ISOLATION OF NUCLEOLI

A Method that Combines High Yield, Structural Integrity, and Biochemical Preservation

J. ZALTA, J.-P. ZALTA, and R. SIMARD. From the Laboratoire de Chimie Biologique, Faculté des Sciences, Toulouse, France, and the Département de Biologie Cellulaire, Faculté de Médecine, Sherbrooke, Canada

INTRODUCTION

Studies on the nucleolus have been extensive in biology and medicine since its first description in eucaryotic cells (14). However, in biochemical terms, definition of nucleolar function requires procedures for massive isolation of nucleoli, a technique which has been refined a great deal since the first attempts were described (15, 20). The nearest approximation of presently employed methods resulted from the work of Maggio et al. (11, 12) and Muramatsu et al. (17): in both cases, it involved the use of sonic oscillation to disrupt purified nuclear preparations. Although alternative procedures have been proposed (4, 7, 19), most of the standard methods known to produce a high yield of purified nucleoli derived from sonication procedures.

A new investigation of the variables of the methods employing sonication led to the procedure described in the present paper. This method has proven to be the most satisfactory in ascites tumor cells as it meets completely the following requirements: a high recovery of purified nucleoli, ultrastructural integrity, and biochemical preservation of macromolecular components.

MATERIALS AND METHODS

The principle of this method is the dispersion of extranucleolar nuclear chromatin by sonication of nuclei in Ficoll (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) in presence of Mg^{++}. The concentration of Mg^{++} is critical for the maximal dispersion of chromatin with minimal nucleolar damage for differential centrifugation.

Isolation and Purification of Nuclei

The cells used for these experiments are ascites tumor cells (23). Wistar female rats are inoculated intraperitoneally with approximatively 2×10^7 tumor cells. 4 days later, the ascites are collected (15–20 ml, 4×10^8 cells), washed several times at 4°C in saline phosphate buffer (PBS), and deposited by centrifugation. Nuclei are prepared immediately according to a modification (2) of a standard technique based on the use of nonionic detergents (24).

8×10^7 cells from the cell pellet obtained after washing are resuspended in 5 ml of the following media: sucrose, 0.25 M; Tris HCl, 10^{-2} M, pH 7.4; $MgCl_2$, 2.5×10^{-3} M; $CaCl_2$, 10^{-4} M; polyvinylsulfate, 20 μg/ml. To this solution is added Cemulsol NPT 10, 0.5% (vol/vol), 0.5 mg of Collagenase (Calbiochem, Los Angeles, Calif.), and Celanol 251, 0.1% (vol/vol). Cemulsol NPT 10 and Celanol 251 are both polyethylenated nonionic detergents obtained from Melle-Bezons, Neuilly-sur-Seine, France. Both Cemulsol and Collagenase are used for digestion of the cell wall whereas Celanol removes tags of cytoplasm around the nucleus.

This suspension is homogenized with an ultra-Turrax (Janke and Kunkel, Staufen, Germany) used with a rheostat to control the shaft to 10,000 rpm. The nuclear suspension is then centrifuged 5 min at 600 *g*. Each preparation is controlled under the phase-contrast microscope and the speed of the homogenizer is adapted accordingly. The pellet is then resuspended and washed in the same media without detergents and Collagenase.

Isolation of Nucleoli

SOLUTION A	SOLUTION B
Ficoll 2.1% (w/vol), Tris HCl 10^{-2} M, pH 7.4, $MgCl_2$ 5.5×10^{-3} M, Mercaptoethanol 2.5×10^{-4} M, Polyvinylsulfate 10 μg/ml.	Identical except $MgCl_2$ 5×10^{-4} M.

The viscosity of both solutions A and B is equivalent to 0.3 M sucrose, but Ficoll prevents swelling of nucleoli. The nuclear pellet is suspended for sonication in 3 ml of solution A in a conic 15 ml Pyrex tube. An MSE 100-watt ultrasonic desintegrator was used at 22 kc/sec with a titanium probe of 0.3 cm in diameter inserted 1 cm deep in the solution. The tube is kept in an icebox. Complete disruption of nuclei is obtained through 24 successive waves of sonication of 5 sec each with 5 sec rest in between. 3 ml of ice-cold solution B are then added and 18 other sonications (5 sec with 5 sec rest) are carried out: this latter step permits dispersion of most chromatin aggregates. The sonicate is centrifuged at 1600 rpm (400*g*) in Sorvall's HB4 rotor, for 5 min. If examination of the suspension under the phase-contrast microscope indicates that further purification is required, the nucleoli are resuspended in solution A and exposed again to five other waves of sonication.

The Raytheon Sonic Oscillator model DF 101 (Raytheon Co., Waltham, Mass.) (10 kc, 200 w.) can be used with slightly modified conditions. Nuclei are then suspended in 6 ml of solution A in a 50 ml polypropylene tube (Sorval No. 218) refrigerated through circulating ethylic alcohol at −15°C. Disruption of nuclei is obtained with 10 successive waves of sonication of 10 sec separated by periods of 20 sec rest. 6 ml of solution B is then added and 5–10 other similar waves are required to obtain purified nucleoli.

Extraction and Analysis of Nucleic Acids

Nucleolar RNAs are extracted according to Nakamura et al. (18) with hot phenol at 60°C. DNA is extracted by a modified Kirby procedure (8). Polyacrylamide gel electrophoresis was performed as described by Loening (10) modified by Weinberg and Penman (21). Ultraviolet scanning was done with Joyce Loebl Polyfrac system.

Electron Microscopy

The purity of nucleolar fraction was checked by electron microscopy after double fixation in phosphate-buffered glutaraldehyde 2.5%, 20 min, followed by osmium tetroxide 2%, 1 hr, and embedding in Epon 812. Ultrastructural cytochemistry was carried out after fixation with glutaraldehyde alone and embedding in glycolmethacrylate. Ultrathin sections were floated with a plastic ring on the following solutions: pepsin 0.5% at pH 1.5 during 30 min, followed by RNAse (Worthington Biochemical Corp., Freehold, N.J.) 0.1% at pH 6.8 for 1 hr. Thin sections were contrasted with uranyl acetate 0.5% for 10 min, followed by lead citrate 5 min. (9).

RESULTS AND DISCUSSION

The present technique was tested for purity, ultrastructural integrity, yield, and nucleic acid content.

Ultrastructural Analysis

Nucleolar fractions were examined by electron microscopy to determine the degree of contamination and the ultrastructural integrity of their architecture. Fig. 1 is a low-power electron micrograph of purified nucleoli. Such a degree of purity is routine with this procedure. Contamination with unbroken nuclei is incidental and with nonnucleolar dispersed chromatin rare. The ultrastructure of nucleoli is similar to that found *in situ*. Granules are dispersed in a fibrillar reticular network, surrounded by the nucleolus-associated chromatin.

Enzymatic digestions permit identification of the chromatin associated with nucleoli isolated according to the present technique. Pepsin followed by ribonuclease are then used to extract

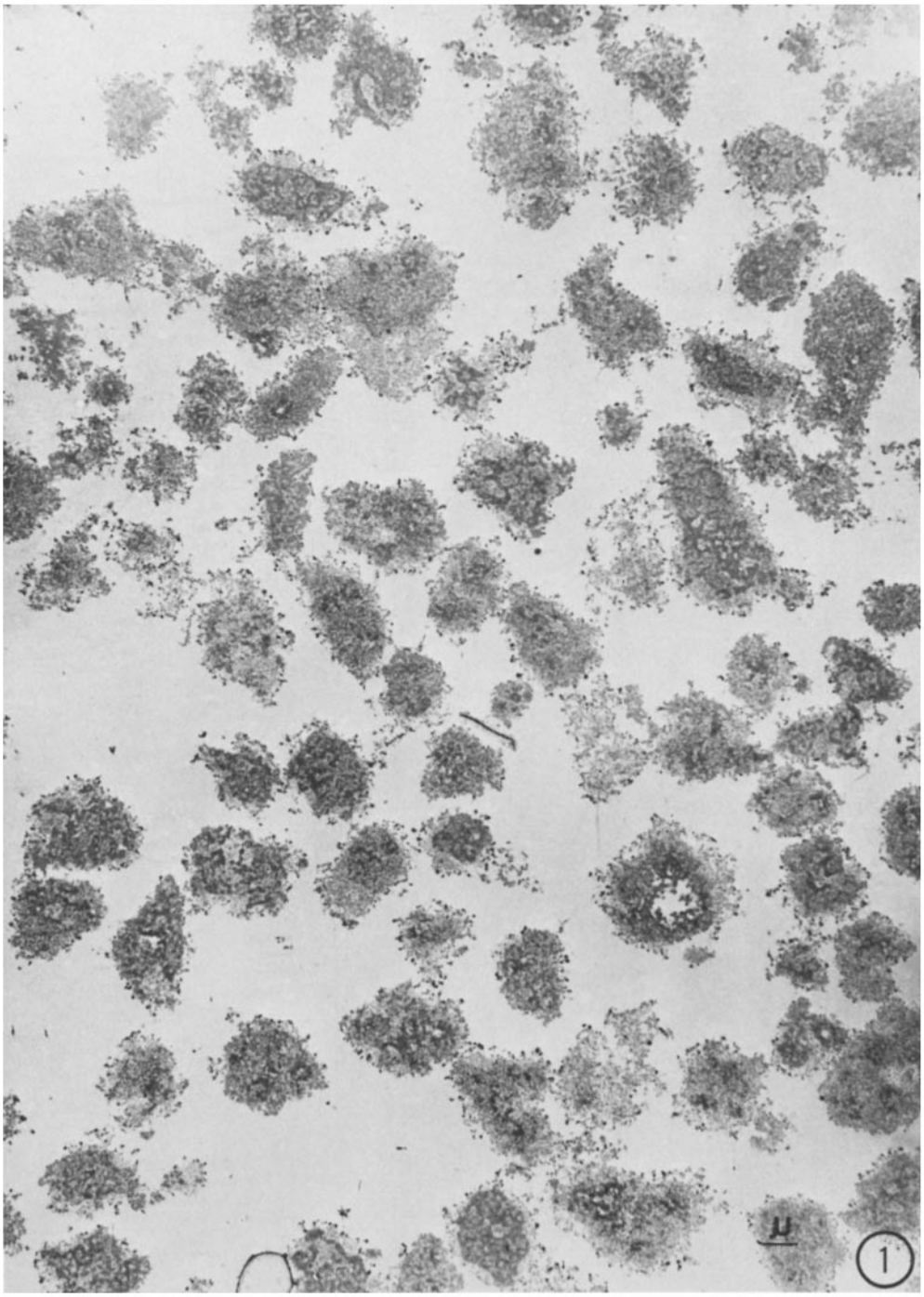

FIGURE 1 Purified nucleoli of ascites tumor cells. The architecture is preserved and there is no swelling of each nucleolus. Double fixation, Epon. Scale marker, 1 μ. × 5000.

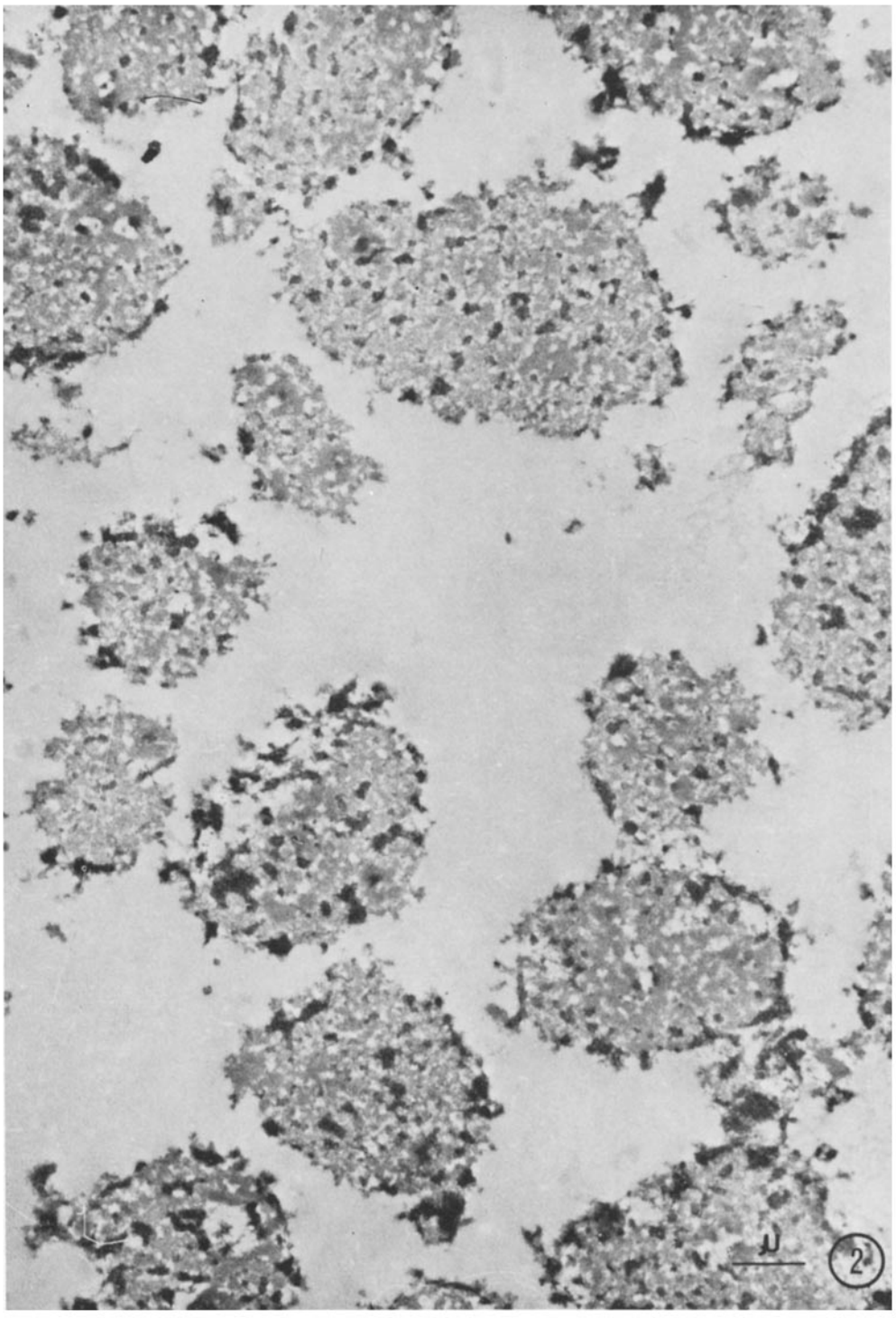

FIGURE 2 Thin section of GMA-embedded nucleolar pellet treated with pepsin and RNAse. The intranucleolar and perinucleolar chromatin is well preserved and heavily contrasted. × 12,000.

ribonucleoproteins and increase the contrast of chromatin. The distinction then becomes easy. Fig. 2 shows that "nucleolar DNA" is located within the perinucleolar chromatin with its intranucleolar ramifications.

Recovery

With this procedure, the recovery of nuclei from whole cells approximates 95–100% and this is probably an important factor for the subsequent yield of nucleoli. Effectively, recovery of nuclei with other methods aimed at purification of nuclei approximates 30–60% (6) and 25–35% (11); by raising the sucrose concentration, Blobel and Potter (5) were able to obtain a recovery of 90% from rat liver. With the present method, nuclei are well preserved but not purified from cytoplasmic contaminants. This contamination is minimal and consists only of portions of the external leaflet of the nuclear membrane with attached ribosomes. If further purification is carried out to remove completely contaminants, the recovery of nuclei falls to 80%, affecting proportionally the yield of nucleoli.

There are few data about recovery of nucleoli from the total number present in intact cell. A value of 20–40% has been obtained by Maramatsu and Busch (16). The routine recovery with the present method averages 70% and is extremely reliable from one experience to another. It is conceivable that large yield of nuclei, presence of Mg^{++}, and sonication procedures are important factors (Table I).

Chemical Composition

Table II shows the average amount of DNA, RNA, and proteins obtained per nucleolus of ascites tumor cells. These values are in agreement with previous results in other laboratories (see reference 16 for a review). Electrophoretic analysis of the RNA extracted from nucleoli revealed no significant changes of profile although the high amount of 41S and 45S is noteworthy (Fig. 3).

This method proved to be, in our hands, the most satisfactory whenever purified nucleoli were required from a limited source of material. At present, none of the nucleoli isolated from mammalian cells are a satisfactory source of ribosomal DNA (rDNA) genes which represent only 0.25–0.5% of total nucleolar DNA (value derived from Attardi et al. [3] and McConkey and Hopkins [13]). However, thanks to the high yield of this method, we were able to prepare highly active, guanine-cytosine–rich, single-stranded DNA from ascites tumor cell nucleoli (1). Preparation of nucleolar enzymes such as RNA polymerase and ribonuclease are other examples that can benefit from this high yield and integrity.

TABLE I

Percentage of Recovery Based on Cell Count

	Count	Recovery
		%
Cells	2.1×10^8	100
Nuclei	2.0×10^8	95
Nucleoli	1.5×10^8	70

The values represent an average of five determinations. Ascites tumor cells have an average of two nucleoli per nuclei.

TABLE II

Composition of Isolated Nucleoli

	Amount (pg/nucleolus)	Per cent
DNA	1.6	6
RNA	3	12
Proteins	23	82

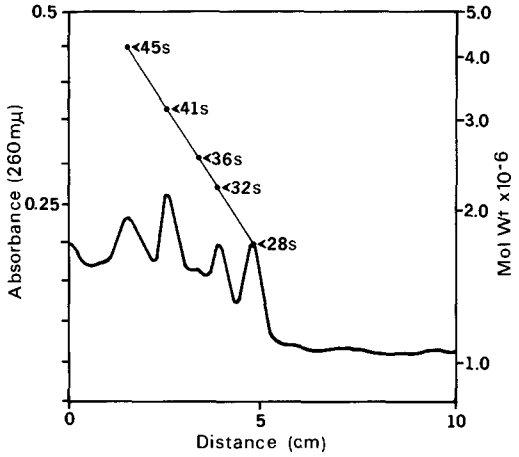

FIGURE 3 Gel electropherogram of nucleolar RNA run 6 hr on a 2.7% gel at 5 mA, with logarithmic calibration of molecular weights *versus* electrophoretic mobility according to Weinberg and Penman (22). Ribosomal 28S and 18S RNA were used as markers.

This study was in part supported by grant MA-3368 from the Medical Research Council to Dr. Simard.

Received for publication 19 April 1971, and in revised form 18 May 1971.

REFERENCES

1. AMALRIC, F., and R. SIMARD. 1970. *J. Cell Biol.* **47**:6 a. (Abstr.)
2. AMALRIC, F., R. SIMARD, and J.-P. ZALTA. 1969. *Exp. Cell Res.* **55**:370.
3. ATTARDI, G., P. C. HUANG, and S. KABAT. 1965. *Proc. Nat. Acad. Sci. U. S. A.* **54**:185.
4. BIRNSTIEL, M. L., M. I. H. CHIPCHASE, and J. BONNER. 1961. *J. Biochem. Biophys. Res. Commun.* **6**:161.
5. BLOBEL, G., and R. VAN POTTER. 1966. *Science (Washington).* **154**:1662.
6. CHAUVEAU, J., Y. MOULE, and C. ROUILLER. 1956. *Exp. Cell Res.* **11**:317.
7. DESJARDINS, R., K. SMETANA, W. J. STEELE, and H. BUSCH. 1963. *Cancer Res.* **23**:1819.
8. KIRBY, K. S. 1964. *Progr. Nucl. Acid. Res. Mol. Biol.* **3**:1.
9. LEDUC, E. H., V. MARINOZZI, and W. BERNHARD. 1963. *J. Roy. Microsc. Soc.* **81**:119.
10. LOENING, U. 1968. *Biochem. J.* **113**:131.
11. MAGGIO, R., P. SIEKEVITZ, and G. E. PALADE. 1963. *J. Cell Biol.* **18**:267.
12. MAGGIO, R., P. SIEKEVITZ, and G. E. PALADE. 1963. *J. Cell Biol.* **18**:293.
13. MCCONKEY, E. H., and J. W. HOPKINS. 1964. *Proc. Nat. Acad. Sci. U. S. A.* **51**:1197.
14. MONTGOMERY, T. H. 1968. *J. Morphol.* **15**:265.
15. MONTY, K. J., M. LITT, E. R. KAY, and A. L. DOUNCE. 1956. *J. Biophys. Biochem. Cytol.* **2**:127.
16. MURAMATSU, M., and H. BUSCH. 1967. *In* Methods in Cancer Research. H. Busch, editor. Academic Press Inc., **2**:303.
17. MURAMATSU, M., K. SMETANA, and H. BUSCH. 1963. *Cancer Res.* **23**:510.
18. NAKAMURA, T., F. RAPP, and H. BUSCH. 1967. *Cancer Res.* **77**:1084.
19. POORT, C. 1961. *Biochim. Biophys. Acta.* **46**:373.
20. VINCENT, W. S. 1952. *Proc. Nat. Acad. Sci. U. S. A.* **38**:139.
21. WEINBERG, R. A., and S. PENMAN. 1968. *J. Mol. Biol.* **38**:289.
22. WEINBERG, R. A., and S. PENMAN. 1970. *J. Mol. Biol.* **47**:169.
23. ZAJDELA, F. 1963. Colloque Franco-Soviétique. Gauthier-Villars, Paris. 49.
24. ZALTA, J.-P., R. ROZENCWAJG, M. CARASSO, and P. FAVARD. 1962. *C. R. Acad. Sci. Paris.* **255**:412.