

Nuclear Synthesis of Cytoplasmic Ribonucleic Acid in *Amoeba proteus*

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ABSTRACT

The enucleation technique has been applied to *Amoeba proteus* by several laboratories in attempts to determine whether the cytoplasm is capable of nucleus-independent ribonucleic acid synthesis. This cell is very convenient for micrurgy, but its use requires a thorough starvation period to eliminate the possibility of metabolic influence by food vacuoles and frequent washings and medium renewal to maintain asepsis. In the experiments described here, amoebae were starved for periods of 24 to 96 hours, cut into nucleated and enucleated halves, and exposed to either C-14 uracil, C-14 adenine, C-14 orotic acid, or a mixture of all three. When the starvation period was short (less than 72 hours), organisms (especially yeast cells) contained within amoeba food vacuoles frequently showed RNA synthesis in both nucleated and enucleated amoebae. When the preperiod of starvation was longer than 72 hours, food vacuole influence was apparently negligible, and a more meaningful comparison between enucleated and nucleated amoebae was possible. Nucleated cells incorporated all three precursors into RNA; enucleated cells were incapable of such incorporation.

The experiments indicate a complete dependence on the nucleus for RNA synthesis. The conflict with the experimental results of others on this problem could possibly stem from differences in culture conditions, starvation treatment, or experimental conditions. For an unequivocal answer in experiments of this design, ideally the cells should be capable of growth on an entirely synthetic medium under aseptic conditions. The use of a synthetic medium (experiments with *A. proteus* are done under starvation conditions) would permit, moreover, a more realistic comparison of metabolic capacities of nucleated and enucleated cells.

INTRODUCTION

One of the functions of the cell nucleus during the interphase period is the synthesis of RNA (9, 1, 7, 17). There are, in addition, good experimental data indicating that such nuclear RNA is transferred to the cytoplasm across the nuclear membrane during interphase (9, 8, 17) and again during mitosis when the nuclear membrane is absent or in a highly altered state (13). Such synthesis and transfer are in full accord with the hypothesized role of RNA as the carrier of genetic information from the nucleus to centers of protein synthesis in the cytoplasm. The scheme is not supported by reports that the cytoplasm is capable of independent RNA production (10, 3), and, as correlative evidence of this, that there are differences in purine-pyrimidine ratios between cytoplasmic and nuclear RNA (16, 6). These data could

be reconciled with the proposition that nucleus-synthesized RNA carries genetic information by postulating a distinctly different function for RNA synthesized in the cytoplasm. Alternatively, genetic specificity for the synthesis of RNA in the cytoplasm might be assumed to be imparted by RNA derived from the nucleus.

There is still no final agreement, however, concerning a cytoplasmic capacity for RNA synthesis. Brachet and Szafarz (4) and Brachet *et al.* (3) have reported RNA synthesis in enucleated fragments of the unicellular alga, *Acetabularia*. Richter (14), however, was unable to detect any increase in the RNA content of enucleated *Acetabularia*. Previously, the author reported that enucleated *Amoeba proteus* were incapable of incorporating C-14 uracil into RNA, while nucleated cells showed strong incorporation (12). It was concluded that

no RNA synthesis was possible in an amoeba without a nucleus. C-14 uracil incorporation is not so vigorous as C-14 adenine incorporation, and in very similar experiments Plaut and Rustad (10) obtained contradictory data using C-14 adenine as a precursor; most of the enucleated amoebae were clearly capable of adenine incorporation, and therefore presumably of RNA synthesis. These latter results on amoeba have been confirmed in Brachet's laboratory (15) and by the author, not only with C-14 adenine but also with C-14 orotic acid. Further studies, to be described in this report, indicate that the results of these experiments are not unequivocal, and synthesis of RNA by enucleated cells has not been established with finality in amoeba.

Materials and Methods

A. proteus was cultured as described previously (11). The amoebae were starved at 18°C. for periods varying from 24 to 96 hours, cut by hand into enucleated and nucleated halves, and exposed for 1 to 5 days to either C-14 uracil, C-14 adenine, C-14 orotic acid, or a mixture of all three in inorganic amoeba medium with a concentration of 1 to 2 microcuries per ml. in each case. The length of the starvation period has an important bearing on the results of the experiment (see below). During the starvation period great care was exercised to eliminate all contaminating organisms through frequent washings in sterile, inorganic amoeba medium containing penicillin and tetracycline (both at 1 mg./ml.). Yeast cells were the most troublesome contaminants. Concentrations of an antimycotic (mycostatin; Squibb and Sons, New York) sufficient to prevent yeast proliferation also cause cytolysis of the amoebae. Viable yeast cells are sometimes released from amoeba food vacuoles even after 3 days of starvation.

During the exposure to the radioactive solution the experimental cultures were frequently checked for the appearance of contaminating organisms, and, as an added safety measure, the amoebae were transferred to a fresh, sterile radioactive solution every 10 to 14 hours with an intervening wash in the antibiotic solution. These detailed, precautionary procedures are necessary in order to minimize the possibility that contaminating organisms contribute by their metabolism to the experimental results. Following the exposure to the radioactive precursors the amoebae were squashed on slides, fixed with cold acetic acid-alcohol (1:3) for 2 minutes, and washed in a large volume of distilled water at 2°C. for 1 hour to remove unincorporated precursor. In one experiment the cells were extracted with 5 per cent perchloric acid for 30 minutes at 0°C., but the results were not detectably different from those obtained with simple cold water extraction. As a con-

trol in each experiment, a few nucleated and enucleated amoebae were treated with ribonuclease (Sigma Chemical Co.; 5x crystallized; 0.1 mg./ml., pH 7.0) for 30 minutes at 22°C. Incorporated radioactivity is 90 per cent to 100 per cent removed by this treatment. Incorporated radioactivity was assessed by autoradiography with exposure times of 2 to 6 weeks.

EXPERIMENTAL RESULTS AND DISCUSSION

Nucleated amoebae incorporate all three precursors into RNA. The autoradiograph in each case is always much heavier over the nucleus than over the cytoplasm (see Figs. 1, 2). C-14 adenine incorporation is consistently the heaviest and uracil the lightest. Even after 96 hours of previous starvation the incorporation of C-14 precursors into RNA is active enough to give excellent autoradiographs.

Amoebae starved for 24 hours still contain numerous food vacuoles. When such amoebae are exposed to the C-14 precursors, the incorporation into RNA contained in a certain number of the food vacuoles is much heavier than that of the surrounding amoeba cytoplasm (see Fig. 2) and sometimes more concentrated than the activity of the amoeba nucleus. Starvation of amoebae for 48 hours reduced markedly the number and size of the food vacuoles, but the situation in many of the amoebae is qualitatively the same; *i.e.* there is light but definite incorporation of radioactivity into food vacuole material. After 72 hours of starvation the food vacuoles are few and small, and incorporation of radioactivity into these is either very slight or entirely absent. With 96 hours of starvation the food vacuoles are very small, and only rarely is there a suggestion of radioactivity in a food vacuole. Incorporation into the amoeba nucleus, even after this extended starvation, remains strong (Fig. 1).

The food vacuoles originally contain living organisms, and under the particular culture conditions used in this case these are either *Tetrahymena*, bacteria, or yeast cells. The food organisms are gradually digested over a period of several days, but viable yeast cells (presumably undigestible) are sometimes released from an amoeba after a sojourn of 2 or even 3 days in a food vacuole. The radioactivity taken up by food vacuoles is not extractable with cold water or cold perchloric acid (5 per cent for 5 minutes), but is almost completely removed by ribonuclease digestion. Presumably, these organisms contained within vacuoles continue to incorporate precursors into

RNA at least during the first 2 days of starvation, and, in this manner, contribute to the over-all radioactivity of RNA that is observed within either nucleated or enucleated amoebae. It is obvious that a clear distinction must be made between the activities of the amoeba itself and the activities of the organisms (or possibly even partially digested organisms) within the amoeba food vacuoles or contained in the medium as trace contaminants.

The essential point is to be able to compare the activities of amoebae with and without nuclei, and to do this unequivocally requires that contributions to synthesis by contaminants in food vacuoles or elsewhere be eliminated with certainty from both nucleated and enucleated cells. A starvation period in excess of 72 hours and careful washing of the amoebae before the initiation of the isotope feeding appear to be the minimum requirements in the case of RNA studies. Although food vacuole remnants certainly persist beyond 3 or 4 days of starvation, their independent metabolic abilities are reduced to an undetectable level. As pointed out by Cohen (5), however, the possibility of various metabolic contributions by food vacuole organisms to the over-all amoeba metabolism has been too often ignored or overlooked. Cohen has also demonstrated the virtual impossibility of obtaining an amoeba which is completely free of food vacuoles. After many days of starvation, at which time the amoebae contain no food vacuoles which can be identified under light microscopy, food vacuole remnants are still detectable with electron microscopy.

Enucleated amoebae obtained from whole amoebae starved less than 72 hours frequently show incorporation of radioactive precursors into RNA. The shorter the starvation period, and therefore, presumably, the greater the possibility for synthetically active food vacuole organisms to be present, the larger is the proportion of enucleated amoebae that shows apparent RNA synthesis. Fig. 3 is an autoradiograph of an enucleated cell that was starved for 24 hours and exposed to C-14 adenine for only 24 hours, and shows incorporation at the level of food vacuoles. Any detectable radioactivity in enucleated amoebae exposed to C-14 adenine for longer periods (48 hours or more) is not heavily localized in food vacuoles, as in Fig. 3, but is more evenly dispersed through the cytoplasm, possibly as a result of digestion. Considering the situations in which very large molecules move across cell membranes (pinocytosis; the

uptake of RNA by amoebae (2)), it seems possible that transfer of RNA molecules or fragments from food vacuole to amoeba cytoplasm may occur as a part of the digestive process. In any case, there is a correlation between the degree of starvation of an enucleated amoeba and the amount of apparent RNA synthesis. The objection that starvation of the whole amoeba prior to the experiment reduces the ability of the subsequent enucleated amoeba to incorporate precursors into RNA is incompatible with the strong incorporation activity of the nucleated half derived from the same starved cell.

The effectiveness of a starvation period in the elimination of food organism contributions depends upon such variables in stock cultures as the type and supply of food organisms. In a rapidly growing culture (amoeba generation time of 22 to 24 hours at 23°C.) the amoebae tend to be small, contain appreciably fewer food organisms, and are definitely more easily starved free of major food vacuoles. The efficiency of a given starvation period will, therefore, vary from one culture to another. This circumstance could possibly contribute to the conflict of results obtained with C-14 precursors of RNA in the enucleate-nucleate studies with *A. proteus*. Plaut and Rustad (10) also found considerable variability within their experiment; the activity incorporated by enucleated cells varied from none to a level equivalent to that found in the least active nucleated cells. This variability could conceivably be related to various states of starvation.

It should be understood also that food vacuoles are not always easily visible in starved amoebae. Some vacuoles contain only a few bacteria or a few yeast cells. The latter are easily mistaken for cytoplasmic granules. Furthermore, even after 96 hours of starvation it is unusual to find an amoeba which does not have at least a few very small Feulgen positive bodies scattered in the cytoplasm. Presumably these are food vacuole remnants.

Fig. 1 shows an autoradiograph of a nucleated and an enucleated cell derived from amoebae starved for 96 hours and then exposed to a mixture of C-14 uracil, C-14 orotic acid, and C-14 adenine for 3 days. Starvation of this duration has yielded uniform incorporation results; of 47 enucleated cells all except one were completely free of detectable activity; the exception contained a small amount of radioactivity. Nucleated cells all showed approximately the same level of nuclear and cytoplasmic radioactivity. With the exception of lighter

radioactivity incorporation, the results were the same with C-14 precursors employed singly.

In view of the complications introduced by the possibility of contributions by organisms included in food vacuoles to the synthetic events in *A. proteus* and the difficulties of maintaining aseptic conditions, it may be advisable to repeat certain of the nucleated-enucleated amoeba studies dealing with other metabolic activities with more attention to asepsis and appropriate starvation. Alternatively, nucleate-enucleate studies with a cell which can be grown aseptically in a complete nutrient medium would offer certain opportunities for more conclusive results. *Acanthamoeba sp.*, for example, fills these requirements, and efforts to apply micrurgy to this cell are underway in this laboratory. Unfortunately, the small size of *Acanthamoeba* (20 to 30 microns in length) greatly increases technical difficulties.

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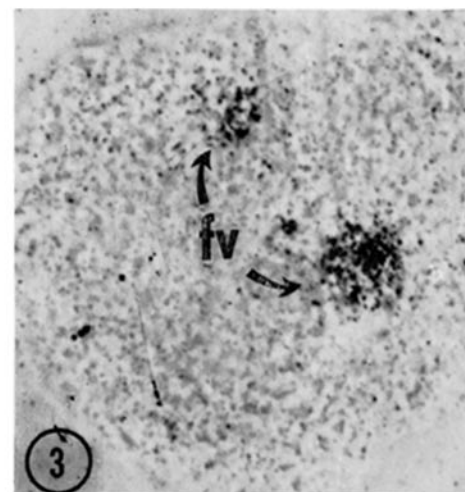
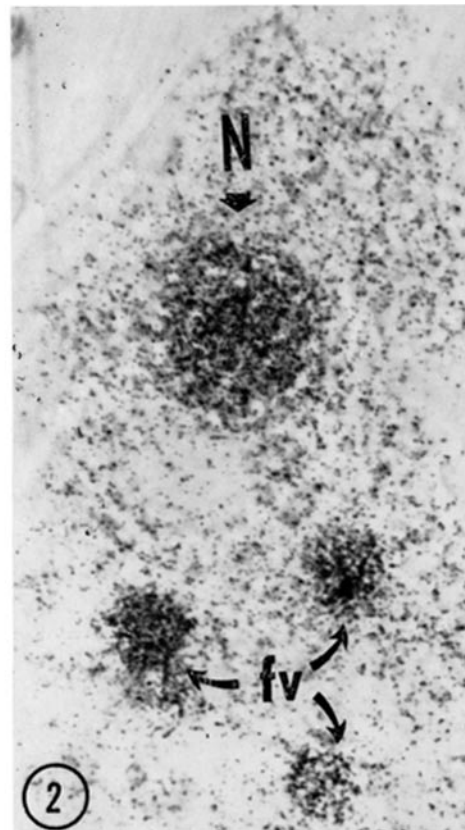
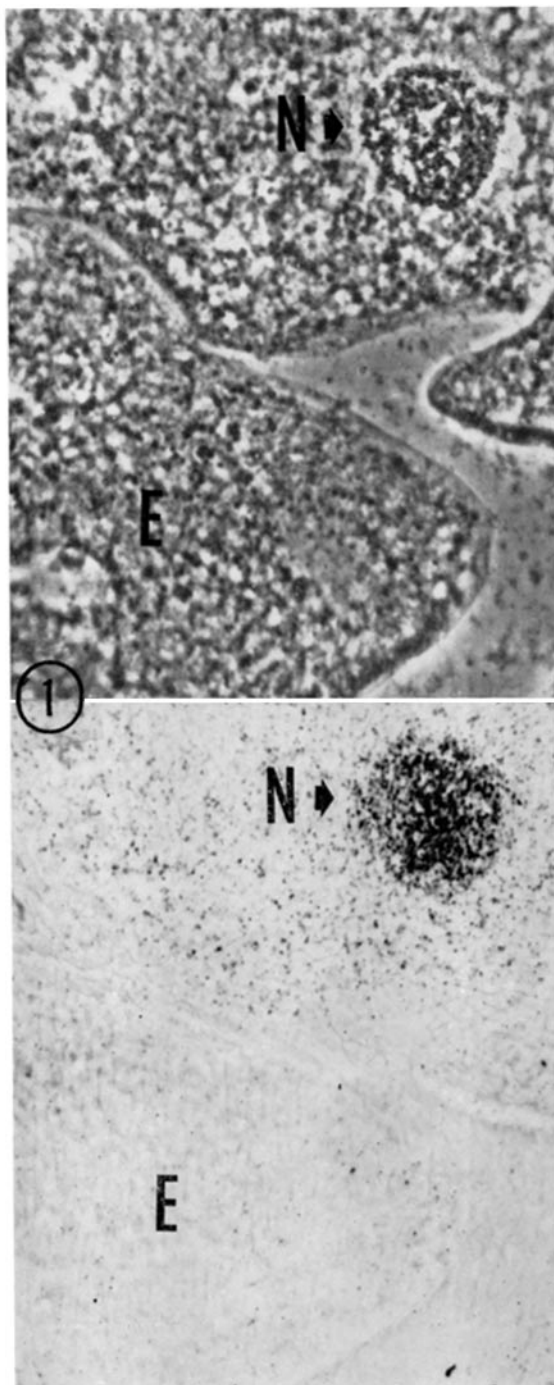
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EXPLANATION OF PLATE 115

FIG. 1. The lower photograph shows the autoradiograph over the cells which are shown by the phase contrast photograph immediately above; *E*, enucleated half amoeba; *N*, nucleus within a nucleated half. These amoeba fragments were incubated for 3 days in a mixture of C-14 adenine, C-14 orotic acid, and C-14 uracil. The incorporation of radioactivity is heavy in the nucleus, moderate in nucleated cytoplasm, and equivalent to background in the enucleated cell.

FIG. 2. An autoradiograph of a nucleated half of *A. proteus* incubated for 24 hours in C-14 adenine. The preperiod of starvation was 24 hours. There is heavy radioactivity incorporation into the nucleus (*N*) and into food vacuoles (*fv*).

FIG. 3. An autoradiograph of an enucleated half of *A. proteus* incubated for 24 hours in C-14 adenine medium. The preperiod of starvation was 24 hours. The concentrations of silver grains are over food vacuoles (*fv*).



(Prescott: Nuclear control of RNA synthesis)