

STUDIES ON NUCLEAR STRUCTURE AND FUNCTION IN *TETRAHYMENA PYRIFORMIS*

II. Isolation of Macro- and Micronuclei

MARTIN A. GOROVSKY

From the Whitman Laboratory, the Department of Biology, The University of Chicago, Chicago, Illinois 60637. Dr. Gorovsky's present address is the Department of Biology, The University of Rochester, Rochester, New York 14627

ABSTRACT

This report describes a rapid, efficient method for isolating macronuclei from *Tetrahymena*. The macronuclear fraction contains only small amounts of micronuclear material and little detectable whole cell or cytoplasmic contamination. A method is also described for preparing a "micronuclear fraction" which contains 20-40 micronuclei for every macronucleus present. Electron microscope observations indicate that the ultrastructure of the nuclei in the macronuclear fraction closely resembles that of nuclei *in situ*. The presence of ribosomes on the outer membrane of micronuclei and of pores in the micronuclear envelope is also described.

INTRODUCTION

Tetrahymena is a ciliated protozoan that can be cultured axenically and in defined media, and it is possible to manipulate culture conditions to obtain large numbers of cells in different physiological states. *Tetrahymena* can also be rapidly labeled with radioactive molecules under a variety of growth and experimental conditions (Gorovsky and Woodard, 1969; Kumar, 1970; Cameron, 1966; Cameron and Guile, 1965; Prescott, 1960), and is sensitive to many of the inhibitors frequently employed in metabolic studies of the synthesis and assembly of macromolecules (Frankel, 1967; Rosenbaum and Carlson, 1969; Gorovsky, 1969). In short, the growth and metabolism of *Tetrahymena* can be controlled and studied with an ease usually associated with studies of prokaryotic organisms. However, *Tetrahymena* is a eukaryote. Like most ciliates, each cell contains two nuclei, a macro- and a micronucleus. Both nuclei are surrounded by a nuclear envelope (Elliott et al., 1962; Flick-

inger, 1965; Cameron et al., 1966) and contain DNA¹ which is associated with histones (Alfert and Goldstein, 1955; Gorovsky, 1968). The macronucleus contains typical nucleoli (Elliott, 1963; Flickinger, 1965; Swift et al., 1964; Cameron et al., 1966; Gorovsky, 1968) and numerous ribonucleoprotein particles (Swift et al., 1964; Gorovsky, 1968), and appears to synthesize large amounts of RNA (Gorovsky and Woodard, 1969). In addition, the synthesis and processing of ribosomal RNA in macronuclei is not unlike that of mammalian cells (Kumar, 1970). Although the macronucleus undergoes amitotic division, the micronucleus contains chromosomes (Alfert and Balamuth, 1957; Elliott, 1963; Ray, 1956) and divides mitotically with an intranuclear spindle (Elliott, 1963). Taken together, then, these two

¹ Abbreviations: RNA, ribonucleic acid; DNA, deoxyribonucleic acid.

nuclei have many, if not all, of the properties associated with eukaryotic nuclei (Ris and Chandler, 1963). *Tetrahymena*, therefore, combines many of the growth characteristics of bacteria with a nuclear apparatus similar to that of higher organisms, and would seem to be an excellent cell for studying nuclear functions.

Equally important, however, is the fact that the macronucleus and micronucleus of *Tetrahymena* (and perhaps of other ciliates) present a unique opportunity to explore the underlying molecular mechanisms by which the same genetic information acquires and maintains very different morphological and functional properties. Both the macronucleus and micronucleus arise from the division of a single nucleus during conjugation, and probably contain similar, if not identical, genomes. Nonetheless, they differ markedly in structure, ploidy level (Gorovsky and Woodard, 1969; Woodard et al., 1968), time of DNA synthesis in the cell cycle (Flickinger, 1965; Woodard, Gorovsky and Kaneshiro, unpublished observations), and RNA synthetic capacity. The micronucleus, unlike the macronucleus, does not contain a nucleolus or any ribonucleoprotein particles, and appears to synthesize little, if any, RNA (Gorovsky, 1968; Gorovsky and Woodard, 1969).

For these reasons, it is of interest to study and to compare the structure, chemistry, and function of *Tetrahymena* macro- and micronuclei. One approach to studying cell organelles is to examine their properties, *in vitro*, after isolation. This report describes a rapid, efficient method for isolation of a macronuclear fraction from *Tetrahymena* and for preparing a micronucleus-rich fraction which is approximately 50% pure. Observations on the ultrastructure of the nuclei in the macronuclear fraction and of nuclei *in situ* are also presented.

MATERIALS AND METHODS

Culture Methods

Cells of mating type I, variety I of *Tetrahymena pyriformis* were grown axenically in an enriched proteose peptone medium (Dr. Frank Child, personal communication) containing 2.0% proteose peptone, 0.2% glucose, 0.1% yeast extract, and 0.003% sesquetrine.² Stock cultures were maintained in 5 ml of medium in

² An iron-EDTA complex, available from Geigy Chemical Corporation, Ardsley, New York.

10-ml culture tubes and were transferred as necessary. For actual use, a 5 ml culture was used to inoculate 25 ml of medium in a 250 ml Erlenmeyer flask. After 48 hr of growth, this 30 ml culture, containing $1-2 \times 10^6$ cells/ml, was used to inoculate 3 liters of medium in a 5 liter diphtheria toxin bottle. The cells were grown for 24-72 hr at 23-28°C under vigorous aeration produced by bubbling air through a gas dispersion tube.³ Cell densities as high as 2×10^6 cells/ml can be obtained under these conditions. Cultures were routinely used at densities of $0.5-1.2 \times 10^6$ cells/ml.

Collection of Cells

6 liters of culture were concentrated to 500 ml by passing the medium through a modified cream separator (Model 100, DeLaval Separator Co., Chicago, Ill.). The cells were then pelleted by centrifugation at 1000 *g* for 10 min.

Electron Microscopy

Whole cell or "macronuclear" pellets were fixed in either 10% formalin (3.7-3.8% formaldehyde) in 0.18 M phosphate buffer, pH 7.4, for 1 hr at 0-5°C, or in 6.0% glutaraldehyde in 0.15 M phosphate buffer, pH 6.8, followed by 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.4, for 2 hr. The pellets were broken up in the fixative, and the fragments were dehydrated in a graded series of alcohols, and were embedded in Epon 812 (Luft, 1961). Sections were cut on a Porter-Blum ultramicrotome, stained in lead citrate (Reynolds, 1963) and/or uranyl acetate (Swift et al., 1964), and examined with an RCA EMU 3C or a Siemens Elmiskop I electron microscope.

Light Microscopy of Isolated Nuclei

So as to determine the effects of methodological modifications on recovery of nuclei, whole cells and isolated fractions were fixed and stained in 0.4% methyl green in 6.0% (v/v) acetic acid containing 2×10^{-3} M CaCl₂ (Kuehl, 1964), and were counted in a hemocytometer. Isolated fractions also were examined with phase contrast optics.

Cytochemical "spot" tests were performed on nuclei which had been smeared on slides, air dried, and postfixed in 100% ethanol. These tests included the Feulgen reaction for DNA, alkaline fast green for histones, Sakaguchi reaction for arginine, azure B with and without prior extraction of nucleic acids with nucleases, and low pH fast green for total protein.

³ Sterile Dow-Corning Antifoam B was added as necessary for preventing foaming.

RESULTS AND DISCUSSION

Isolation of the "Macronuclear Fraction"

All operations were performed at 0–5°C, using an International PR-2 or PR-6 centrifuge, swinging bucket head No. 253. The isolation method was a modification of that used by Kuehl (1964) for plant nuclei. The packed cells were resuspended in 500 ml of Medium A (0.1 M sucrose, 4.0% gum arabic,⁴ 1.5 mM MgCl₂, pH 6.75 with NaOH) and were washed twice by centrifugation at 1000 g for 5 min. The cells were then resuspended in Medium B (0.63 ml of n-octyl alcohol per 100 ml of Medium A) at a concentration of 1/2–4 ml cells/40 ml Medium B, and were homogenized in 40-ml aliquots in the semimicro attachment of a Waring blender. Two-6 sec at the higher of the two blender speeds usually were sufficient to disrupt the cells. Approximately 10% of the nuclei were lost during homogenization.

The homogenate was then centrifuged at 1000 g for 10 min, sedimenting the nuclei while most of the nonnuclear components either remained in suspension or formed a "skin" at the top of the tube. The supernatants and "skins" were pooled, shaken vigorously, and nuclei were collected again. Three such centrifugations served to collect most nuclei. The crude nuclear fractions were pooled, resuspended in 80 ml of Medium A, and washed by centrifugation at 1000 g for 10 min (two times) and then at 250 g for 15 min. The final pellet contains both macro- and micronuclei, but, owing to the 12–24-fold excess of DNA in macro- over micronuclei (Gorovsky and Woodard, 1969; Woodard, Gorovsky, and Kaneshiro, 1968), contains 90–95% macronuclear DNA and only 5–10% micronuclear DNA. This pellet is referred to as the "macronuclear fraction." Recoveries of macronuclei in this fraction averaged 56% (10 isolations, range 43–71%).

Purity of the "Macronuclear Fraction"

A number of criteria attest to the purity of the isolated macronuclei. By phase contrast, this fraction was free of whole cells and contaminating cytoplasmic organelles (Fig. 1). Electron micrographs indicated that the occasional contaminants were whorls of ribosome-studded membranes,

⁴ Medium A or a stock solution of 20% gum arabic was centrifuged at 3,000 g for 1 hr for removing large contaminants found in commercial gum arabic.

small ciliary and pellicular fragments, and food vacuoles. Layering the resuspended nuclei over denser sucrose solutions (0.5–1.0 M) and centrifuging at 1000 g (15–30 min) removed most of these remaining cytoplasmic elements. However, since they were present in small amounts, and losses of nuclei by these methods were considerable, we have not routinely attempted to remove them.

Although nuclei were generally free of large, adhering cytoplasmic tabs, ribosomes which were attached to the outside of the nuclear envelopes (Fig. 5) of both the macro- and the micronuclei probably represented the major cytoplasmic contaminants. These perinuclear ribosomes are obligatory contaminants of nuclei isolated by aqueous methods which leave nuclear membrane intact (Maggio et al., 1963). They can be removed from *Tetrahymena* nuclei by treating the nuclei with dilute detergents (Gorovsky, 1969) in a manner similar to that used to produce cytoplasm-free nuclei in HeLa cells (Holtzman et al., 1966).

Kumar (1970) has shown that nuclei isolated by these techniques contain no detectable peak of newly synthesized 17s ribosomal RNA, even when this type of RNA is present in large amounts in the cytoplasm, and DNA and RNA determinations (Spirin, 1958) have shown a preponderance of DNA over RNA (RNA/DNA = 0.2–0.4) in these macronuclear fractions. Therefore, chemical as well as morphological criteria attest to the purity of the macronuclear fraction isolated by this method.

Light Microscope Cytochemistry of the Isolated Macronuclear Fraction

Isolated macro- and micronuclei both gave strongly positive cytochemical reactions for DNA (Feulgen), histone (alkaline fast green), total protein (fast green, pH 2.0), and arginine (Sakaguchi). The macronucleus, but not the micronucleus, stained strongly with azure B after deoxyribonuclease digestion, indicating the presence of RNA.

Morphology of the Isolated Macronuclear Fraction

When viewed by phase contrast, the macronuclear fraction contained apparently intact, spheroidal macronuclei (Figs. 1 and 2). Many were associated with a micronucleus (Fig. 2) which

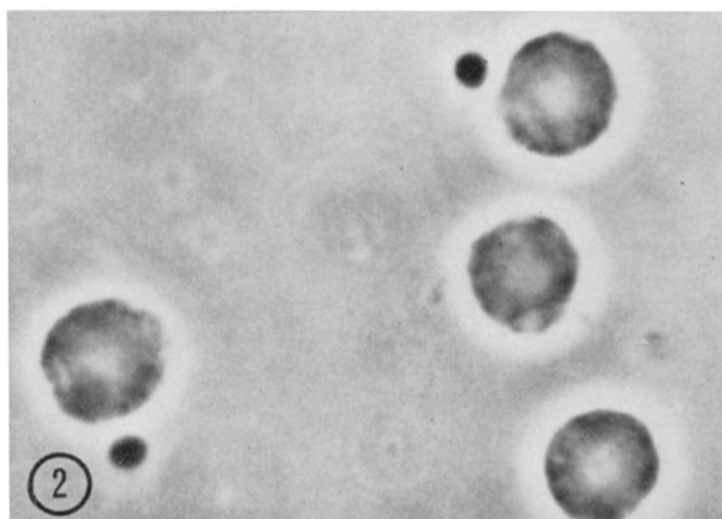
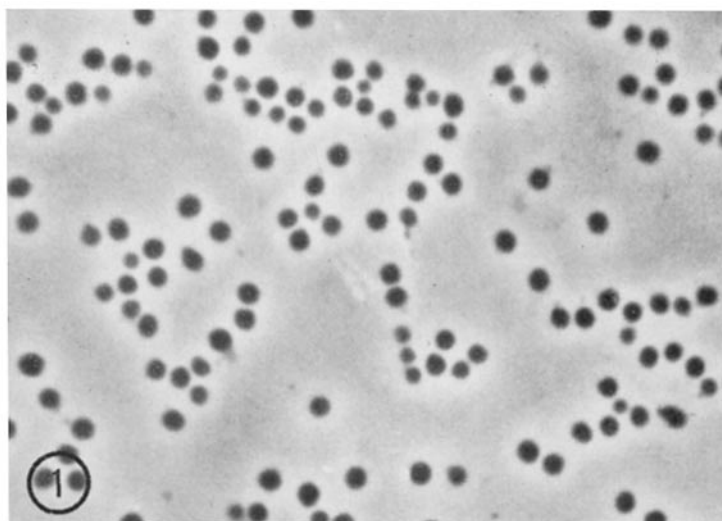


FIGURE 1 Low-magnification phase micrograph of isolated macronuclear fraction. About $\times 200$.

FIGURE 2 High-magnification phase micrograph of isolated macronuclear fraction showing macronuclei and micronuclei. About $\times 2300$.

was often located within a small inpocketing of the macronucleus.

Electron microscope examination revealed that many of the nuclear cross-sections had damaged or folded nuclear envelopes (Fig. 4). Where intact, the outer aspect of the macronuclear envelope was covered with cytoplasmic ribosomes (Fig. 5). The bulk of the macronucleus was filled with the dense, spheroidal bodies typical of ciliate nuclei (Figs. 4-6). In the nucleoplasm between the chromatin bodies lie irregular clumps of inter-

chromatin granules of various sizes, ranging from 200 to 600 A in diameter (Figs. 5, 6).

The numerous nucleoli were located just inside the macronuclear membrane (Figs. 4-6). They were somewhat irregular in size and shape, but were consistently larger than the chromatin bodies. Nucleoli were made up of three distinct components: (a) an outermost granular region containing particles slightly smaller than the cytoplasmic ribosomes seen adhering to the outer nuclear membrane (Fig. 5); (b) a middle amor-

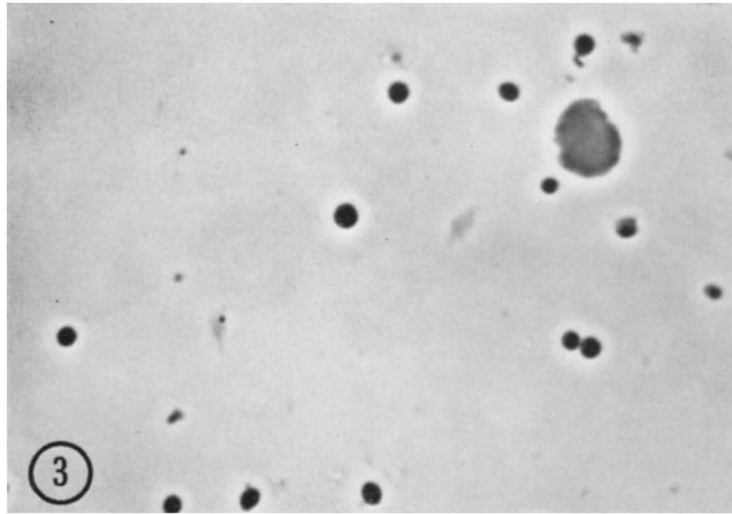


FIGURE 3 Light micrograph of micronuclear fraction stained with methyl green. A macronuclear fragment has been deliberately included for comparison with the micronuclei. These preparations contained approximately one such macronuclear fragment for every 20–40 micronuclei. About $\times 1200$.

phous region (Fig. 5); (c) a small inner region of low density containing a clump of electron-opaque, chromatin-like material (arrow, Fig. 6).

Micronuclei present in the “macronuclear fraction” were bounded by an envelope which was studded with ribosomes on its outer surface and was indistinguishable from the macronuclear membrane. In contrast to the wealth of subnuclear organelles (nucleoli, chromatin bodies) and heterogeneous particles found in macronuclei, micronuclei consisted largely of highly condensed chromosome-like threads (Fig. 5).

Morphology of Nuclei In Situ

Macronuclei *in situ* contain nucleoli, chromatin bodies, and interchromatin granules similar to those described above for isolated nuclei (Fig. 7). It is clear, however, that nuclei *in situ* were not so compact as the isolated nuclei, and the subnuclear organelles appeared to be more dispersed in the nucleoplasm. It is not known if these volume differences resulted from marked shrinkage of nuclei during the isolation itself or from a differential response of nuclei to fixation under different conditions (i.e. *in situ* compared to isolated).

Micronuclei fixed *in situ* contained the chromosome-like coil observed in isolated micronuclei (Fig. 7). The micronuclear membrane *in situ* was

also extensively covered with cytoplasmic ribosomes (Fig. 7), and clearly showed the presence of nuclear envelope “pores” (Fig. 8). The presence of ribosomes on the outer membrane of the micronuclear envelope raises interesting questions regarding the origin and function of these ribosomes since the absence of a nucleolus and of any detectable ribonucleoprotein particles in the micronucleus makes it unlikely that the ribosomes are synthesized by the micronucleus itself. Similarly, the presence of pores in the micronuclear envelope is of interest, since it is also unlikely that the micronucleus synthesizes any RNA for transport to the cytoplasm.

Isolation of the “Micronuclear Fraction”

“Micronuclear fractions” were prepared from pooled, purified macronuclear fractions (which contained micronuclei) which had been stored at -25°C for 1 day–4 wk. No differences were noted between nuclei which had been frozen for various times.

Macronuclear fractions were resuspended in 40–80 ml of Medium B and were homogenized for 15 sec at high speed in the Waring blender. This procedure served to break a large number of the previously intact macronuclei into very small fragments, but had little effect on the structure of micronuclei (at the light microscope level).

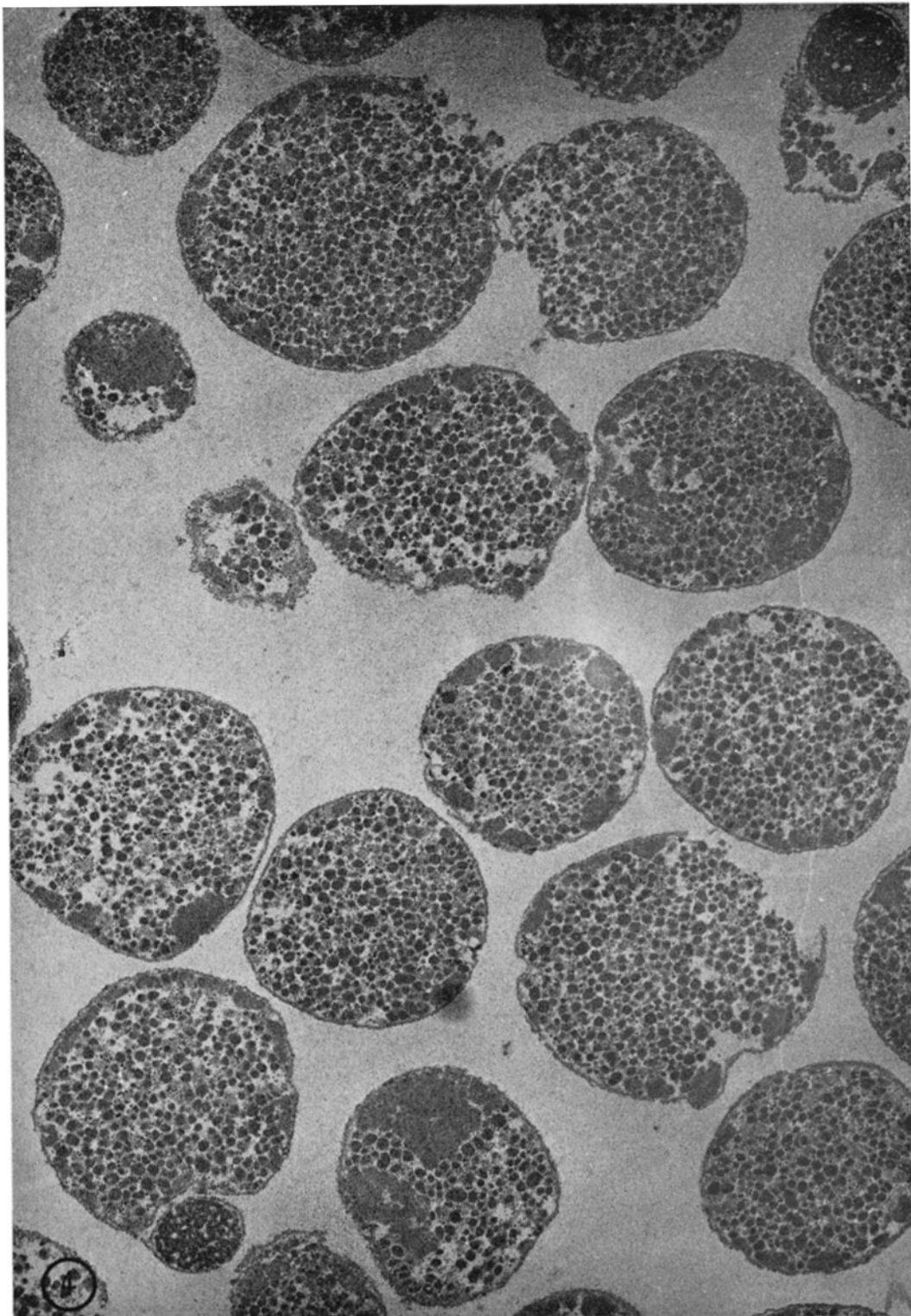


FIGURE 4 Electron micrograph of isolated macronuclear fraction (glutaraldehyde- OsO_4 , uranyl acetate, lead citrate; about $\times 7400$.)

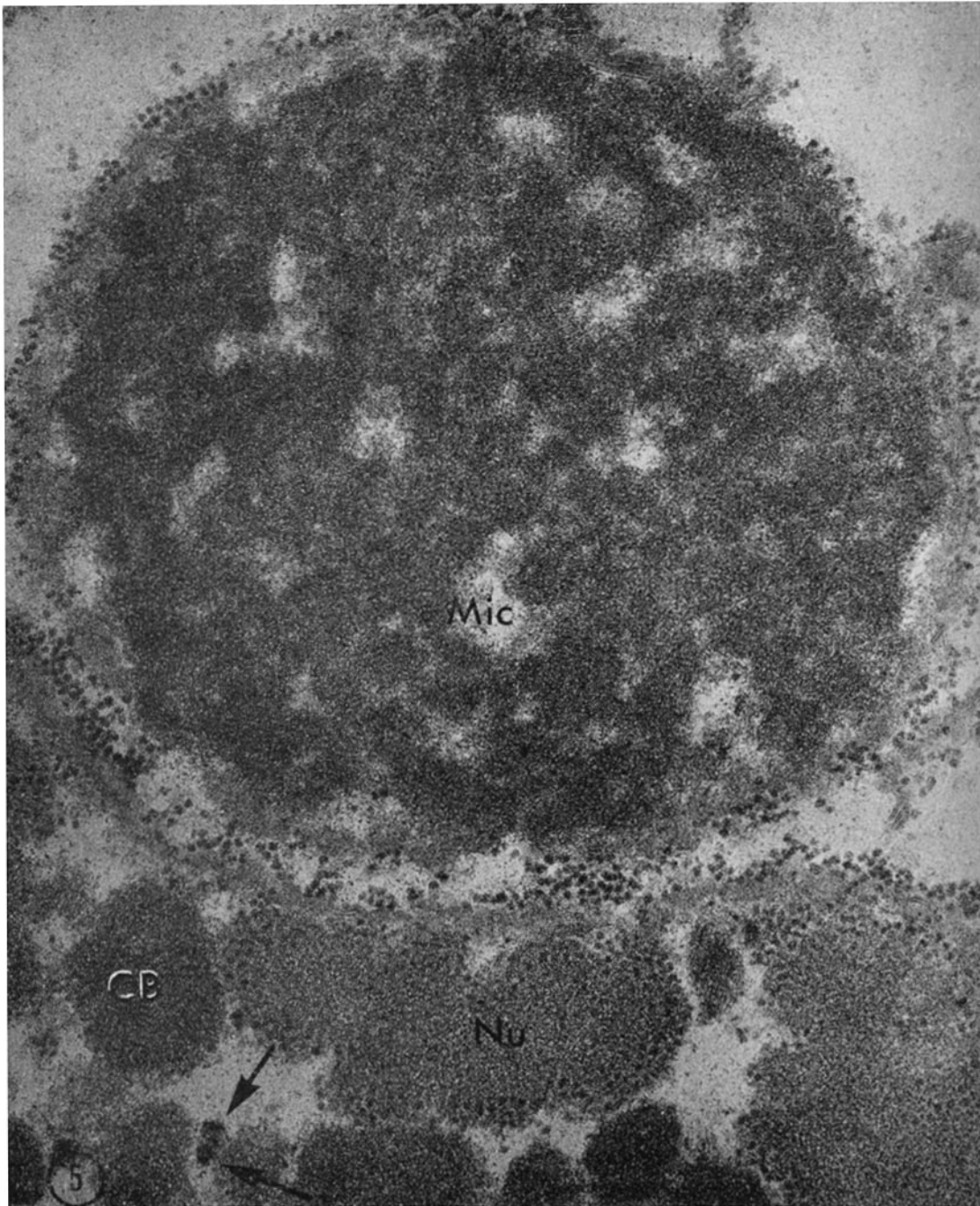


FIGURE 5 Electron micrograph of portions of an isolated macronucleus and micronucleus. Both nuclei have ribosome-studded nuclear envelopes. The macronucleus contains nucleoli (*Nu*); chromatin bodies (*CB*); and interchromatin granules (arrows). The micronucleus (*Mic*) appears to contain only a chromosome-like coil (glutaraldehyde- OsO_4 , uranyl acetate, lead citrate; about $\times 70,000$.)

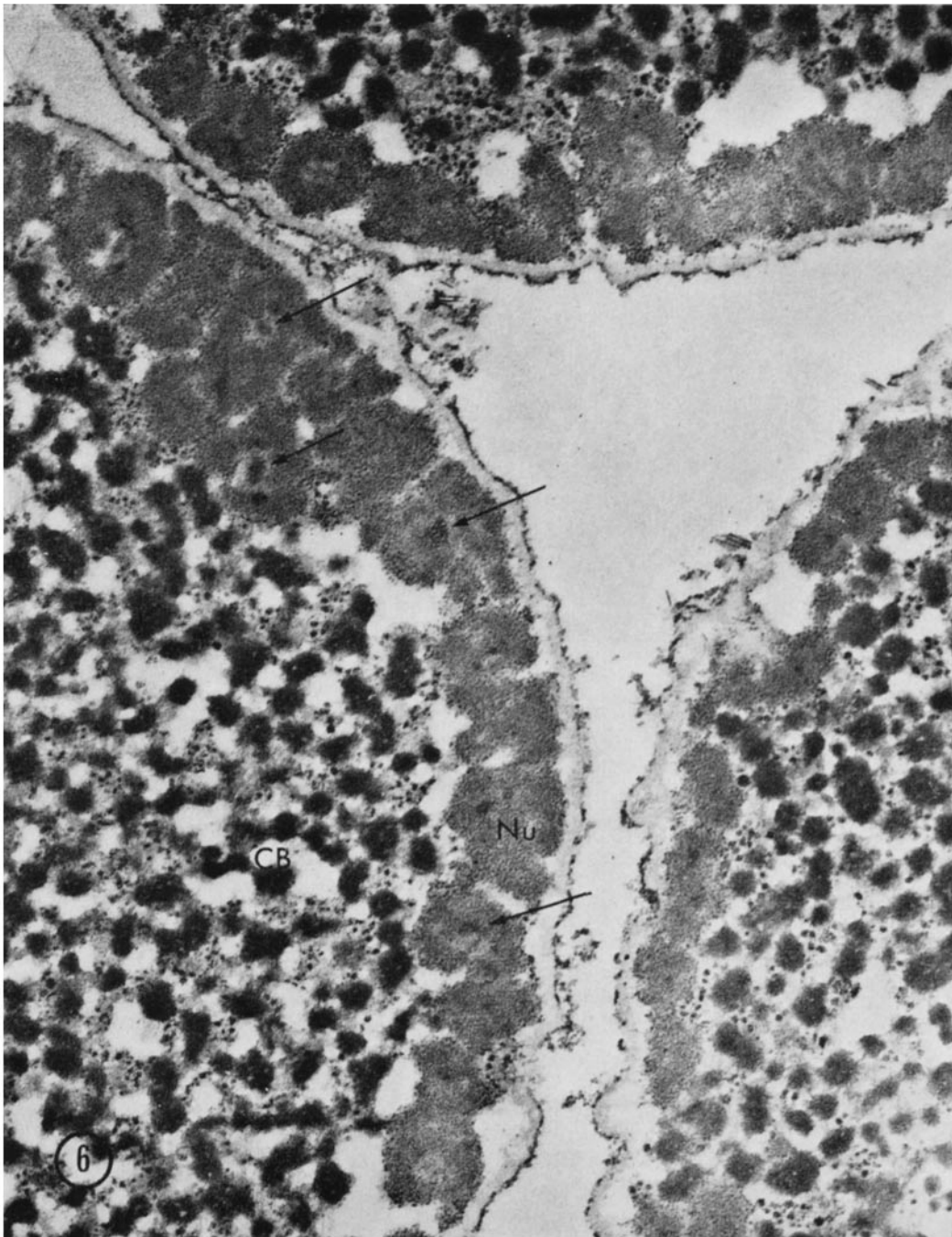


FIGURE 6 Electron micrograph of the edges of three isolated macronuclei. The arrows indicate small clumps of chromatin-like material which are located in clear areas within the nucleoli. *Nu*, nucleolus; *CB*, chromatin body. (Formalin; uranyl acetate; about $\times 33,000$.)

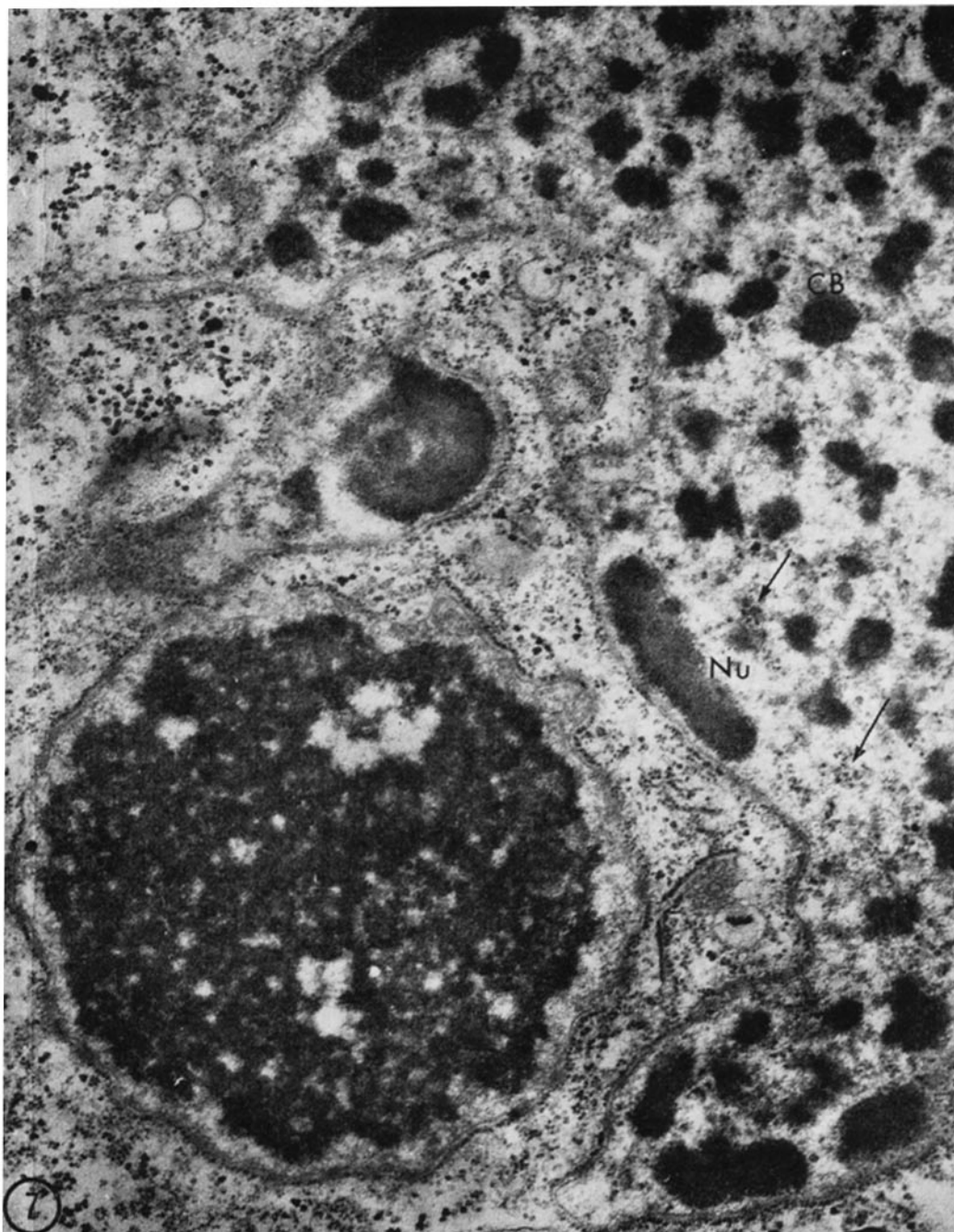


FIGURE 7 Electron micrograph of a macronucleus and micronucleus *in situ*. As in the isolated nuclei, the macronucleus contains nucleoli (*Nu*), chromatin bodies (*CB*), and interchromatin granules (arrows), and the micronucleus consists of a chromosome-like coil and a small amount of amorphous material of low electron opacity. Note the ribosome-studded nuclear envelopes of both macro- and micronuclei. The micronucleus is located in an inpocketing of the macronucleus (glutaraldehyde-OsO₄; uranyl acetate; about $\times 32,000$.)

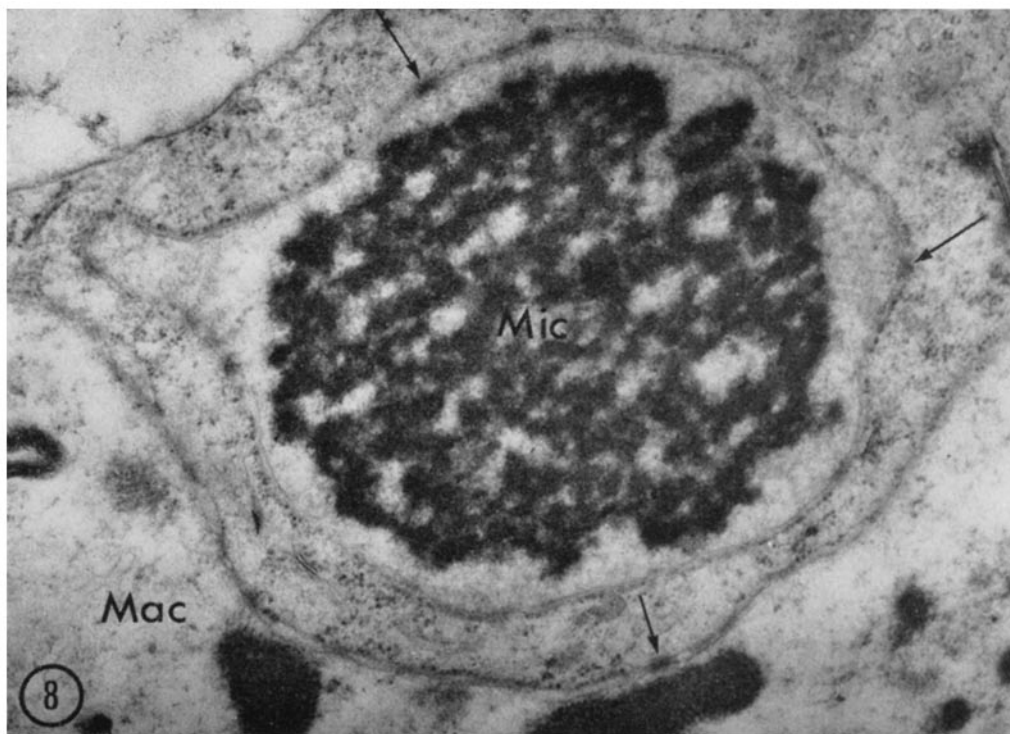


FIGURE 8 Electron micrograph of a micronucleus and a portion of a macronucleus (*Mac*) *in situ*. Note the pores (arrows) in the macro- and micronuclear envelopes. (Glutaraldehyde-OsO₄; uranyl acetate; about $\times 32,000$.)

The homogenate was then centrifuged at 250 *g* for 10 min, preferentially pelleting macronuclei and large macronuclear fragments. The supernatant was shaken vigorously and spun again at 250 *g* for 10 min. This procedure was performed a total of three times. The three pellets were resuspended in Medium B, rehomogenized, and were added back to the 250 *g* supernatant. The resulting mixture, containing all of the material of the original macronuclear fraction, was spun at 1000 *g* for 10 min so as to pellet the micronuclei, macronuclei, and larger fragments. The pellets from three to four such collections were resuspended in 40 ml of Medium A and were spun at 250 *g* for 10 min (three times) so as to pellet macronuclei and macronuclear fragments. The supernatant which contained 20-40 micronuclei per large macronuclear fragment was spun at 1000 *g* for 30 min to yield the micronuclear fraction which contained 20-40 micronuclei for every large macronuclear fragment. This fraction was also slightly contaminated with smaller

particulates (Fig. 3). Since macronuclei contain 12-24 times as much DNA as do micronuclei (Gorovsky and Woodard, 1969; Woodard, Gorovsky, and Kaneshiro, 1968), this micronuclear fraction contains approximately 50-75% micronuclear DNA and 25-50% macronuclear DNA, and although impure, represents a marked enrichment for micronuclei when compared to the original macronuclear fractions.

Methodological Variables

CULTURE CONDITIONS: Nuclei can be routinely isolated by this method only from growing populations in well aerated cultures. Recently, we have been able to isolate nuclei successfully from stationary phase cells by including 0.01% of spermidine (Rosenbaum and Holz, 1966) in the homogenization medium and extending homogenization times, or by doubling the octanol concentration in Medium B.

CELL DENSITY DURING HOMOGENIZA-

TION: At high cell densities (4 ml of cells/40 ml of Medium B), more than three centrifugations were necessary to collect and purify the nuclei. Low cell densities ($\frac{1}{2}$ –1 ml of cells/40 ml of Medium B) were used in all isolations in which the efficiencies of recovery were determined.

HOMOGENIZATION: Since whole cells, if present, were found in the nuclear pellets, homogenization conditions were chosen which favored complete breakage of cells, but which resulted in some damage to the nuclear envelope. Better nuclear integrity was obtained if spermidine (0.01%) was used to stabilize nuclear structure. However, the presence of spermidine in the isolation media made it impossible to isolate micronuclei by the methods described here since macronuclei treated with spermidine were resistant to breakage by subsequent homogenization. However, the use of spermidine is strongly advised to preserve nuclear integrity where its use is not contraindicated by the particular requirements of the experiment, since nucleus isolations performed with spermidine in the media routinely result in recoveries of over 70% of the macronuclei.

OCTANOL CONCENTRATION: Although successful nuclear isolations were obtained using 0.024 or 0.032 M octanol, 0.040 M octanol was used to insure reproducible, efficient isolations from log-phase cells. In the presence of octanol, the nuclear membranes contained numerous small blebs which disappeared if nuclei were resuspended in octanol-free solutions.

DIVALENT IONS: Recovery and appearance (phase contrast) of isolated nuclei were unaffected by calcium concentrations from 0.02 to 0.0002 M or by 0.001 M EDTA. Nuclei were routinely isolated in solutions containing 0.0015 M $MgCl_2$ which allowed subsequent isolation of ribosomes from postnuclear supernatants and from nuclear membranes (Gorovsky, 1969).

pH: At the pH's tested (6.0, 6.5, 7.0), phosphate-buffered solutions (0.02 M) were less satisfactory than Tris (0.02 M) or unbuffered solutions. Efficiency of release and recovery was similar at pH 6.5 and 7.0, but was markedly reduced at pH 6.0.

GUM ARABIC: If gum arabic was left out of the isolation media, macronuclei were swollen and badly damaged, and few nuclei were recovered. However, after isolation, nuclei can be washed repeatedly and resuspended in gum arabic-free solutions. Gum arabic has been found

to contaminate both RNA and histone preparations from isolated nuclei if the nuclei were not previously washed in gum arabic-free solutions.

In summary, methods have been described for the isolation and purification of a macronuclear fraction from *Tetrahymena*, and for the preparation of a fraction which was markedly enriched in micronuclei. Most of the ultrastructural components observed in the nuclei in intact cells can be seen clearly in the isolated nuclei. Coupled with the ease with which *Tetrahymena* can be cultured, synchronized, and labeled with radioactive precursors, these fractionation procedures should prove useful in further studies of nuclear structure and function, as well as of the underlying cause(s) of the marked structural and functional differences between macro- and micronuclei. In fact, these methods have already been used to study RNA synthesis in *Tetrahymena* (Kumar, 1970), in a comparison of histones of macro- and micronuclei (Gorovsky, 1968; Gorovsky, 1970), and of protein synthesis in isolated nuclei (Gorovsky, 1969).

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