Protoplasma 128, 190–200 (1985)

PROTOPLASMA

Isolation and Characterization of *Euglena* Nuclei

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Received March 16, 1985
Accepted April 17, 1985

Summary

A novel method for isolating *Euglena gracilis* Z. nuclei, based on pretreatment of cells in concentrated glycerol buffer before homogenization, is described. Such a treatment weakens the tough cell pellicle facilitating cell disruption, and avoids nuclear damage induced by detergents and by freezing and thawing the cells in aqueous media. Nuclei, purified by centrifugation in dense sucrose, are obtained with a 30% yield, and only small amounts of cell wall fragments contaminate the nuclear pellets. The purified nuclei retain their ultrastructural characteristics. High molecular weight DNA, as well as undegraded RNA species and histones, can be extracted from these nuclei. Nuclease digestions and spread preparations show an unaltered nucleosomal structure of chromatin. This method has been applied to cell samples at any stage of the cell cycle, including mitosis, since in *Euglena* the nuclear envelope persists during cell division.

Keywords: Chromatin spreading; *Euglena;* Macromolecular components; Nuclease digestions; Nuclei isolation; Nucleus ultrastructure.

1. Introduction

Euglena nuclei, like those of many plant cells, are difficult to isolate owing to the tough pellicle and the presence of chloroplasts and paramylon granules. Nevertheless, this microorganism possesses a number of advantages for molecular biology studies. It can be grown autotrophically in the light or heterotrophically in darkness and therefore it has been used as a model for chloroplast morphogenesis (NIGON and HEIZMANN 1978). When grown under alternative periods of light and darkness, it naturally divides with a very high degree of synchrony (EDMUNDS 1965), and since nuclear envelope, in *Euglena,* does not disappear during mitosis, nuclei can in principle be isolated even during the mitotic stage. Also, when deprived of their essential growth factor, the vitamin B_{12}, *Euglena* cells exhibit anomalies similar to those which occur in megaloblasts—typical cells described in human pernicious anemia (see VALENCIA 1974 for review).

Several methods for isolating *Euglena* nuclei have already been described (PARENTI *et al.* 1969, APRILLE and BUETOW 1973, LYNCH and BUETOW 1975, JARDINE and LEAVER 1977, MAGNAVAL *et al.* 1979). In spite of recent improvements, they still remain rather harsh for preserving the integrity of nuclear components since they include several of the following treatments: freezing and thawing cycles, proteolytic digestion of the pellicle, incubation of cells in high detergent concentration, cell disruption by sonication or by high pressure forces, and acidic isolation media. The earliest method was reported by PARENTI *et al.* (1969), and preparations using this procedure reveal that the ultrastructure of nuclei was not preserved (MAGNAVAL *et al.* 1979). LYNCH and BUETOW (1975) were the first to control the ultrastructural integrity of nuclei obtained from a bleached strain of *Euglena* by a relatively mild procedure. These nuclei were used to study chromatin fractionation (LYNCH *et al.* 1975). More recently, two other methods have been described (JARDINE and LEAVER 1977, MAGNAVAL *et al.* 1979). Following the first one, and in spite of the acidity of the isolating medium, the presence of histones was confirmed in

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Euglena cells (JARDINE and LEAVER 1978). The second one consists of improvements brought to the PARENTI *et al.* procedure in order to obtain a better preservation of nuclear structures (MAGNAVAL *et al.* 1979). A nuclease digestion study of nuclei obtained with both methods has revealed the repetitive substructure of *Euglena* chromatin (BRÉ *et al.* 1980, MAGNAVAL *et al.* 1980). However, studies in our laboratory on chromatin ultrastructure, using spread preparations of nuclei obtained by the method of MAGNAVAL *et al.* (1979), have revealed rearrangements of nucleoproteins, possibly due to the high detergent concentration used during nuclei isolation.

The aim of the present study was to develop a method for isolating nuclei while avoiding any treatment which precludes good preservation of chromatin components. This is obtained by pretreatment of cells in a concentrated glycerol buffer which induces the weakening of the cell wall and permits cell disruption at low pressure without apparent nuclear damage. The proposed method provides well-preserved nuclear structures and appears to be appropriate for biochemical studies of *Euglena* nuclear components.

2. Materials and Methods

2.1. Culture Conditions

Autotroph cultures of *Euglena gracilis* Z. (Cambridge no. 1224-5 D) were grown synchronously in 2 L toxin flasks under alternative periods of 14 hours light/10 hours darkness, as previously described (BERTAUX *et al.* 1978). Cells were collected in exponential phase of growth, when the concentration reached 2.5–5.0 × 10^5 cells/ml. Cell concentration and cellular mean volumes were monitored with a Coulter counter, model F.

2.2. Buffers

Composition of the buffers was as follows: *preservation buffer*— 10 mM Tris-HCl (pH 7.4 at 25 °C), 6 mM $MgCl_2$, 0.13 mM $MnCl_2$, 0.6 mg% polyvinylsulfate, 1 mM phenylmethylsulfonylfluoride (PMSF), 5 mM $Na_2S_2O_5$, and 70% (v/v) glycerol. *Homogenization buffer* and *dense sucrose buffers* have a similar composition as preservation buffer, but they contain sucrose instead of 70% glycerol; sucrose concentration of homogenization buffer is 10%, while it ranges from 74 to 80% for dense sucrose buffers.

The concentration of metal ions in the buffers was chosen according to MAGNAVAL *et al.* (1979); metal ions are required to maintain *Euglena* chromatin condensation, otherwise nuclei swell and are lysed during cell disruption. In addition to protease and RNase inhibitors, metabisulfite was introduced in the buffers to inhibit phenol oxydases which lead to the denaturation of proteins and nucleic acids (LOOMIS 1974). This antioxydant has been widely used in isolating plant enzymes and organelles, and a 5 mM concentration proves optimal in *Euglena* to isolate nuclei. It must be noted that metabisulfite used at this concentration slightly reduces the final pH (to about 6.0) of the buffers, which were used without further adjustment.

2.3. Glycerol Pretreatment of Cells

About 2.5 × 10^8 synchronized cells were washed with fresh culture medium (CRAMER and MYERS 1952) and once more with 10 ml of the preservation buffer before storage at —20 °C in 5 ml of the same buffer. Cells must be stored at least 6 days and up to 3 weeks in this glycerol buffer, which induces the weakening of the cell pellicle before nuclei isolation.

2.4. Isolation of Nuclei

Nuclei were obtained following the flow chart shown in Fig. 1. The procedure first involves the rehydration of cells at 0 °C in the homogenization buffer. For this purpose, the viscous glycerol buffer containing the preserved cells was diluted by addition of 2 vol. of homogenization buffer and the cells were pelleted at 1,100 g for 5 minutes. The cells were then incubated for 30 minutes in 15 ml homogenization buffer, with occasional shaking and a buffer change at 15 minutes for better extraction of glycerol from the cells. At the end of the incubation time, the rehydrated cells look like big spheroblasts in which the nucleus seems to be detached from other cytoplasmic organelles, as could be observed under UV light after acridine orange staining. After a centrifugation at 700 g for 15 minutes, the cells were resuspended in the homogenization buffer at the optimal density of 2 × 10^7 cells/ml and broken at 1,500 psi (10 MPa) using a French pressure cell refrigerated at 0 °C. Two or three strokes were necessary to ensure a complete breakage of cells.

Nuclei were separated from chloroplasts and other cellular organelles and debris by sedimentation in dense sucrose, made in the same buffer. In this way, the cell lysate was first centrifuged at 400 g for 10 minutes and the soft pellet was carefully homogenized in 75 ml of 77% sucrose buffer, using a loose-fitting Dounce homogenizer (pestle A, 15 ml capacity). The suspension was centrifuged for 1 hour at 15,000 rpm (40,000 g) in a Beckman SW 27 rotor. Chloroplasts, cellular debris and some trapped nuclei migrate to the top, making a dark-green layer easily removed in a piece with a spatula. The supernatant was poured off by suction, and the tube wall wiped dry with filter-paper. The white or slightly green pellet was further washed in the homogenization buffer, using a tight-fitting Dounce homogenizer (pestle B), with 0.25% of detergent Nonidet P-40 and twice more without detergent. The yield of nuclei in the final nuclear pellets was calculated by the DNA recovery using the diphenylamine test (GILES and MYERS 1965).

2.5. Microscopy

2.5.1. Light Microscopy

A Zeiss Jena fluorescence microscope (Fluoval), equipped with a phase contrast system and a camera, was used to observe cells and nuclei during the isolation procedure. Nuclei were better observed under UV light with acridine orange staining: some µl of 0.1% acridine orange dissolved in water were mixed on the microscope slide with a drop of the sample. Nuclei fluoresced green, principally on chromatin masses surrounding the rose nucleolus, while chloroplasts are dark red.

2.5.2. Electron Microscopy

For thin sections, nuclei were fixed in their isolation medium (10% sucrose buffer) with 4% glutaraldehyde for 1 hour, postfixed with 1% osmium tetroxide in 0.1 M Sörensen phosphate buffer, pH 7.3, before dehydration and Epon embedding. Whole cells were fixed and

Harvested cells

Washing of cells
- 2.5×10^8 normal cells washed once in fresh culture medium;
- centrifuge 700 g, 10 minutes;
- wash cell pellet in 10 ml preservation buffer;
- centrifuge 1,100 g, 10 minutes.

Washed cells

Weakening of the cell pellicle
- resuspend cell pellet in 5 ml preservation buffer;
- store 6 days, and up to 3 weeks, at −20 °C.

Glycerol treated cells

Rehydration of cells
Spheroblasts formation
- dilute preservation buffer with 10 ml homogenization buffer;
- centrifuge 1,100 g, 5 minutes,
- incubate 2 times, 15 minutes with 15 ml homogenization buffer, and
- centrifuge 700 g, 5 minutes each time.

Spheroblasts

Homogenization step
- resuspend cell pellet in 12.5 ml homogenization buffer;
- pass through a French press at 1,500 psi, 2–3 strokes;
- control cell breakage under UV light with acridine orange staining.

Cell lysate

Purification of nuclei
- centrifuge 400 g, 10 minutes;
- resuspend pellet in 75 ml 77% sucrose-buffer with Dounce homogenizer (pestle A);
- centrifuge 40,000 g, 1 hour in SW 27 rotor.

Nuclear pellet (Fig. 2 *a*)

Washing of nuclei
- resuspend nuclei, with Dounce homogenizer (pestle B), in 10 ml homogenization buffer containing 0.25% Nonidet P-40;
- centrifuge 700 g, 10 minutes;
- resuspend nuclear pellet 2 times in homogenization buffer (Dounce B), and
- centrifuge 700 g, 10 minutes each time.

Purified nuclei (Fig. 2 *b*)

Fig. 1. Flow chart for the isolation and purification of *Euglena* nuclei

embedded as previously described (MOYNE *et al.* 1975). Thin sections were stained with uranyl acetate and lead citrate.

For spread preparations, nuclei were previously washed in 75 mM Tris-HCl, pH 7.4, 25 mM EDTA buffer, and swollen in 0.5 mM borate buffer adjusted to pH 7.6, prior to spreading according to MILLER and BAKKEN (1972).

2.6. Characterization of Nuclear Proteins

Nuclei either fresh or frozen were resuspended in 10% sucrose, 0.4 mM PMSF, 30 mM KCl, 1 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.4. The nuclear pellets were easily resuspended in this buffer by using a gentle Potter homogenization. Nuclear suspensions were filtered through nylon gauze and adjusted to DNA concentrations around 0.5 mg/ml, as measured by UV absorption at 260 nm of nuclei exploded with 0.5% SDS. For total nuclear protein analysis, aliquots of nuclei, equivalent to 12 µg of DNA, were mixed with an equal volume of electrophoresis sample buffer (LAEMMLI 1970), sonicated and directly loaded onto the electrophoresis gel. Histones were also extracted with 0.25 N HCl. In this case, a volume of 0.5 N HCl was added to a volume of the nuclear suspension and the mixture left with agitation overnight at 4 °C; proteins were precipitated with TCA at the final concentration of 20%, washed once with acetone-HCl 0.1%, once with acetone, dried and histones redissolved in sample buffer. Slab gel electrophoresis was run according to LAEMMLI (1970).

2.7. Digestion of Nuclei by Micrococcal Nuclease

Suspensions of nuclei, in the same buffer as for protein analysis, were made 1 mM $CaCl_2$. Micrococcal nuclease (15,000 units/mg, Boehringer Mannheim) was added at a relation of 60 units per mg DNA. Digestions were allowed to proceed for different periods of time (0, 5, 15, 45, and 60 minutes) at 37 °C, and were stopped by addition of EDTA to a final concentration of 2.5 mM. DNA was deproteinized by proteinase K and phenol-chloroform treatments and analyzed in 1.6% agarose gel made in Tris-borate-EDTA buffer (90 mM Tris-borate, pH 8.3, 2 mM EDTA). Vertical agarose gels, 20 cm long, 3 mm wide, were run at 100 volts for 8 hours, stained with ethidium bromide and photographed with a Polaroid MP-4 camera under UV illumination.

2.8. Isolation and Characterization of Nuclear DNA

On account of important losses of DNA, conventional DNA isolation procedures, using enzymatic digestions and multiple extractions of proteins with organic solvents, were avoided. *Euglena* nuclear DNA has been quantitatively recovered by CsCl isopycnic centrifugation following nuclear lysis in hypotonic buffer and dissociation of nucleoprotein complexes in detergent.

Practically, 150 to 300 µg nuclear DNA were recovered from about 5×10^7 nuclei isolated respectively during G_1 or G_2 phase. According to a procedure described for plant nuclei (LIBERATI-LANGENBUCH *et al.* 1980), *Euglena* nuclei were lysed by 3 successive incubations, each for 30 minutes, in the following buffer: 1 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 0.5 mM dithiothreitol, 12.5% glycerol and 0.5 mM PMSF. The resulting chromatin gel, recovered by centrifugation at 2,500 g for 10 minutes, was dissociated overnight at 0 °C with occasional swirling in 5 ml SSC buffer (0.15 M NaCl, 0.015 M sodium citrate, pH 7.8) containing 2% sodium N-lauroyl sarcosinate and 0.1 M EDTA. CsCl was added to the lysate up to a refractive index of 1.3995 and the DNA banded at 25 °C and 35,000 rpm (107,000 g) for 24 hours in a Beckman 65 rotor. The protein upper fractions and RNA lower ones were discarded and, to assure purity of DNA, the DNA intermediate fractions rerun for 72 hours under the same conditions. After dilution of the DNA containing fractions with 3 vol. SSC, the DNA was concentrated by overnight precipitation at — 20 °C with 0.1 vol. 3 M sodium acetate and 2.0 vol. 95% ethanol. The precipitate was collected by centrifugation at 10,000 g for 15 minutes, washed with 70% ethanol, air dried and rehydrated in TE buffer (10 mM Tris-HCl, pH 8.0, and 1 mM EDTA). The molecular weight of DNA was determined by horizontal agarose gel electrophoresis using λDNA as a marker. Gels, containing 0.8% or 0.4% agarose, were prepared in TAE buffer (40 mM Tris-base, pH 7.8, 5 mM sodium acetate and 2 mM EDTA), and run at room temperature and 1.0 volt/cm for 16 hours. They were stained with ethidium bromide and photographed under UV light.

2.9. Extraction and Analysis of Nuclear RNA

Bulk RNA was extracted from purified nuclei by the hot SDS-phenol method of SCHERRER and DARNELL (1962), or at 0 °C according to the HEIZMANN procedure (1970). Zone velocity centrifugation was used to analyse the extracted RNA. About 100 µg nuclear RNA were layered onto 37 ml convex exponential gradient of the type $C = 1.4\text{–}1.1\ \exp^{-37/29}$ (NOLL 1967). Gradients were made in 0.1 M NaCl, 10 mM Tris-HCl, pH 5.0, and 1.0 mM EDTA with RNase-free sucrose. The acidic pH of the gradients was chosen owing to a much greater stability of *Euglena* RNA molecules. Centrifugation was carried out at 26,000 rpm (122,000 g) for 22.5 hours at 4 °C in a Beckman SW 27 rotor. Sedimentation patterns were scanned automatically using an ISCO gradient analysing system.

3. Results and Discussion

3.1. Cell Preservation

Storage of *Euglena* cells in 70% glycerol buffer proved to be an essential step of the nuclei isolation procedure. Glycerol solubilizes the bulk of cytoplasm, as observed in glycerol-treated cells by electron microscopy (not shown). The destruction of connections between cytoplasm and nucleus is certainly one of the first causes of the protection of nuclei during cell disruption. The minimum delay to obtain this effect by the glycerol pretreatment is 6 days, but cells could be stored up to 3 weeks at — 20 °C in the preservation medium without changes in yield and characteristics of isolated nuclei. Finally, the major advantage of this preservation step is to avoid the treatments normally used before cell disruption to weaken *Euglena* cell pellicle, such as proteolytic enzymes, freezing and thawing cycles, and incubation of cells in high detergent concentration. In addition, glycerol allows to avoid the adverse effects of freezing in aqueous media (WIST and KROKAN 1978). It has already been used, pure or in aqueous solution to isolate nuclei which retain their protein and nucleic acid content (KIRSCH *et al.* 1970, GURNEY and FOSTER 1977, YANG and STREET 1978, KENNEDY and MATHIAS 1979, MARKOV *et al.* 1979); it is a powerful inhibitor of DNase and protease activities, and nuclei stored in 70% glycerol buffer retain their synthetic capacity (WIST and KROKAN 1978), as well as RNA polymerase activity (READ and MAURITZEN 1970).

It should be noted that nuclei prepared by this procedure would not be adequate for studies of the nuclear envelope, owing to lipoprotein modifications induced by glycerol. However, none of the published methods for *Euglena* nuclei isolation preserve the integrity of the nuclear envelope, since the milder ones use too high detergent concentrations (PARENTI *et al.* 1969, LYNCH and BUETOW 1975, MAGNAVAL *et al.* 1979) sometimes along with acidic pH (JARDINE and LEAVER 1977).

3.2. Disruption of Cells and Purification of Nuclei

The major problem for isolating *Euglena* nuclei is to succeed cell disruption under conditions which preserve nuclei. Having discarded proteolysis of the cell wall, only three of the homogenization methods tried were efficient for breaking *Euglena* cells (see BUETOW 1978 for review): sonication (LYNCH and BUETOW 1975), vortex mixer in the presence of glass beads (MCLENNAN and KEIR 1975) and the French press (PARENTI *et al.* 1969, JARDINE and LEAVER 1977, MAGNAVAL *et al.* 1979); however, if the cell pellicle has not previously been damaged before cell disruption, these three methods lead to extensive destruction of nuclei. Thus, in the mentioned procedures, the weakening of the pellicle was obtained both by freezing and thawing cycles and incubating of cells with high concentration of Triton, 1.25 to 10% (PARENTI *et al.* 1969, LYNCH and BUETOW 1975, JARDINE and LEAVER 1977, MAGNAVAL *et al.* 1979).

Among these homogenization procedures, the French press is certainly the most reproducible, and the milder

if low pressures are used. Up to now, breakage of cells at low pressure (1,500 psi) was only obtained after 1 hour incubation of cells in 10% Triton (PARENTI *et al.* 1969, MAGNAVAL *et al.* 1979). On the other hand, preservation of cells a few days in 70% glycerol-buffer also induces the weakening of *Euglena* cell pellicle. Thus, when the cells are transferred from the glycerol-buffer and rehydrated in the 10% sucrose-buffer, the pellicle largely expands and the cells form big spheroblasts which could be broken at 1,500 psi without damage for the bulk of nuclei. The reduction of the cell breakage pressure obtained after treatment of cells with Triton or glycerol probably results from the same mechanism: (i) the extraction of lipids from the membranes and (ii) the solubilization of cytoplasmic components, leaving nuclei free of attachments inside the swollen cells, thus avoiding wrenching forces when the cells burst open.

When working on synchronous *Euglena* cells, the same pressure forces (1,500 psi) could be used at every step of the cell cycle, and since nuclear envelope persists around the dividing nuclei, the latter have also been successfully isolated. In spite of their greater size and elongated shape, they do not seem more fragile than interphase nuclei. After their purification in dense sucrose, they keep their elongated bended shape, like those observed *in situ* (BERTAUX *et al.* 1985). This implies that the nuclear skeletal elements are not essentially altered by the nuclei isolation procedure.

In the initial attempts to purify nuclei of unsynchronized cells by centrifugation in dense sucrose, an optimum concentration of 77% (2.25 M) has been determined. Owing to changes occurring in the size and density of the nuclei of synchronized cells during G_1, S, G_2 and M phases, this concentration has to be adjusted. For cells in early G_1, it must be lowered to 74% (2.16 M), and increased to 80% (2.35 M) for those in G_2 phase. After this single centrifugation, and in spite of the fact that detergent has not yet been used, a good quantity of fairly clean nuclei was obtained (Fig. 2 *a*), with an average yield of 30%. The bulk of chloroplasts, unbroken cells and cytoplasmic debris floats over the dense sucrose cushion as a dark green layer within which some nuclei remain trapped. After a rapid wash of the nuclear pellets with detergent (0.25%), the residual contaminating material only consists of some pellicular fragments and some paramylon granules. The purest preparations arise from cells collected in G_1 phase and during cell divisions in the dark period. Indeed, *Euglena gracilis* Z. cells in autotroph cultures contain very few paramylon granules, particularly at these two steps of the cell cycle. Constrastingly, there is some more contamination with paramylon at the end of the light period (G_2 phase). Therefore, this simple purification procedure would not be sufficient for cells containing a bulk of paramylon granules, such as heterotrophic *Euglena* cultures. Additional purification steps would be necessary, such as floating of nuclei upon Percoll cushion (WILLMITZER and WAGNER 1981) or upon colloidal silica (HENDRIKS 1972). The slight contamination of nuclei observed, when autotrophic *Euglena* cells are used, has never been cumbersome either for electron microscopy or for biochemical experiments.

3.3. Characterization of the Purified Nuclei

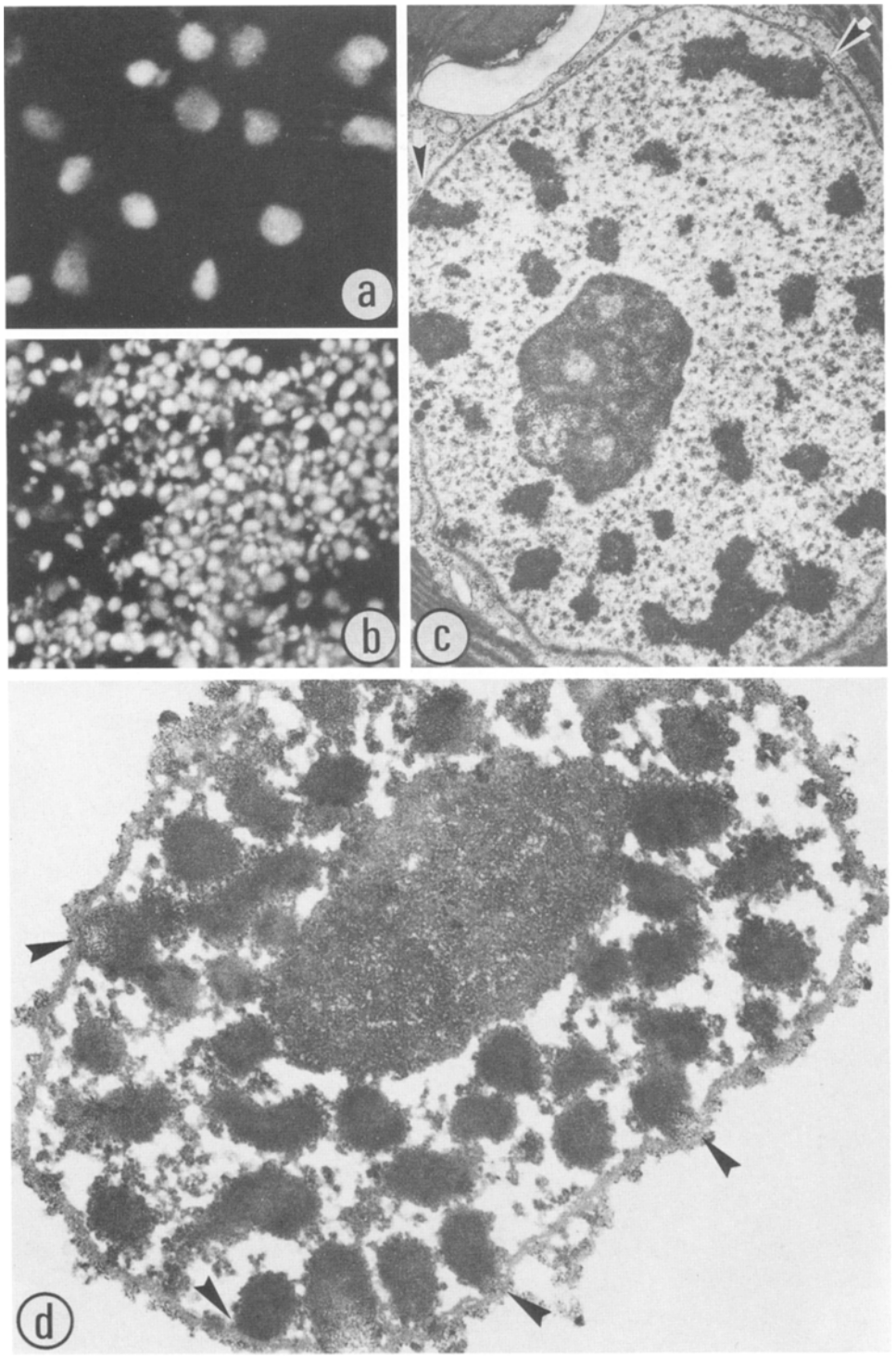
3.3.1. Morphology

Fig. 2 shows, in light microscopy, nuclei purified in dense sucrose after the 40,000 g centrifugation step (see flow chart, Fig. 1). In spite of the presence of metal ions in the medium, nuclei are well-dispersed (Fig. 2 *a*) and there is no aggregation in dense sucrose buffer. This perfect dispersion of particles has allowed the separation of nuclei from chloroplasts and other cellular debris by isopycnic density. Contrastingly, after the final washing step with detergent in isotonic sucrose buffer, some aggregation of nuclei occurs (Fig. 2 *b*).

As observed in electron microscopy, the ultrastructure of isolated nuclei is essentially maintained (Fig. 2 *d*). In comparison with a nucleus *in situ* (Fig. 2 *c*), the isolated nuclei retain condensed chromatin masses with a paracrystalline substructure, a nucleolus with a fibrillo-granular appearance, and a thick nuclear envelope remnant to which some chromatin masses remain attached. As for the nucleoplasm, it is clumped around and between chromatin masses.

3.3.2. Nuclear Proteins

Analysis of total nuclear proteins extracted with SDS shows a typical histone pattern (Fig. 3 *a*). The electrophoretical mobilities of these proteins are very similar to rat liver histones used as a marker but different from higher plant histones (LIBERATI-LANGENBUCH *et al.* 1980). As has been observed with acid-extracted histones from *Euglena* (BRÉ *et al.* 1980), histone H 3 has a slightly lower mobility than rat liver H 3, and H 1 runs faster than rat liver H 1. In any case, histones extracted from glycerol-treated nuclei keep their relative proportions with any appreciable sign of degradation. This is specially clear in HCl-extracted histones (Fig. 3 *b*) where no band moving in front of the easily degraded



Figs. 2 *a* and *b*. Nuclei observed under UV light after acridine orange staining

Fig. 2 *a*. Nuclei observed after the 40,000 g centrifugation step in dense sucrose-buffer. × 950

Fig. 2 *b*. Nuclei from final nuclear pellet observed in isotonic sucrose-buffer. × 512

Fig. 2 *c*. *Euglena* nucleus *in situ;* note the highly condensed state of chromatin masses; some of them are attached to the inner leaflet of the nuclear envelope, arrows. × 12,700

Fig. 2 *d*. Isolated *Euglena* nucleus with well-preserved nucleolus and chromatin masses; note that some chromatin masses remain attached to the nuclear envelope remnant, arrowheads. × 24,500

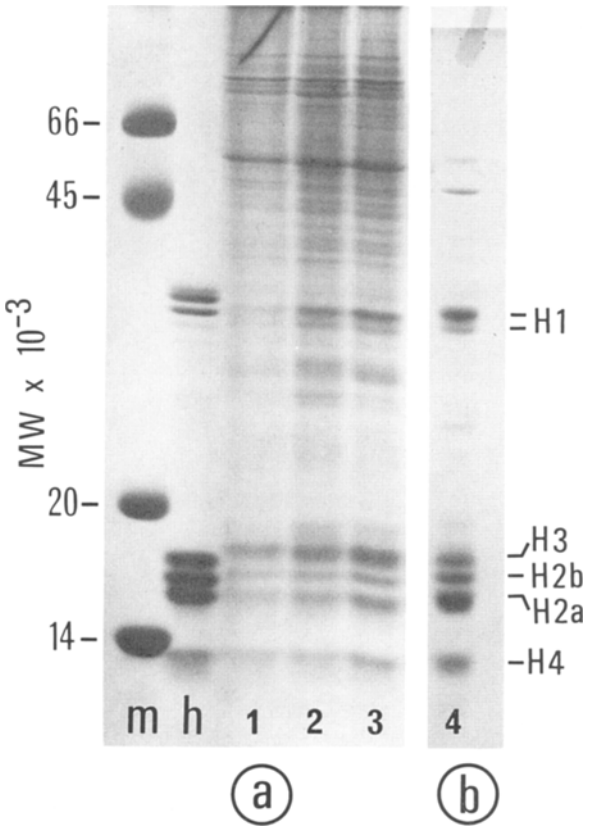


Fig. 3 *a*. Electrophoretical analysis of the total protein content of *Euglena* nuclei. nuclei equivalent to 12 µg DNA were sonicated in electrophoresis sample buffer and directly loaded onto the electrophoresis gel. Samples *1, 2,* and *3* correspond to nuclei from cells harvested after 0, 5, and 7 hours illumination. Samples *h* and *m* correspond respectively to histones from rat liver and to molecular weight markers (lysozyme, soybean trypsin inhibitor, ovalbumine, and bovine serum albumine)

Fig. 3 *b*. HCl-extracted histones from *Euglena* nuclei (*lane 4*)

histone H 1 is observed. This indicates that the present method of preparation of nuclei does not lead to proteolysis of nuclear proteins.

3.3.3. Nucleosomal Organization of Chromatin

The preservation of chromatin structure may be confirmed by analysing the nucleosomal repetitive structure. This analysis has been carried out with *Euglena* nuclei in two different ways, nuclease digestion and electron microscopy of spread nuclei. Agarose gel electrophoresis of DNA (Fig. 4 *a*) from micrococcal nuclease digestion of *Euglena* nuclei shows a typical oligonucleosome pattern. At time 0 only high molecular weight DNA and residual RNA is observed indicating that no nucleolytic activity was present. After the nuclease is allowed to act, the oligonucleosome ladder appears giving rise to oligomers of decreasing multiplicity. Digestion proceeds in a continuous way without any indication of heterogeneity. Such a fact indicates that neither shearing of the chromatin (NOLL *et al.* 1975) nor aggregation of the nuclear material occurs as a consequence of the preparation procedure. Nuclease digestion was also carried out at 4 °C. At this temperature, used in methods devised to preserve higher order structure of chromatin (RUIZ-CARRILLO *et al.* 1980), an increase in digestion time resulted in no appreciable digestion by the nuclease (not shown) indicating a highly condensed state of chromatin within the isolated nuclei.

Electron microscopic observations using spread preparations of nuclei confirm these results. The highly condensed state of *Euglena* chromatin makes spreading experiments rather difficult. Decondensation of this chromatin first requires washing of nuclei in an EDTA-buffer prior to their incubation in low ionic strength buffer. Before nuclear lysis, chromatin fibers are observed to escape all around the nuclear periphery as loops of nucleosomal beaded fibers (Fig. 4 *b*). Similar observations were made using mouse L-929 cells (RATTNER and HAMKALO 1979) or sea urchin sperm cells (ZENTGRAF *et al.* 1980); the DNA loops in mouse cells, 20 to 200 kbp length, would be attached to the skeletal elements of nuclei (see HANCOCK 1982 for review).

3.3.4. DNA

Total DNA of *Euglena* cells displays a symmetrical sedimentation profile in CsCl gradient, and nuclear DNA bands at a density of 1.708 g/cm^3 (THIERY *et al.* 1976), while chloroplastic and mitochondrial DNAs band respectively at 1.683 and 1.691 (GIBSON and HERSHBERGER 1975). Contrastingly, the DNA isolated from our purified nuclei bands in CsCl as an asymmetrical peak with a very steep slope on the light side, no chloroplastic and mitochondrial DNAs being detected at their typical densities (not shown). Analysis in agarose gel of DNA extracted from the nuclei shows that it has a high molecular weight whatever the phase of the cell cycle (Fig. 5 *a*). A better resolution obtained in 0.4% agarose gel shows per comparison with undigested λDNA that the size of the extracted DNA exceeds 50 kbp (Fig. 5 *b*).

3.3.5. RNA

PARENTI *et al.* (1969) have shown that RNA extracted from *Euglena gracilis* Z. nuclei by the hot phenol-SDS

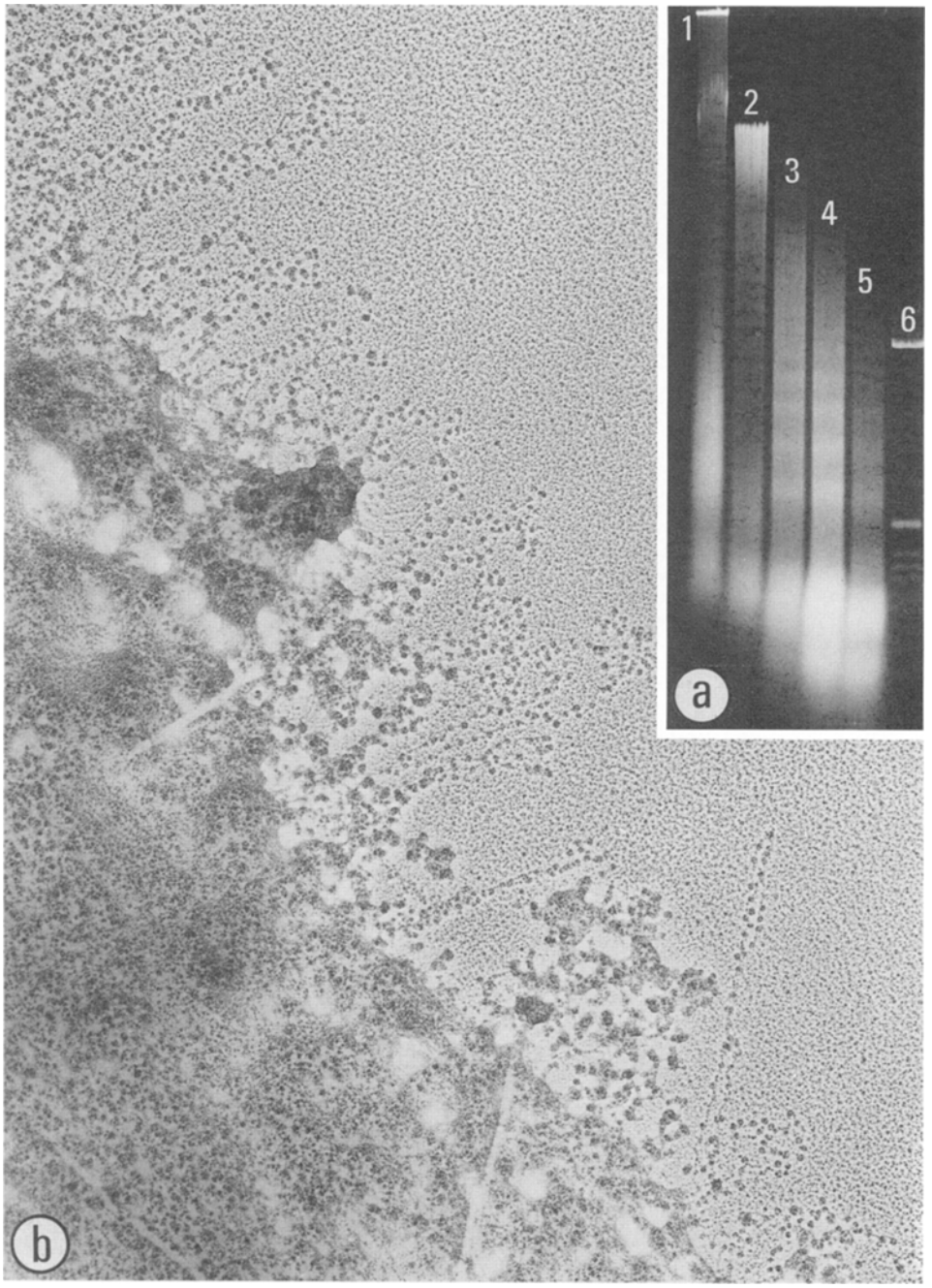


Fig. 4 *a*. Nucleosome repeat pattern obtained after micrococcal nuclease digestion of *Euglena* nuclei for 0, 5, 15, 45, and 60 minutes at 37 °C (*lanes 1 to 5*). A Hinf I digest of plasmid pBR 322 was used as a marker (*distinct fragment sizes: 1,631, 517, 506, 396, and 304 bp; lane 6*). 1.6% agarose gels were run for 8 hours at 5 volts/cm

Fig. 4 *b*. Spread preparation of *Euglena* nuclei swollen in hypotonic buffer; nucleosomal beaded fibers escape as loops all around the nuclear mass. Spread preparations were rotary-shadowed with platinum at an angle of 8°. × 50,000

procedure is composed of three RNA species sedimenting in sucrose gradient at 6, 11, and 19 S. Owing to the absence of faster-sedimenting RNA species, they suggest that some degree of degradation occurred during the nuclei isolation procedure. Contrastingly, when total nuclear RNA is extracted from the glycerol-treated nuclei by a similar hot phenol procedure (SCHERRER and DARNELL 1962), an undegraded RNA species sedimenting at about 33 S and probably also a small amount of a heavier one are observed (Fig. 6,

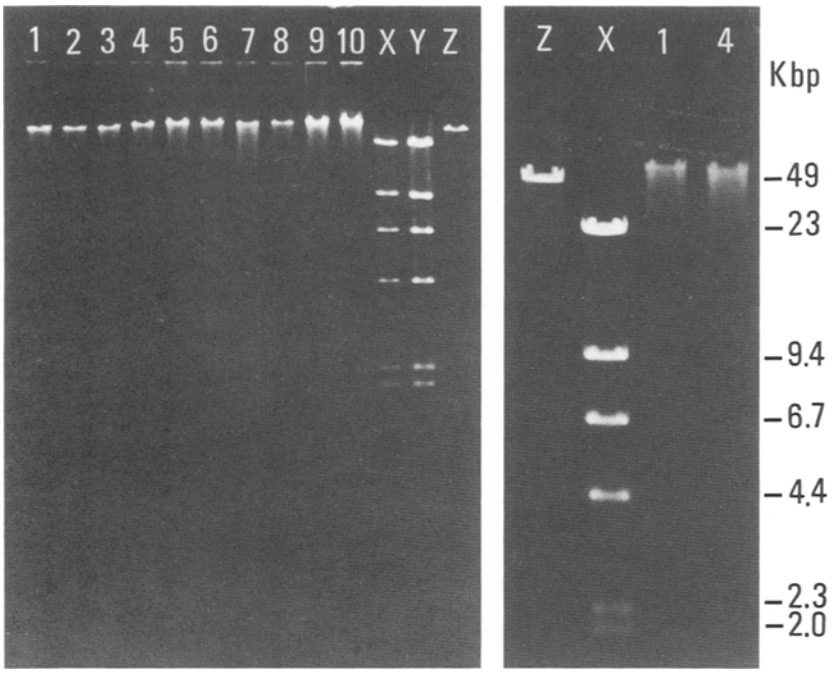


Fig. 5 *a*. Analysis in 0.8% agarose gel of the molecular weight of DNA extracted from nuclei at several steps of the cell cycle: G_1 phase, 2 and 6 hours of light (*lanes 1 and 2*); S phase, 8 and 13 hours of light (*lanes 3 and 4*); G_2 phase, 14 hours of light (*lane 5*); mitosis, 2, 4, 6, 8, and 10 hours darkness (*lanes 6 to 10*). A Hind III digest of λDNA (*125 and 250 ng respectively, lanes X and Y*) and undigested λDNA (*lane Z*) were used as markers

Fig. 5 *b*. Samples *1* and *4*, *X* and *Z* were also analysed in 0.4% agarose gel. The size of DNA extracted from nuclei exceeds 50 kbp

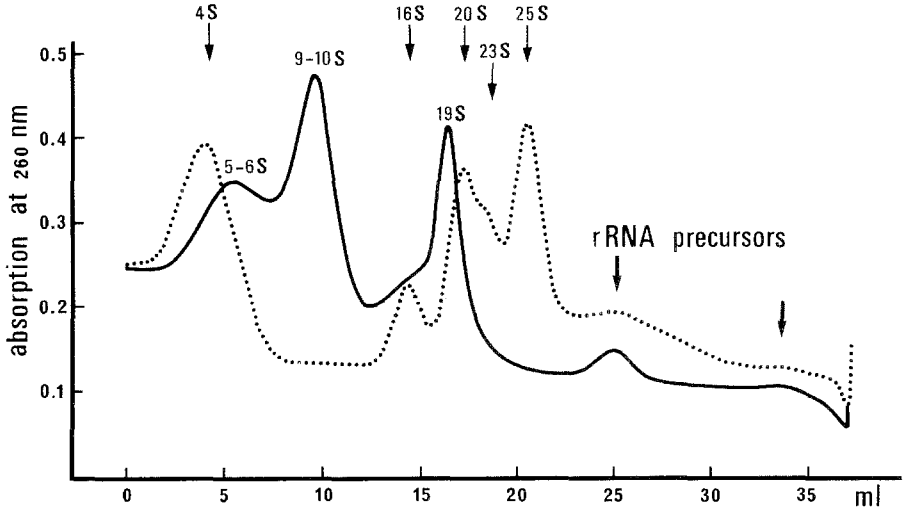


Fig. 6. Comparison of the sedimentation profiles in isokinetic sucrose gradients of total nuclear RNA (*solid line*) extracted by a hot phenol procedure, and cellular RNA extracted at 0 °C (*dotted line*). When extracted by the hot phenol procedure, the rRNA precursors are heat stable, while the mature 25 S rRNA produces specific degradation fragments in the 10 S regions

solid line). They probably correspond to the 2.2×10^6 and 3.5×10^6 molecular weight ribosomal RNA precursors which have been identified with ^{32}P labelled total RNA using pulse-chase experiments (BROWN and HASELKORN 1971). These rRNA precursors are heat stable (BROWN and HASELKORN 1971), while the mature ribosomal RNA, especially the 25 S subunit, is extremely unstable when isolated in low ionic strength medium or at high temperature; specific degradation fragments are obtained, namely in the 20 S and 10 S regions

(HEIZMANN 1970, RAWSON *et al.* 1971, BERTAUX *et al.* 1979).

For comparison, Fig. 6 (dotted line) shows the position in the gradient of cytoplasmic (25 and 20 S), chloroplastic (23 and 16 S), and 4 S RNAs when RNA is extracted at 0 °C from whole cells according to HEIZMANN (1970).

4. Conclusions

The present method for isolating *Euglena* nuclei has been developed with the specific aim of preserving nuclear components, both ultrastructurally and biochemically. This method is mild and simple compared to previous ones, and the complete procedure requires about 3 hours. Drastic treatments to weaken the tough cell pellicle are totally avoided by pretreatment of cells with glycerol buffer. The procedure includes three essential steps: the spheroblast formation, the controlled disruption of cells and the purification of nuclei in dense sucrose, without using detergent at any of these steps. A 30% yield is considered acceptable taking into account the difficulties encountered to separate the numerous cytoplasmic organelles and cellular debris. The major contamination concerns fragments of the cell pellicle and some paramylon granules. This contamination is easily removed during DNA and RNA isolation, and does not interfere with chromatin preparation and nuclease digestions.

The isolated nuclei keep well-preserved chromatin masses and nucleoli. The nuclear envelope retains its two leaflets in spite of the lipid extraction by the glycerol pretreatment of cells. Controlled nuclease digestions and spread preparations indicate that the structural organization of chromatin is preserved. The integrity of chromatin components (protein, DNA, RNA) is evidenced by electrophoretic and biochemical analyses. Therefore, these nuclei may be useful for molecular biology studies.

Acknowledgements

This work was supported by the CNRS (ER 197), the CSIC (Institut de Biologia de Barcelona), and grants from INSERM (CRL no. 802015) and Fondation pour la Recherche Médicale, Paris.

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