either for 4 hours at 39°C or overnight at room temperature by placing washed slides in distilled water with 0.3 mg/ml of crystalline ribonuclease (Worthington) adjusted to pH 7.0 with Na_2HPO_4. Deoxyribonuclease treatment involved a 4 hour incubation at 39°C with 0.3 to 0.5 mg/ml of crystalline enzyme (Worthington) in ¼ strength McIlvaine buffer at pH 7, with 3×10^{-3} M $MgSO_4$. The The specificity of both enzymatic extractions was controlled through the parallel use of the same solutions with the enzymes omitted. The DNase buffer was found to remove considerable RNA, presumably because of its appreciable salt concentration (approx. 4.7×10^{-2} M).

Staining Procedures

Several stains were employed: gallocyanin-chromalum (17); toluidine blue (18); crystal violet (19); acridine orange (0.03 mg/ml, in 0.1 M acetate buffer, pH 4.5, for 30 minutes, followed by two changes of buffer alone, 3 to 5 minutes each, and semipermanent mounting in buffer sealed with Gurr's Laktoseal); basic fuchsin (8.3 mg/100 ml in 0.02 M acetate buffer, pH 4.6, rapid differentiation in buffer alone, air drying, and mounting in immersion oil; staining in basic fuchsin was usually preceded by a 5 to 10 minute treatment at 4°C with 6 per cent $HClO_4$ to remove metachromatically staining substances). Coverslip seals, immersion oil, and stains were removed from slides to be analyzed autoradiographically by immersion in xylene and dilute alcohol.

Autoradiography

Kodak Ltd.'s AR-10 emulsion, applied at 24°C, was used throughout (20). Exposure periods ranged from 60 to 80 days. Development and fixation were carried out at 18°C in D-19b and Kodak's standard Fixer, respectively. The analysis of developed autoradiographs was performed by means of phase, brightfield, and darkfield microscopy.

Fluorescence Microscopy

Cells stained with acridine orange were examined with xenon arc illumination, a BG-12 exciter filter, and a Bausch and Lomb barrier filter. For all detailed observations a Leitz 54 × fluorite oil immersion objective (N.A. 0.95) was employed.

RESULTS

A. Non-centrifuged Amebae, Stained with Acridine Orange after Ribonuclease Digestion

All the cells examined (200 to 300) contained small (0.3 to 0.5 μ) particles with a distinct yellowish green fluorescence color against a dull greenish gray background. In cells digested with

deoxyribonuclease as well as RNase the fluorescence of the particles was completely absent. DNase buffer incubation, on the other hand, had no detectable effect on the yellowish green fluorescence of these bodies. In sharp contrast to the particles, the bacteria or molds found within or in association with vacuoles showed a yellowish green fluorescence which was not abolished by DNase digestion. The small particles were present in large but variable numbers; aside from a tendency to occur in pairs, they were fairly uniformly distributed in the cytoplasm of most cells. Uncentrifuged cells stained with acridine orange halfway through the cell; centripetally with respect to this band, there is very little affinity for basic dyes (Fig. 1). All the dyes used on stratified cells revealed the 0.3 to 0.5 μ particles in the slightly basophilic region containing the nucleus and in the centrifugal part of the densely basophilic band. Few particles were present in the most densely stained part of this band and none above it. In stratified cells acridine orange staining permitted the detection of the particles without RNase digestion: they were clearly visible as reddish orange granules against a paler background of similar fluorescence color. Basic fuchsin and

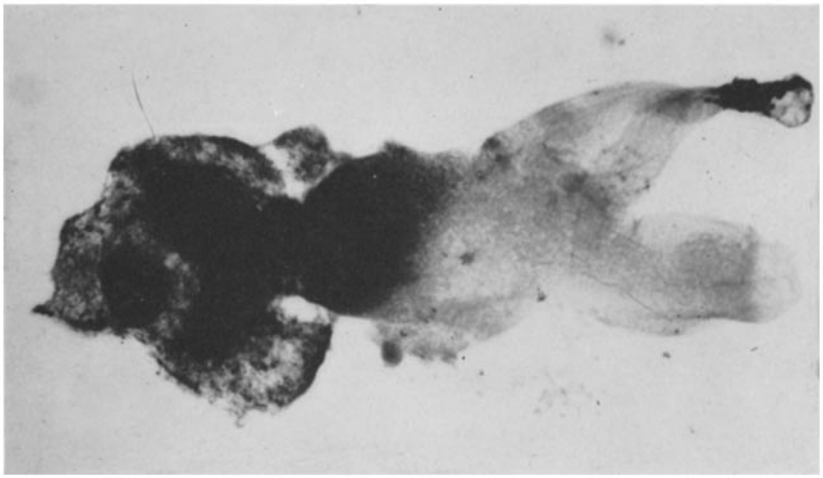

FIGURE 1

Amoeba proteus, centrifuged. Basic fuchsin stain. Centrifugal pole on the left. Note nucleus near extreme left and dark basophilic band; centrifugal half almost unstained. × about 250.

without prior RNase digestion did not permit detection of the particles by fluorescence microscopy. The cytoplasm of these cells showed a fairly uniform orange-red fluorescence background against which only the nuclei of larger ingested organisms (*i.e.*, *Tetrahymena*) and less distinctly the ameba's own nucleus could be detected as yellowish orange regions.

B. Centrifuged Amebae

Centrifuged cells stained with basic dyes showed the stratification pattern described by Mast and Doyle in 1935 (21). Crystal vacuoles are sedimented at the centrifugal pole; above these the nucleus comes to rest in a zone of slight basophilia; a very dense band of basophilic material extends from this region approximately gallocyanin-chromalum, as well as toluidine blue and crystal violet, also showed the particles with reasonable clarity. RNase digestion, but not incubation with the control solution, removed both the ameba's basophilic pattern and the detectable affinity of the particles for basic fuchsin, crystal violet, and toluidine blue; the particles could still be seen faintly with gallocyanin-chromalum and quite distinctly with acridine orange. Both of the latter two dye affinities were abolished by digestion with DNase. (Several attempts to demonstrate the particles with the Feulgen reaction were unsuccessful; only the nucleus of the ameba and the nuclei of ingested organisms were visibly stained.)

In summary, these observations indicate that *Amoeba proteus* contains in its cytoplasm small

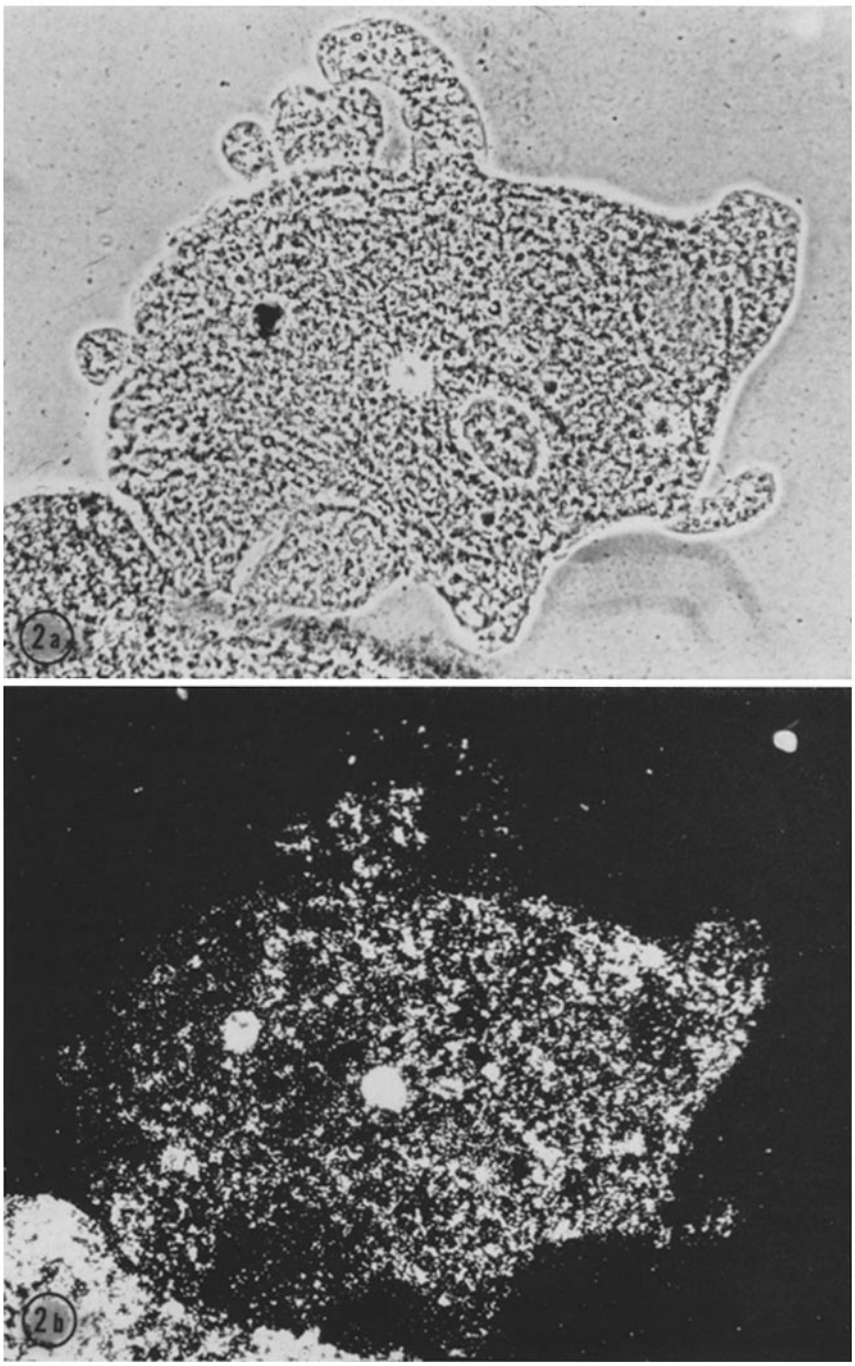

FIGURE 2

Non-centrifuged ameba, phase contrast (Fig. 2 *a*) and darkfield (Fig. 2 *b*) photographs of autoradiograph. Note relatively uniform distribution of label; nucleus is not obviously labeled. × approx. 300.

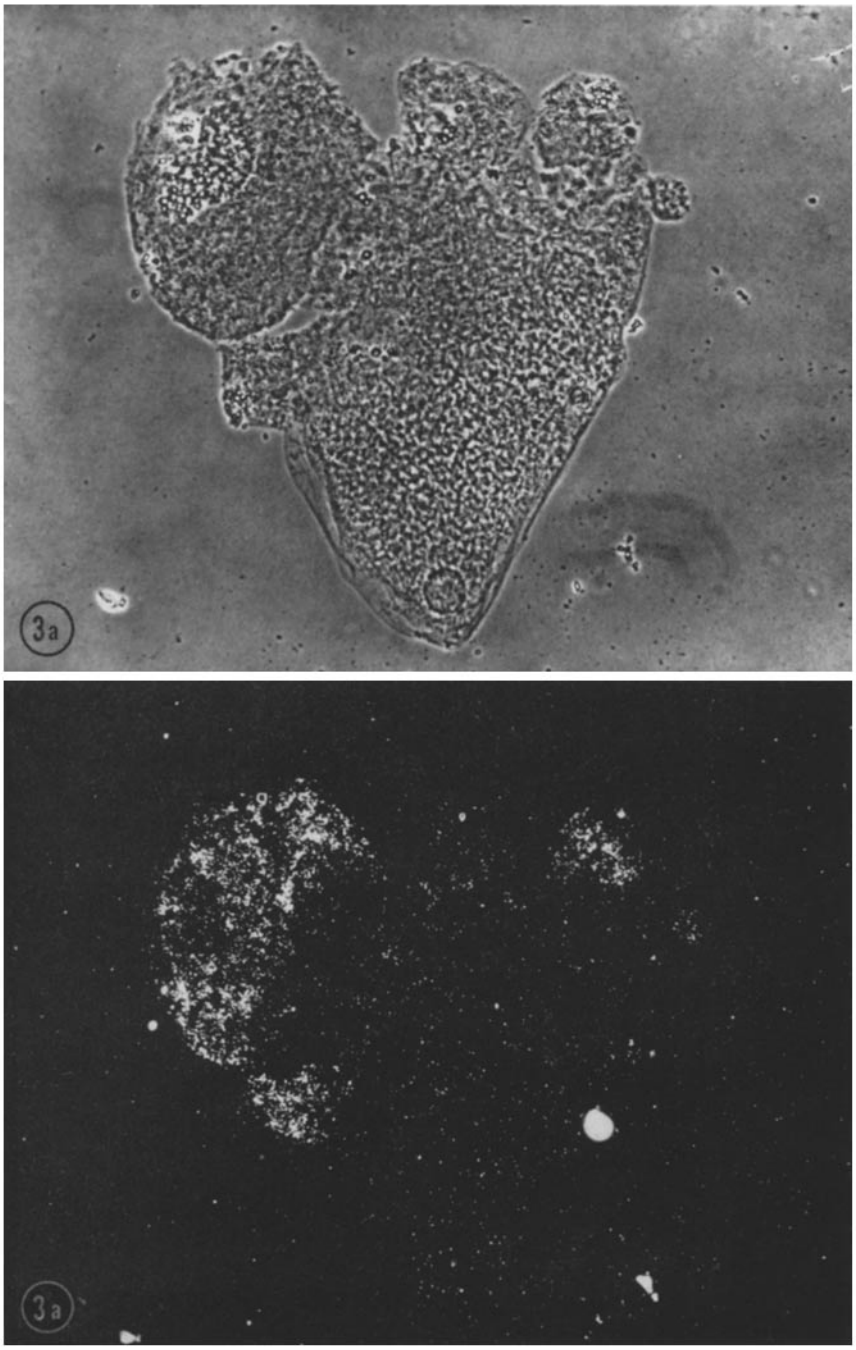

FIGURE 3

Centrifuged ameba; centrifugal pole at upper left. Phase contrast (Fig. 3 *a*) and darkfield (Fig. 3 *b*) photographs of autoradiograph. Note that most of the label is found at the extreme centrifugal pole and in two extensions of the cell. × approx. 300.

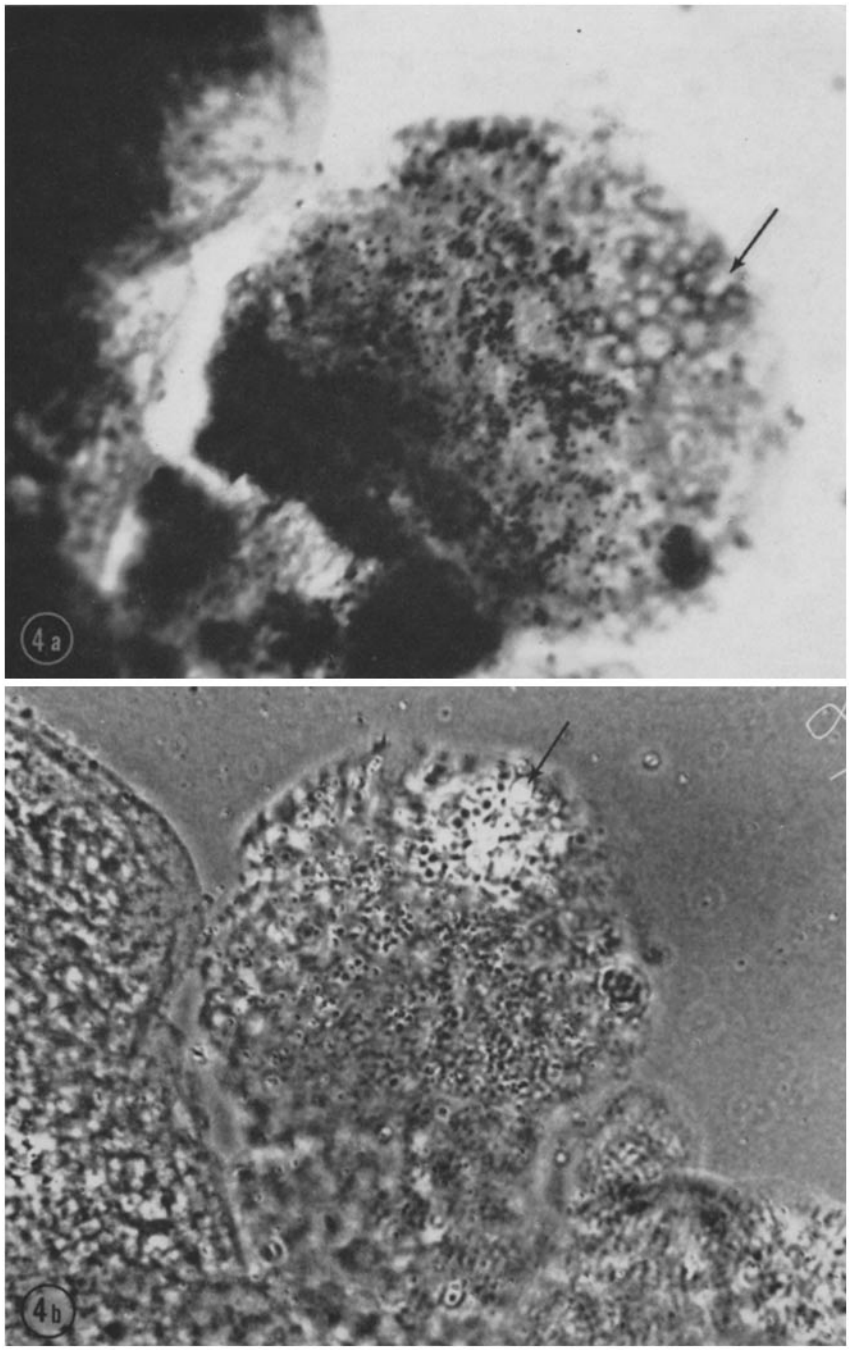

FIGURE 4

Higher magnification of one of the cell extensions pictured in Fig. 3, after basic fuchsin staining Fig. 4 *a*). The particles and their frequent paired arrangement are evident. Figs. 4 *b* and 4 *c* are, respectively, phase contrast and darkfield photographs of the destained and autoradiographed preparation. Fig. 4 *a* should be turned approximately 30 degrees counterclockwise for registration with *b* and *c*. On Fig. 4 *c* the contour of the cell extension is indicated. (The final magnifications of the three photographs are not identical.) Arrows indicate common points of reference. × about 1500.

particles which are distinct from ingested bacteria, molds, or food organisms in their distribution and their sensitivity to DNase digestion. The affinity of these particles for a wide range of dyes and the effect of nuclease treatments on this affinity indicate the presence of DNA and probably RNA as well. (The lack of a demonstrable Feulgen reaction can be tentatively attributed to the difficulty of visually detecting small amounts of DNA stained with this reagent.)

among the autoradiographs following the latter group of treatments or between them and preparations subjected only to water washing. Most cells contained one or more small areas of very intense label concentration superimposed on the diffuse pattern. The radioactivity of these "hot spots" appeared to be insensitive to enzymatic or TCA extraction. (Some of these "hot spots" could be identified with vacuolar inclusions in cells which had been stained and examined prior to auto-

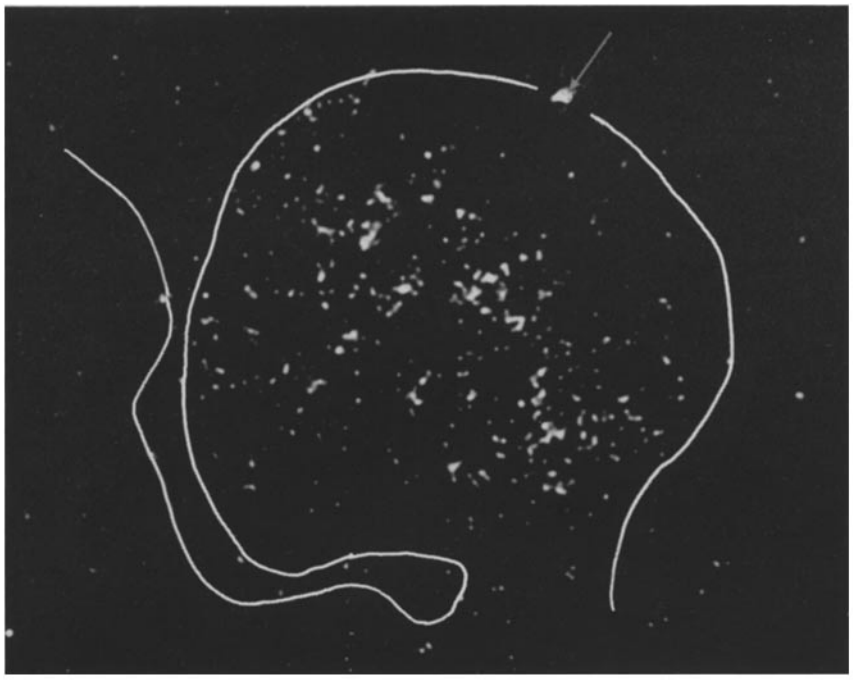

Fig. 4 *c*

C. Autoradiography of Tritiated Thymidine–Labeled, Uncentrifuged Amebae

The results obtained agree with those previously published from this laboratory (10, 11). All the cells examined (over 30) showed diffuse labeling of the cytoplasm (Fig. 2 *a* and *b*). Approximately one-third of the cells also had distinct nuclear autoradiographs. Digestion with DNase or extraction with hot trichloroacetic acid (10 per cent, at 90°C, for 20 minutes) led to preparations in which the diffusely distributed cytoplasmic labeling and the nuclear radioactivity were drastically reduced in comparison with slides incubated with RNase, RNase control solution, or DNase buffer. No obvious difference was noted

radiography.) The amount of diffuse labeling varied considerably from cell to cell. Some variation was also found in the homogeneity of label distribution: the more heavily labeled cells showed a pronounced tendency toward clustering of the grains in the autoradiographic emulsion.

D. Autoradiography of Tritiated Thymidine–Labeled, Centrifuged Amebae

Thirteen labeled amebae, incubated with RNase and stained with acridine orange, were examined with the fluorescence microscope and sketched, and their regions of high concentrations of particles mapped. This involved registration of the position and form of the granule band near

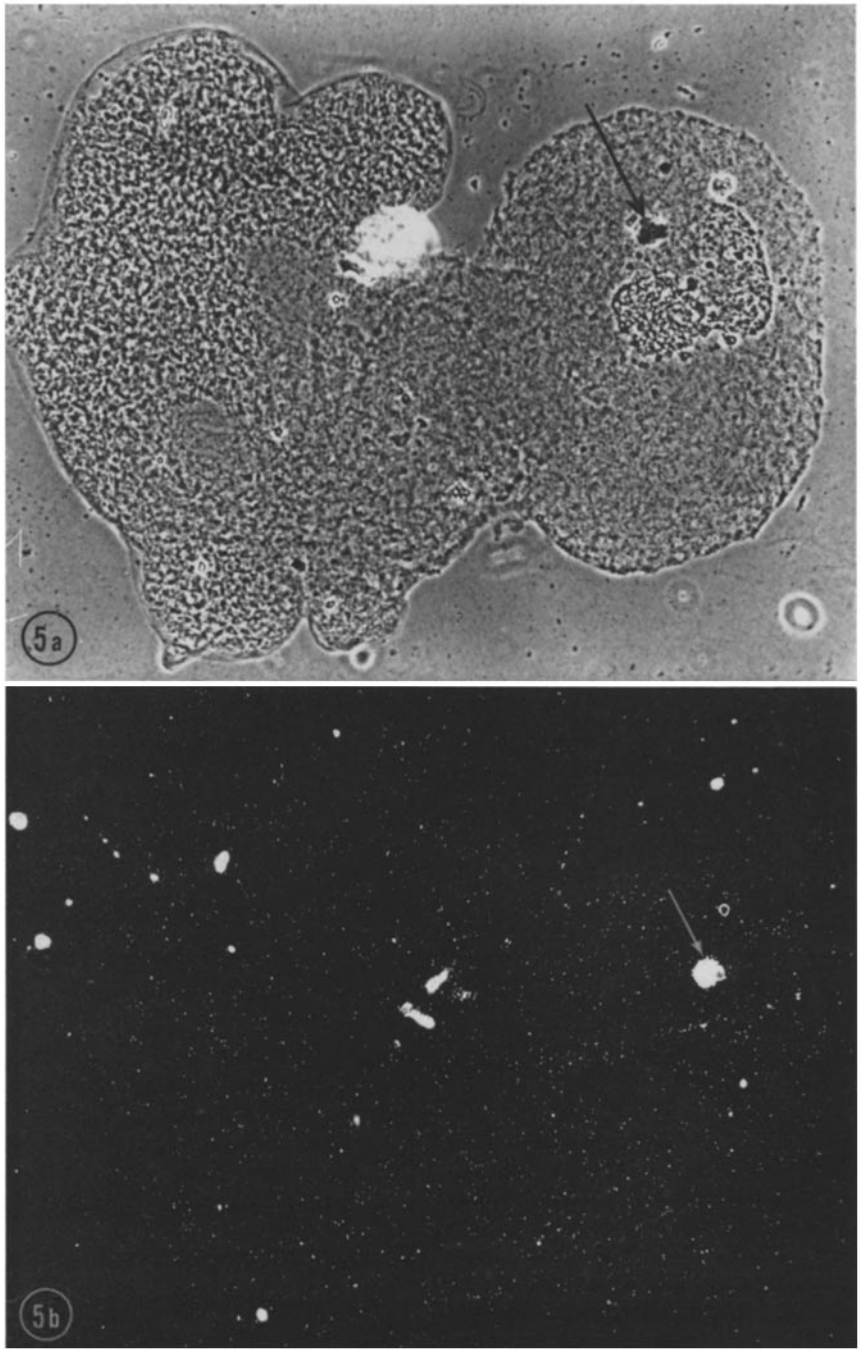

FIGURE 5

Centrifuged ameba; DNase-treated before autoradiography. Phase contrast (Fig. 5 *a*) and darkfield (Fig. 5 *b*) photographs. Arrows indicate common points of reference. Compare with Fig. 3 to assess the effect of DNase treatment.

the centrifugal pole, and recording the position of the nucleus and inclusion bodies and the position of cell extensions ("quasi pseudopods" formed during centrifugation) with and without granules. The autoradiographs of the mapped cells were then examined to establish the degree of coincidence between the positions of silver grains and those of granule concentrations. In all cases the positional correlation was extremely high. Not only was most of the label concentrated over the cell regions where the majority of granules was located, but even in cell extensions where perhaps a few dozen granules had been seen the autoradiographs showed a definite concentration of silver grains. Little if any label was found in the centripetal half of the cells (Fig. 3 *a* and *b*).

A group of 25 labeled amebae, either undigested or treated with the RNase control solution, were briefly stained with basic fuchsin and either mapped as above or photographed. The preparations were then destained, autoradiographed, and analyzed for label distribution. As with the acridine orange preparations, a high degree of correspondence between granules and label position was found (Fig. 4 *a*, *b*, and *c*).

A further group of 33 labeled and centrifuged cells were autoradiographed without previous staining or examination. These cells showed a label distribution which fitted the granule stratification pattern previously described, in that most of the cytoplasmic radioactivity was localized over the region corresponding to the lightly basophilic band above the crystal vacuoles but below the center of the densely basophilic zone. The ratio of autoradiographic grain densities between the granule band and the centripetal pole varied from about 3 to about 15 in 5 cells counted.

Centrifuged cells which were digested with DNase or extracted with TCA before autoradiography (40 cells) showed either no labeling (except over some inclusion bodies) or very light residual labeling (Fig. 5 *a* and *b*). Most cells in which residual label was evident did not show any centrifugal-centripetal gradient in their autoradiograph; however, a slight gradient was discernible in some cases. Several of the DNase- or TCA-treated cells were stained with acridine orange and examined for fluorescence before autoradiography. In none of these cells could particles be detected. There was, however, some yellowish green fluorescence in evidence in several vacuoles. The fact that some of the vacuoles showed labeling which was resistant to extraction or digestion suggests the possible presence there of DNA which is either resistant or not accessible to the degradative reagents employed.

DISCUSSION

The question raised in the introduction to this report was whether or not the incorporation of thymidine into an acid-insoluble, DNase-sensitive molecule in the cytoplasm of *Amoeba proteus* could be traced to a structural organization beyond the level of the polymer itself. The evidence presented argues strongly for a positive answer to the question posed. The mere demonstration that most of the incorporated thymidine can be stratified in a gravitational field of 10,000 *g* indicates that we are not dealing with free individual molecules. The closely parallel sedimentation behavior of demonstrable particles and radioactivity and the DNase-sensitive affinity of these particles for dyes suggest that the thymidine is in fact associated with these structures. (Though it could not be claimed that all the acid-insoluble cytoplasmic radioactivity was restricted to regions where granules were seen or expected in all cells, not all the radioactivity was found to be sensitive to DNase or hot TCA extraction. We believe that it is reasonable to conclude that all the DNase-sensitive fraction of the incorporated thymidine is in or on the granular elements.)

The identification of the labeled molecule as DNA remains tentative: our only knowledge of its chemical composition derives from the presence of thymidine and its behavior toward a series of basic dyes. The negative results obtained with the Feulgen reaction, though not a strong argument against its identification as DNA, prevent us from inferring the presence of hydrolyzable purines. However, the consistent sensitivity of the molecule to nuclease digestion and the characteristic fluorescence color obtained after acridine orange staining define it as at least DNA-like and perhaps permit us to speak of it as DNA.

One might ask whether the particles observed are the actual site of DNA synthesis or simply accumulations of DNA molecules synthesized elsewhere in the cytoplasm. The uniformity of the particles in centrifuged as well as uncentrifuged cells, though not conclusive, argues against

the supposition that the particles are haphazard collections of DNA molecules. A stronger argument for the organized nature of the granules arises from their probable RNA content as well as from their numerical behavior in relation to the ameba's division cycle (22).

Beyond demonstrating the highly probably association of non-nuclear synthesis of DNA-like molecules with a visible structure in *Amoeba proteus*, the present data provide no information on the precise nature of this association. It will be of considerable interest to subject this question to reexamination with the now available methods of electron microscopic autoradiography.

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