A Method for Isolation of Nuclei from Cells of the Walker 256 Carcinosarcoma*

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Recent reports from this laboratory (1, 2) have indicated the desirability of a method for the preparation of nuclei free from cytoplasmic contamination which would be applicable to tumors as well as other tissues. A variety of methods have been reported to produce satisfactory preparations of nuclei of liver cells, but Dounce has emphasized the difficulties in obtaining preparations from tumor cells of nuclei which are not contaminated with a large number of whole cells or cytoplasmic fractions (5). The serious problems in isolation procedures requiring the use of organic solvents have been discussed (1). The use of strongly acidic media would increase the possibility of losses of acid-soluble nuclear proteins into which L-lysine-U-C₁⁴ is rapidly incorporated (2). Accordingly, a number of procedures involving the use of sucrose media were tested, and the method of Chauveau et al. (3) was found to be most useful when modifications were made. The main modifications of the procedure of Chauveau et al. were: (a) definition of the limits of pestle clearance for removal of adherent cytoplasm from the nucleus in the course of homogenization in isotonic sucrose; (b) use of 2.0 M sucrose for resuspension of the 600 g precipitate prior to sedimentation in a force field of 40,000 g for 30 minutes. With optimal conditions, nuclei of the Walker 256 carcinosarcoma could be isolated which were essentially free of adherent cytoplasm, whole cells, or cytoplasmic granules. Following the injection of L-lysine-U-C₁⁴ into tumor-bearing rats, histones were extracted from nuclei of tumor cells obtained by this method and were found to have approximately the same specific activities as the histones obtained by methods used previously (2).

Homogenization.—For the purpose of this report, the pestle clearance is defined as the difference between the maximum outer diameter of the pestle and the minimum inner diameter of the homogenizer tube. The maximum outer diameter of the pestle was determined to ±0.0005 inch by means of a micrometer caliper. The minimum inner diameter of the tube was determined by means of a telescoping gauge, 1/2 inch, fitted with a 9-inch handle. All pestles and tubes used in this study were calibrated in this way before use in the experiment.

The initial homogenization of the tissues was carried out in isotonic sucrose (9:1, v:w) with ten strokes of a Teflon pestle in a glass homogenizer tube. The pestle clearance for this step was 12 x 10⁻³ inch. The speed of the homogenizer motor was 1200 r.p.m.

Homogenization at defined pestle clearance.—After the initial homogenization, the sample was filtered through a 120-mesh stainless steel screen with the aid of gentle suction and then was transferred to one of a series of all-glass homogenizers (6) with pestle clearances ranging from 0.5 to 7 x 10⁻⁹ inch; the homogenate was then subjected to five up and down strokes of the pestle. The homogenate was transferred to a 12-ml glass centrifuge tube and subjected to a force field of 600 X g for 10 minutes (7) in an International refrigerated centrifuge at 5°C. The supernatant solution was decanted and discarded.

Centrifugation in hypertonic sucrose.—To the precipitate, 9 volumes of sucrose were added. In various experiments, the molarity of sucrose ranged from 1.5 to 2.5 M. After a few minutes, most of the tissue rose to the top, leaving a small layer of packed red cells at the bottom of the centrifuge tube. The homogenate and sucrose were decanted into glass homogenizer tubes and re-

MATERIALS AND METHODS

Animals and preparation of tissues.—The techniques of transplantation of the tumors, anesthesia,

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homogenized with glass pestles with a pestle clearance of 7-10 × 10⁻³ inch. This preparation was centrifuged at 40,000 × g for 30 minutes in the Spinco Model L ultracentrifuge. The supernatant solution was decanted from the clear jelly-like pellet at the bottom of the tube. To prevent aggregates of granules or the supernatant solution from sliding into the pellet, the centrifuge tubes were cut with scissors just above the pellet.

*Phase microscopy.*—Because of the reported difficulties in the detection of cytoplasmic aggregates adherent to the nuclei by means of the light microscope (4), the phase microscope (A-O Spencer Microstar) was used throughout this study. The bright-contrast oil immersion lens was found to be particularly useful for detection of cytoplasmic granules.

*Purity of the preparations of nuclei.*—In the preparations obtained, differential counts were made of (a) nuclei completely isolated from cytoplasmic granules, (b) nuclei with adherent cytoplasm and granules, and (c) nuclei in intact cells.

Several counts of this type were made on each preparation in ten or more fields to a total of 150 nuclei per count. The preparation was repeated 7 times.

The purity of the preparation was also determined by division of the volume of the nuclei by the volume of nuclei plus granules. The nuclei and the granules were assumed to be spheres, and their diameters were determined by means of a calibrated ocular. The diameter of the nuclei was found to average 18 μ, while the average diameter of the cytoplasmic granules was found to be 0.5 μ.

**RESULTS**

**VARIABLES**

Three variables were found to affect the yield and purity of the nuclear preparation. In order, these were pestle clearance, number of up-and-down strokes of the pestle, and molarity of the sucrose used for the fractional sedimentation of the initial 600 × g precipitate.

*Pestle clearance.*—All-glass homogenizers with pestle clearances ranging from 0.5 × 10⁻³ inch to 7 × 10⁻³ inch were studied for their effectiveness in removing the cytoplasm from the nuclei. As indicated in Chart 1, the greatest ratio of isolated nuclei to nuclei in intact cells was produced by homogenizers with a pestle clearance of 3 × 10⁻³ inch.

As indicated in Chart 1, homogenizers with pestle clearances of greater than 3 × 10⁻³ inch produced a smaller ratio of isolated nuclei to nuclei in cells. Homogenizers with smaller pestle clearances resulted in significant destruction of the nuclei as evidenced by the fact that relatively few if any nuclei were found in each field even with a pestle clearance of 1.5 × 10⁻³ inch. With a pestle clearance of 0.5 × 10⁻³ inch, essentially no nuclei were found, and a gelatinous mass was noted. In a number of experiments with such tight homogenizers, the initial 600 × g precipitate was greyish, as contrasted with the pink color of the precipitate obtained after use of homogenizers with pestle clearances of 3 × 10⁻³ inch or greater.

*Strokes of the pestle.*—Each preparation was subjected to five or more up-and-down strokes of the homogenizer pestle through the suspension. Improvement of the preparation resulted from a greater number of strokes only when it was observed that the initial nuclear preparation contained excess cytoplasm adherent to the nuclei.

When the preparation was subjected to more than 40 strokes of the pestle through the suspension, the preparation was largely denatured.

*Molarity of sucrose for resuspension of the 600 × g precipitate.*—Molarities of sucrose ranging from 1.5 to 2.2 m were tested in increments of 0.1 m. At molarities of sucrose ranging from 1.5 to 1.8 m, the entire sample settled to the bottom of the tube. Conversely, at molarities greater than 2.1 m, most of the initial 600 × g precipitate remained suspended at the top of the medium. In the intervening range of 1.9-2.1 m the nuclei settled to the bottom of the tube, and highly purified preparations...
could be obtained which were largely free of cytoplasmic granules. However, the yield at 2.1 M was less than that obtained at 2.0 M; hence, the latter concentration was selected as the standard concentration for these experiments.

**Effectiveness of the procedure.**—From the studies above, the following conditions were selected for isolation of nuclei of the Walker tumor: (a) rehomogenization of the sample filtered through the stainless steel screen (120 mesh) with an all-glass homogenizer having a pestle clearance of $3 \times 10^{-3}$ inch; (b) five up-and-down strokes of the pestle; (c) fractional centrifugation of the initial 600 X g precipitate in 2.0 M sucrose.

The effectiveness of this over-all procedure in producing isolated nuclei is noted in Figures 1–3. Figure 1 is a photograph of a 600 X g precipitate obtained by the Schneider procedure (7) with a homogenizer having a pestle clearance of $7 \times 10^{-3}$ inch. It can be seen that isolated nuclei are rare, and whole cells are common. A number of the nuclei are surrounded by cytoplasmic granules which are adherent to the nucleus or are embedded in semicircular bits of cytoplasm. Figures 2 and 3 present the appearance of a microscopic field of nuclei obtained by the method outlined above. The few dark granules interspersed among the nuclei are ground glass particles. Most of the nuclei are completely free of adherent cytoplasmic granules.

**Glass.**—In all preparations made in glass homogenizers, highly refractile particles were found which ranged in size from that of small intracellular granules to one-fourth the diameter of the nucleus. The amount of such particles markedly increased as the pestle clearance diminished. Figure 3 indicates the appearance of such particles when viewed through a bright-contrast oil immersion lens. The nuclei in this field are very similar to those seen in Figure 2, which is a photograph through a dark-contrast oil immersion lens. When

1 It was noted that a number of the nuclei of tumor cells had a slight invagination of the nuclear membrane slightly off the center of the nucleus. Because of this slight folding, some of these nuclei appeared to be kidney bean-shaped.

**DISCUSSION**

From the data indicated above, it is apparent that requirements for isolation of the nuclei of the Walker 256 carcinosarcoma include (a) an all-glass homogenizer with a pestle clearance of $3 \times 10^{-3}$ inch, (b) five up and down strokes of the pestle (motor speed, 1200 r.p.m.) and (c) recentrifugation of the initial 600 X g precipitate in 2.0 M sucrose. The preparations of nuclei varied in purity from 88 to 99 per cent when counts were made of completely isolated nuclei, nuclei with adherent cytoplasm, and nuclei in intact cells. Isolated nuclei comprised 99 per cent of the total volume occupied by cellular particles in the best preparations.

**Limitations.**—With this method, excellent preparations of nuclei of Walker tumor and liver were obtained. However, the preparations of nuclei of kidney and spleen were considerably more contaminated, even under the best conditions tested. It should be noted that the conditions required for isolation of good preparations of the nuclei of the liver were not nearly so stringent as those required for the tumor. For example, a Teflon® pestle in a glass tube sufficed to produce a preparation in
which a large number of the nuclei of the liver were isolated from the cytoplasm. When these preparations were subjected to centrifugation in the 2.0 M sucrose, nuclei were obtained in moderately good yield, i.e., about 30–50 per cent of the nuclei either in cells or with adherent cytoplasm and granules. It remains to be determined whether the ease of separation of nuclei from cells is a biological characteristic of different tissues.

Confirmation of data on uptake of L-lysine-U-C\textsuperscript{14} into the histones.—In a recent study (2) data were obtained which indicated that the uptake of isotope into the histones of the Walker and Jensen tumor exceeded that of most other tissues when radioactive lysine was used as the precursor. Following the development of the present method, this problem has been further investigated, and it has been found that the specific activities of the histones obtained from nuclei of the liver and of the Walker tumor do not vary significantly, i.e., 5 per cent or less, from the data reported previously. With the present method, the total weight of histones recovered from the nuclei of the cells of the Walker tumor was 95–100 per cent of the total histones obtained in previous experiments (2), while the total weight of histones recovered from the liver was 40–53 per cent of the total previously obtained.

SUMMARY

1. To obtain isolated nuclei of the Walker 256 carcinosarcoma, samples of the tumor were subjected to initial homogenization in 0.25 M sucrose in a glass homogenizer tube fitted loosely with a Teflon\textsuperscript{R} pestle and having a pestle clearance of 9–12 × 10\textsuperscript{-3} inch. The homogenate was rehomogenized in an all-glass homogenizer with a pestle clearance of 3 × 10\textsuperscript{-3} inch, and then centrifuged at 600 × g for 10 minutes. The precipitated nuclei and debris were resuspended in 2.0 M sucrose and recentrifuged at 40,000 × g for 30 minutes.

2. The nuclei were obtained in high yield with a purity of 99 per cent by volume and 88–99 per cent by differential count of isolated nuclei, nuclei with fragments of adherent cytoplasm, and nuclei in intact cells.

REFERENCES

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