Isolation of Nucleoli of the Walker Carcinosarcoma and Liver of the Rat Following Nuclear Disruption in a French Pressure Cell*

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SUMMARY

A method is presented for the isolation of nucleoli of the rat liver and the Walker tumor in which the tissues were initially homogenized in a solution containing 0.25 M sucrose and 0.005 M CaCl₂ and then were compressed and rapidly decompressed in a French pressure cell. Approximately 60 minutes after homogenization the sample was subjected to 5000–7000 p.s.i. in a French pressure cell. The product was layered over discontinuous gradient consisting of 1.5 and 2.2 M sucrose solutions and was centrifuged at 64,000 × g for periods of time ranging from 5 to 25 minutes. Under optimal conditions the recoveries of nucleoli in the sediment were 75 and 55 per cent in the tumor and liver, respectively.

The average protein, RNA, and DNA contents of the nucleoli of the Walker tumor were 7.8, 1.3, and 1.1 pg. and in the nucleoli of the liver were 5.0, 0.3, and 0.15 pg., respectively. These data support previous analytical studies (5) that showed there was a significantly greater content of nucleic acids and protein in nucleoli of the Walker tumor than in nucleoli of rat liver cells.

There is an increasing interest in the function and composition of the nucleolus of the cancer cell and other types of cells (2). For accurate studies on nucleoli, adequate methods for the isolation of highly purified nucleolar preparations are required. Recently, Muramatsu et al. (5) have developed a procedure for the isolation of nucleoli of the Walker tumor and the liver that provided yields ranging from 12 to 24 per cent of the total nucleoli in the whole homogenates. Moreover, the appearance of the isolated nucleoli on electron microscopy was similar to the nucleoli in situ, with the exception of the fact that the spacing between the nucleolonemas was somewhat larger in some isolated nucleoli. The nucleoli isolated by the technic employing sonication differed morphologically from those seen in suspensions of tumor cells in that they were generally oval or round. Nucleoli of tumor cells have irregular shapes and frequently have small projections from their surfaces (4) that may be part of the nuclear ribonucleoprotein network (7).

The present study was designed to explore the possibility of developing a simplified technic that would provide a higher yield of nucleoli with a morphology similar to the nucleoli in tumor cells. In essence, the technic developed consists of compression and decompression of whole homogenates of the Walker tumor in a French pressure cell and subsequent centrifugation of the product layered over concentrated sucrose solutions. With this technic, nucleolar preparations have been obtained with very little contamination. The recoveries of nucleoli were 75 and 55 per cent of the total in the original whole homogenates of the Walker tumor and liver when optimal conditions were employed.

MATERIALS AND METHODS

Animals.—The animals used in these experiments were male albino rats, weighing 180–220 gm., obtained from the Holtzman Rat Company.

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(Madison, Wisconsin) and fed ad libitum on Purina Laboratory Chow. The tumor studied was the Walker 256 carcinosarcoma transplanted 7–10 days prior to the experiment. The animals were anesthetized with diethylether and sacrificed by exsanguination following aortic section. The excised tissues were placed in ice-cold 0.25 M sucrose containing 0.005 M CaCl₂. All subsequent procedures were carried out at 4°C. After a fine dissection to remove connective tissue, hemorrhagic or necrotic masses, and adherent muscle, the tissues were weighed and homogenized in a homogenizer with a Teflon pestle with a pestle clearance of 0.005–0.006 inch containing 0.25 M sucrose and 0.005 M CaCl₂ (1:10, w/v). After ten up-and-down strokes, the homogenate was filtered through eight layers of gauze and a 90-mesh stainless-steel screen.

Disruption of the nuclei.—Forty ml. of the whole homogenate was placed in a French pressure cell which was subjected to 7000 p.s.i. in a Carver hydraulic press. When the pressure reached 7000 p.s.i., the needle value was opened with caution; release of the filtrate in the cell caused the pressure to drop slowly. The pressure was maintained between 5000 and 7000 p.s.i. In the initial experiments, different pressures were tried, ranging from 3000 to 12,000 p.s.i. At 3000 p.s.i., the disruption of nuclei was incomplete. Increasing the pressure increased the destruction of the nuclei, but at 9000 p.s.i. a grayish sediment appeared on the bottom of the nucleolar preparations, suggesting the possibility that denaturation of the proteins was occurring. Accordingly, the pressures indicated above—i.e., 5000–7000 p.s.i.—were used in this study.

Approximately 60 minutes had to elapse between the homogenization and nuclear disruption, since the data of Charts 1 and 2 show that the ratio of nucleoli to nuclei was maximal when compression and rapid decompression were carried out at this time.

Isolation of the nucleoli.—Approximately 20 ml. of pressed product was placed into 30-ml. tubes of the S.W. 25.1 rotor of the Spinco Model L ultracentrifuge. With the use of a Cornwall continuous pipettor with a 4-inch 14-gauge needle, 5 ml. of 1.5 M sucrose (pH 4.5–6.2) was layered on the bottom of each tube. With another Cornwall pipettor, 3 ml. of 2.2 M sucrose (pH 4.7–6.7) was layered under the 1.5 M sucrose. The samples were centrifuged at 25,000 r.p.m. (av., 64,000 X g) for 10 minutes for the tumor and 20 minutes for liver. After centrifugation, the supernatant solutions were aspirated with a Pasteur pipette connected to a water pump until only the sedimented pellet remained at the bottom of the tube. When necessary, a clean tissue was used to wipe the wall of the tube where the interphases were formed. The remaining pellet was the nucleolar preparation.

Identification of nucleoli.—Smears prepared from homogenates and nucleolar preparations were stained with the Giemsa stain for general orientation and with toluidine blue for staining of nucleoli (6). Isolated nucleoli and nucleoli in nuclei were also studied by phase-contrast microscopy before and after “supravital” staining with 10 per cent Giemsa solution in 0.25 M sucrose.

Electron microscopy.—Pellets of nucleolar preparations were fixed 2–3 hours at pH 5–9.5 with buffered 2 per cent OsO₄ according to Palade; some were postfixed in 10 per cent neutral formalin. Some pellets were fixed in 10 per cent neutral formalin and postfixed with 2 per cent OsO₄ at pH 7.5. The dehydration, embedding, and other
procedures used were essentially the same as those described previously (6).

**Staining and counting.**—The number of the nucleoli in the pressed product was determined on an aliquot by direct counting in a hemacytometer, with either Azure C (0.1 per cent in 0.25 m sucrose) or Giemsa (10 per cent solution in 0.25 m sucrose and 0.15 m NaCl) as the diluent. Counting of the nucleoli in the final nucleolar preparation was carried out by the same procedure after dilution of an aliquot with 0.25 m sucrose containing 0.005 m CaCl₂.

**Analytical procedures.**—Each fraction was precipitated with 0.5 N perchloric acid and centrifuged. The precipitate was washed successively with 10 ml. of 95 per cent ethanol and then with 10 ml. of absolute ethanol and centrifuged. To the residue 5 ml. of 0.5 N perchloric acid was added, and the sample was digested at 70° C. for 15 minutes (5). The extract was used for the determination of nucleic acid. The residue was then washed with 95 per cent ethanol, 100 per cent ethanol, 2:1 chloroform: methanol, benzene, and ether, and desiccated. The dry protein powder obtained was weighed. Deoxyribonucleic acid (DNA) was determined by the Burton (1) modification of the diphenylamine procedure. Ribonucleic acid (RNA) was determined by the orcinol procedure (3). The readings for the orcinol reaction were corrected for the values which would account for DNA. Yeast RNA and calf thymus DNA (Worthington Biochemical Corporation) were used as standards.

**RESULTS**

**Morphology of isolated nucleoli.**—Nucleoli in nuclei of liver were either round or oval (Fig. 1), and the nucleoli in nuclear preparations of the Walker tumor were irregular in shape in many instances (Fig. 2). In the nucleolar preparations, isolated nucleoli as well as nucleoli surrounded by a halo of nucleolus-associated chromatin were observed (Figs. 3, 4). Nucleoli isolated from liver nuclei were similar in morphology to those present in situ (Fig. 3). Nucleoli isolated from Walker tumor cells showed variability in size and shape similar to that noted in whole cells or nuclear preparations (Fig. 4). The toluidine blue used for staining the preparations did not stain the chromatin. The stainability of isolated nucleoli was essentially the same as that of nucleoli in nuclear preparations.¹

The ultrastructure of the isolated nucleoli (Figs. 5, 6) was essentially the same as the ultrastructure of nucleoli in situ (5). However, the surfaces of the isolated nucleoli appeared to be more compact, and the light areas between the nucleolonemata appeared to be somewhat smaller. A thin, dense layer was found around isolated nucleoli after formaldehyde fixation that apparently represents the proximal portion of the nucleolus-associated chromatin. By comparison with nucleoli isolated after sonication (5), the nucleoli were more compact and had segments of nucleolus-associated chromatin on the surfaces.

**Recovery of nucleoli.**—Recovery is a function of the logarithm of the time of centrifugation as shown in Chart 3. In view of the fact that contaminating particles increased as the time of centrifugation was prolonged, the maximum time employed was 20 minutes for the liver and 10 minutes for the tumor. This is the optimal time for minimal contamination and maximal recovery. The contamination was less than 20 per cent by direct particle count when 60 per cent of the nucleoli of the tumor were recovered. From the rate of sedimentation of nucleoli of tumor which was greater than that of the liver, it seems possible that the density of the nucleoli of the tumor was significantly greater than that of the nucleoli of the liver.

As noted previously (5), divalent ions are required to maintain the integrity of the nucleoli. In the present study, a range of concentrations of Ca⁺⁺ from 0.0015 to 0.10 m was added to the homogenizing medium. At concentrations below 0.0018 m, intact nucleoli were not recovered. Under the standard conditions of these experiments, maximal recoveries of nucleoli were obtained when the

¹ The Feulgen stain and the Giemsa stain showed the presence of nucleolus-associated chromatin around isolated nucleoli. By phase-contrast microscopy, the surfaces of nucleoli isolated from Walker tumors were seen to be more irregular than the surfaces of nucleoli isolated from liver cells. The shapes of the nucleoli were unchanged by the isolation technic.
concentration of Ca++ in the homogenizing medium was 0.005–0.010 M.

**RNA content of nucleoli.**—The total RNA recovered in nucleolar preparations was 3–5 times larger for the tumor than for the liver (Table 1) and increased as the centrifugation time was increased. The content of RNA per nucleolus (Chart 4) decreased progressively in both the tumor and liver preparations as the centrifugation time progressed, presumably because of the initial sedimentation of larger nucleoli. The RNA : DNA ratio (Table 1) was higher at the earlier times of centrifugation than at the later times, and in the liver it reached a plateau at 15 minutes of centrifugation. The RNA : DNA ratio was approximately 2–4 in the liver and 1–1.5 in the tumor.

**DNA content of nucleoli.**—In the tumor the DNA recovered in the nucleolar preparation increased proportionately to the recovery of the nucleoli (Table 1), and the content per nucleolus (Chart 5) was approximately one-tenth that of the tumor. A substantial amount of the DNA present in these preparations appears to be in the perinucleolar chromatin—i.e., “the nucleolus-associated chromatin,” as shown in Figure 7.

**Protein content of nucleoli.**—The recovery of protein in the nucleolar preparation was also a function of the logarithm of the centrifugation time (Table 1), both for the tumor and for the liver. The content of protein per nucleolus was approximately 10 times that of the nucleic acids in the liver but only 3–4 times that of the nucleic acids in the tumor (Table 1). As shown in Chart 6, the value for the absolute amount of protein per nucleolus

**TABLE 1**

<table>
<thead>
<tr>
<th>Recovery of Protein and Nucleic Acids in Nucleolar Preparations from 10 Grams of Walker Tumor or Rat Liver</th>
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<tbody>
<tr>
<td>The pressed product was centrifuged at 64,000 X g for the times indicated. Each value presented in parentheses is the average of three experimental determinations for one mass preparation. The average deviation in the three determinations was 3 per cent for protein, 5 per cent for DNA, and 4 per cent for RNA. The values before the parentheses are the averages for two mass experiments.</td>
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<table>
<thead>
<tr>
<th>Centrifugation time (min.)</th>
<th>Walker Tumor</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Protein (mg.)</td>
<td>DNA (µg.)</td>
</tr>
<tr>
<td>5</td>
<td>7.2 (6.1, 8.3)</td>
<td>920 (650, 990)</td>
</tr>
<tr>
<td>7½</td>
<td>9.8 (9.5, 10.2)</td>
<td>1660 (1830, 1690)</td>
</tr>
<tr>
<td>10</td>
<td>13.0 (12.5, 13.4)</td>
<td>2200 (1900, 2300)</td>
</tr>
<tr>
<td>15</td>
<td>15.2 (14.4, 15.9)</td>
<td>2600 (2000, 2800)</td>
</tr>
</tbody>
</table>

**CHART 4.**—Average content of RNA per nucleolus in nucleolar preparations obtained by centrifugation of the pressed product at 64,000 X g (av.) for varying periods of time. The data are average values from multiple determinations in two preparatory experiments.

**CHART 5.**—Average content of DNA per nucleolus in nucleolar preparations obtained by centrifugation of the pressed product at 64,000 X g (av.) for varying periods of time. The data are average values from multiple determinations in two preparatory experiments.
Fig. 1—Whole homogenate of rat liver stained with toluidine blue. Nuclei with large nucleoli are visible. X1,200.

Fig. 2.—Homogenate of Walker tumor stained with toluidine blue showing irregularity of the nucleoli. X1,200.

Fig. 3.—Isolated liver nucleoli stained with toluidine blue. X1,200.

Fig. 4.—Isolated nucleoli from the Walker tumor stained with toluidine blue. X1,200.

Fig. 5.—Electron micrograph of nucleoli isolated from rat liver. X34,000. The black line represents 1 micron.
Fig. 6.—Electron micrograph of nucleoli isolated from the Walker tumor. ×34,000. The black line represents 1 micron.
Fig. 7.—Isolated nucleoli of the Walker tumor stained by the Feulgen technic.
was not markedly different for the tumor from that for the liver.

DISCUSSION

The procedure employing compression and rapid decompression would appear to have some significant advantages over the procedure employing sonication (5). The isolated nucleoli appear to have shapes and sizes that are similar to those seen either in whole cells or in isolated nucleoli of the Walker tumor. In addition, the recovery of nucleoli from the Walker tumor was higher than the recovery employing the sonicating technic. From the point of view of time required for the method, it has been found that a preliminary isolation of nucleoli does not markedly improve the final procedure. Hence, this method can be applied directly to the whole homogenate.

The quantitative analysis of the nucleic acids and protein content of the nucleoli in the present studies provides results that do not differ significantly from the results obtained with the procedure employing sonication with the exception of the value for protein content of the nucleoli of the Walker tumor. In the present study the protein content of nucleoli was approximately 8 pg. per nucleolus, whereas in the previous study a value of 81.6 pg. per nucleolus was obtained. These present results support the previous finding (5) that the average RNA content of the nucleoli of the Walker tumor is approximately 5 times that of the nucleolus of the liver cell and that the DNA content is also approximately 5 times that of the liver cell.

It would seem that a combination of the present procedure with that employing sonication might have some advantages in studies of the nucleolus-associated chromatin. The nucleolus-associated chromatin appears to be preserved to a greater degree with the technic employing compression and rapid decompression than it is in the procedure employing sonication. Studies on the nucleoproteins of the nucleolus-associated chromatin could provide some indication as to the types of macromolecular species involved in control of synthesis of the nucleolus and its component parts. It is possible that this component contributes messenger RNA involved in the formation of the nuclear ribonucleoprotein network recently described (7).

Studies on the chemical composition of the nucleoli sediments at varying times of centrifugation might provide additional information on the question of whether nucleoli have a constant function regardless of their size. As seen in Table 1, differences were found in the composition of the nucleoli as centrifugation was continued. The variation in composition of nucleoli of the tumor appears to be less than the variation of nucleoli of the liver. Experiments at present are directed toward determining the macromolecular species involved in biosynthetic regulations leading to synthesis of nucleoli as well as the composition of the nucleolar structures and their role in biosynthesis of cellular components.

The possibility exists that small amounts of RNA and ribonucleoproteins are extracted from nucleoli in the course of centrifugation. Under the conditions employed in the present experiments significant amounts of RNA are not extracted from ribosomal RNA. Studies in this laboratory by Muramatsu et al. (unpublished) have shown that the base ratios of nucleolar RNA are similar to those of cytoplasmic ribosomal RNA. To resolve the question of extraction of RNA or ribonucleoprotein from nucleoli, studies need to be completed on isolation of nucleoli in nonaqueous media.

REFERENCES

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