Summary

Nuclear preparations from the Walker tumor were isolated after homogenization of the tumors in 2.5% citric acid. The recovery of nuclei in the procedure employed was 43%, and the purity of the preparations was 80–90% by phase microscopy and particle counts with the aid of ultracentrifugation. The DNA, RNA, and protein contents of the isolated tumor nuclei were 11.9, 5.7, and 34.4 pg per nucleus, respectively. The RNA/DNA ratio of isolated nuclei of the Walker tumor was 0.48, as compared to 0.28 for the nuclei of the normal liver. In studies on normal liver nuclei, it was found that the amounts of DNA, RNA, and protein recovered after treatment with citric acid were 85, 80, and 70%, respectively, of the amounts recovered in the nuclei isolated with the sucrose-calcium procedure. The RNA/DNA ratio was approximately the same. The amount of DNA recovered in the tumor nuclei isolated with the citric acid procedure was 80% of that found when the nuclei were isolated with the sucrose-calcium procedure.

Sucrose density gradient sedimentation studies showed that the nuclei of tumor cells isolated with the citric acid procedure contained approximately 1 of the total nuclear RNA in the 35 S, 45 S, and 55 S fractions. Following a 20-min pulse of orthophosphate-32P, the radioactivity in the RNA was largely in the high molecular weight RNA. In studies with the zonal ultracentrifuge, fractionation of the RNA was improved, and substantial amounts of tumor nuclear RNA could be separated into various sedimentation classes with this instrument.

Introduction

Isolation of nuclei from cells of transplantable tumors has been more difficult (4, 5, 10, 13) than isolation of nuclei from rat liver cells. With the mild conditions used in the sucrose-calcium procedure described by Chauveau et al. (11) or Widnell and Tata (31), highly purified preparations of nuclei are obtained from liver cells. However, it has not been possible to remove the adherent cytoplasm from nuclei of transplantable tumors by homogenization with 2.2 M sucrose containing 3.3 mM CaCl2, presumably because of the dense perinuclear ribonucleoprotein network (5).

Materials and Methods

Animals. Male Holtzman rats obtained from the Cheek-Jones Company, weighing 180–220 gm, and fed ad libitum on Purina Laboratory Chow were used in these experiments. The Walker 256 carcinosarcoma was implanted 7–10 days prior to the experiment. Some rats were injected i.v. with 1 mc of orthophosphate-32P (carrier-free) 20 min before they were sacrificed.

The livers were perfused via the portal vein with 20 ml of ice-cold 0.14 M NaCl followed by 20 ml of 0.25 M sucrose, and then were rapidly removed, placed in ice-cold 0.25 M sucrose, and transferred to the cold laboratory. The tumors were immediately

1 These studies were supported in part by grants from the American Cancer Society, the National Science Foundation, the Jane Coffin Childs Fund and the USPHS (CA 08182).  
2 As noted recently (8), nuclei of human tumors are less difficult to isolate than those of transplantable rodent tumors.  
3 As indicated previously (5), methods have been developed for isolation of nuclei employing nonionic and other detergents. Unfortunately, the loss of nuclear RNA is very serious when these procedures are employed.
Mass Isolation of Nuclear RNA from Walker 256 Carcinosarcoma

PREPARATION OF NUCLEI FROM LIVER. The livers were disrupted and freed of fibrous tissue with the aid of a Harvard tissue press. The fragmented tissue was suspended in 2.4 M sucrose (1:14 w/v) containing 3.3 mM CaCl₂ and homogenized with 3 strokes of a Teflon pestle (6-9 x 10⁻² inch clearance). The suspension was centrifuged at 40,000 x g for 60 min (11). The pellets were resuspended in 1.0 M sucrose containing 1 mM CaCl₂ (1 ml/gm of liver) and centrifuged at 3,000 x g for 10 min (30).

In the experiments with citric acid, the pellets from 2.4 M sucrose preparation were resuspended in 2.5% citric acid (5 ml/gm of liver) and centrifuged at 600 x g for 10 min.

PREPARATION OF NUCLEI FROM WALKER TUMOR. Tumor tissues were fragmented with the tissue press, weighed, and suspended in cold 2.5% citric acid (1:9 w/v). The suspension was passed through a continuous tissue homogenizer (7) 3 times. The temperature of the homogenate was 3-4°C, and the pH of the homogenate was 2.5-2.8. The homogenate was centrifuged at 600 x g for 10 min, and the sediment was resuspended in 0.25 M sucrose (5 ml/gram of tumor) containing 1.5% citric acid with a Teflon glass homogenizer (3-5 x 10⁻² inch clearance). The suspension was layered over 2 volumes of 0.88 M sucrose containing 1.5% citric acid and centrifuged at 900 x g for 10 min.

In the experiments utilizing the sucrose-calcium procedure, the fragmented tumor tissue was suspended in 2.4 M sucrose containing 3.3 mM CaCl₂ and the same homogenization procedure was followed as for liver nuclei (11).

COUNTING THE NUCLEI. The number of nuclei in the preparation was determined by direct counting of an aliquot in a hemacytometer. The sample was stained by mixing with a solution of azure C (0.05% in 0.5 M sucrose containing 0.5 mM CaCl₂) in a ratio of 9 volumes of staining solution per volume of aliquot (23). The purity of the nuclei was determined by phase microscopy with and without prior staining.

EXTRACTION OF TOTAL NUCLEAR RNA. Isolated nuclei were homogenized in 0.3% sodium dodecyl sulfate (SDS) containing 0.14 M NaCl and 0.05 M sodium acetate buffer at pH 5.0-5.1 (1.0 ml/gm of tissue) for 1 min with a loosely fitting Teflon pestle. After the addition of an equal volume of buffer-saturated phenol containing 0.1% 8-hydroxyquinoline, the suspension was homogenized for another min. The mixture was shaken in a Brunswick water bath for 10 min at 65°C (16). The mixture was then shaken in an Equipoise shaker for 15 min at room temperature (25°C), after which it was centrifuged at 4000 x g for 10 min. The aqueous layer was removed and re-extracted with an equal volume of the phenol solution by shaking for 10 min at room temperature. After centrifugation, the aqueous layer was removed and the RNA was precipitated overnight by addition of 2-2.5 volumes of ethanol containing 2% potassium acetate. The RNA was dissolved in water and reprecipitated twice with ethanol containing potassium acetate. The RNA was then dissolved in a small volume of distilled water and stored at -15°C. Before the RNA was used for further studies, it was chromatographed on Sephadex G-25 buffered with 0.05 M sodium acetate at pH 5.1.

EXTRACTION OF A- AND R-RNA. Isolated nuclei were suspended in 0.02 M sodium phosphate buffer, pH 6.8, containing 0.18 M sodium chloride (17) in the amount of 5 ml/gm of original tissue. The pH of this suspension was adjusted to 4.7-4.8 with either 2.5% citric acid or 0.2 M sodium phosphate buffer (pH 6.8). The suspension was homogenized for 1 min with a loosely fitting glass Teflon homogenizer. After addition of an equal volume of buffer-saturated phenol containing 0.1% 8-hydroxyquinoline, the mixture was homogenized for 1 min, adjusted to pH 5.0-5.1, and then shaken for 30 min at room temperature (25°C). The mixture was centrifuged at 5000 rpm for 20 min in an RC-3 Sorvall centrifuge. The phenolic phase and the interphase were extracted again with the same buffer. The RNA in the pooled aqueous phase was designated as a-RNA (21). After the 2nd extraction at room temperature, the same buffer was added to the phenol phase and interphase, and the mixture was shaken in a Brunswick water bath-shaker at 65°C for 10 min. After shaking again at room temperature for 10 min, the aqueous phase was separated by centrifugation. The hot phenol extraction was repeated once. The RNA in the combined aqueous phase was designated as i-RNA (21). The a-RNA and the i-RNA were re-extracted with phenol. Precipitation and purification of the a-RNA and i-RNA were the same as for the total extractable RNA.

SUCROSE DENSITY GRADIENT SEDIMENTATION STUDIES. For analytic studies, aliquots of nuclear RNA (about 1 mg) were layered on 28-ml linear sucrose gradients (10-40%) containing 0.1 M NaCl, 0.02 M sodium acetate buffer (pH 5.0-5.1), and 1 mM ethylenediaminetetraacetate (EDTA), and the samples were centrifuged at 25,000 rpm for 16 hr in a Spinco SW-25.1 rotor at 4-6°C (27). After centrifugation, the gradients were fractionated into 1.0-ml fractions with an automatic fraction collector (ISCO).

For mass isolation studies on nuclear RNA and its fractions a large scale (1750-ml) linear sucrose gradient (15-40%) was made at 5000 rpm in a zonal ultracentrifuge rotor (Beckman) prior to addition of the sample. Approximately 50 mg of nuclear RNA was pumped in through the center core and centrifuged at 40,000 rpm for 15 hr (2-4°C). The speed was then decelerated to 5000 rpm, at which speed the sample was pumped out with 50% sucrose and divided into 20-ml fractions with an ISCO automatic fractionator.

Individual fractions obtained from 30-ml tubes of the SW-25.1 rotor or aliquots of the fractions of zonal ultracentrifugation were prepared for assay of radioactivity by the addition of 0.05 ml of 5 n perchloric acid, followed by hydrolysis for 15 min at 70°C. Hydrolyzed samples were diluted with 8 ml of the liquid scintillation system (xylene-dioxane-ethyl Cellosolve, 1:4:4 by volume) (2). The efficiency of counting was 53% in an automatic liquid scintillation spectrometer (Packard Tri-Carb, series 3000).

ANALYTICAL METHODS. RNA was determined by the orcinol procedure (15). The readings were corrected for DNA. DNA was determined by Burton’s modification of the diphenylamine procedure (3). For determination of protein, the following procedure was employed (23). Each fraction was precipitated with 5 n perchloric acid and centrifuged. The precipitate was washed successively with 10 ml of 95% ethanol containing potassium acetate and with 10 ml of absolute ethanol; the mixture was then centrifuged. To the residue, 5 ml of 5% trichloroacetic acid (TCA) were added and the sample was digested at 90°C for 30 min; this extraction was repeated once. The extract was used for the determination of nucleic acid. The residue was then washed with 95% ethanol containing 2% potassium acetate, absolute ethanol, 2:1.
chloroform-methanol, benzene and ether and desiccated. The amount of protein was determined gravimetrically.

ELECTRON MICROSCOPY. Pellets of isolated nuclei were fixed by the following procedures: (a) 2 hr at 0–4°C in 2% OsO₄ buffered with Veronal-acetate buffer at pH 7.2–7.4 (25), followed by post-fixation in neutral formalin (18), 30–60 min at 0–4°C; (b) 2 hr at 0–4°C in 6.25% glutaraldehyde in 0.1 M phosphate buffer at pH 7.2–7.4 (26), followed by post-fixation in 2% OsO₄, pH 7.2–7.4 (25) for 30–60 min at 0–4°C; and (c) 2 hr at 0–4°C in 2% OsO₄ in distilled water (12), 2 hr at 0–4°C followed by post-fixation in neutral formalin (18) for 30–60 min at 0–4°C. Dehydration of the pellets was performed routinely in increasing concentrations of ethanol.

### TABLE 2

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Rat Liver</th>
<th>Walker 256 Tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>9.76 (7)</td>
<td>15.4 (2)</td>
</tr>
<tr>
<td>RNA</td>
<td>2.95 (7)</td>
<td>23.54 (2)</td>
</tr>
<tr>
<td>RNA/DNA</td>
<td>0.300</td>
<td>1.534</td>
</tr>
<tr>
<td>Protein</td>
<td>40.5 (7)</td>
<td>171.50 (2)</td>
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### TABLE 3

**PERCENTAGE DISTRIBUTION OF RNA IN VARIOUS SUBFRACTIONS OBTAINED BY SUCROSE DENSITY GRADIENT SEDIMENTATION**

The sedimentation coefficients of RNA's are approximate modal values for the given peak or shoulder and are used to refer conveniently to a given region in the sedimentation profile. Each value was estimated with a planimeter for the area of a given peak. The results are average percentages from 2 to 3 experiments.

<table>
<thead>
<tr>
<th>Subfraction</th>
<th>6 S</th>
<th>18 S</th>
<th>28 S</th>
<th>35 S</th>
<th>45 S</th>
<th>55 S</th>
<th>Total</th>
</tr>
</thead>
</table>

The distribution of the RNA in the subfractions is presented in Table 3. Of the total RNA in the citric acid nuclei, approximately 55% was found in the 18 S and 28 S RNA. The high molecular weight fractions in the 35 S, 45 S, and 55 S peaks and shoulder accounted for approximately 30% of the total nuclear RNA. The amounts of 18 S, 35 S, and 55 S RNA in the a-RNA fraction were much lower than in the i-RNA fraction.

**DISTRIBUTION OF RADIOACTIVITY IN HIGH MOLECULAR WEIGHT RNA**

As noted previously, the RNA in the Walker tumor labeled initially with orthophosphate-32P is in the rapidly sedimenting fraction (24). At 20 min after injection of the isotope, the...
Mass Isolation of Nuclear RNA from Walker 256 Carcinosarcoma

**Chart 2.** Sedimentation profiles of α- and i-RNA from tumor nuclei isolated with citric acid procedure. The pH of the 1st phenol extraction was 5.5 for (a) and (b), or 4.8 for (c) and (d), respectively. The arrow indicates the direction of sedimentation.

**Chart 3.** Sedimentation profiles of α- and i-RNA fractions of tumor nuclear RNA from rats injected i.v. with 1 mc of orthophosphate-32P 20 min prior to death. These sucrose density gradients were prepared in the zonal ultracentrifuge. The arrows indicate the direction of sedimentation, and the numbers above the peaks and shoulders indicate the approximate sedimentation constants.

citric acid contained 43% of the nuclei in the original homogenate.

**pH of Nuclear Suspension of Citric Acid Procedure.** To obtain nuclei free of cytoplasm and undegraded RNA from tumor tissue it was necessary to have 1.5% citric acid in both the 0.25 M sucrose and the 0.88 M sucrose. The pH's of the 1st and 2nd homogenates were 2.6-2.8 and 2.2-2.8, respectively.

**Recovery of DNA, RNA, and Protein.** Table 2 presents the results of determinations of recovery of nuclear components following isolation of nuclei by the citric acid procedure and the sucrose-calcium procedure. For the Walker tumor, a direct comparison of these results is of limited validity inasmuch as the nuclei obtained by the sucrose-calcium procedure are surrounded by a halo of cytoplasm (4-6) and the amount of protein and RNA is excessive. The lower content of RNA and protein in nuclei isolated with the citric acid procedure is a reflection of the loss of perinuclear cytoplasm. The DNA content of isolated nuclei of the Walker tumor was approximately 80% of that of the nuclei obtained with the sucrose-calcium procedure.

To determine the extent of losses of nuclear components following the extractions with citric acid, studies were made on nuclei obtained with procedure of Chauveau et al. (11). Approximately 80-85% of the nucleic acids remained in the nuclei after the isolated nuclei were treated with the citric acid procedure. The protein content was diminished by approximately 30%. However, the ratio of RNA to DNA was not significantly changed following treatment of the nuclei with citric acid.
Ken Higashi, K. Shankar Narayanan, Helen R. Adams, and Harris Busch

PERCENTAGE DISTRIBUTION OF RNA IN VARIOUS SUBFRACTIONS

The sedimentation coefficients of RNA's are approximate modal values for the given peak or shoulder and are used to refer conveniently to a given region in the sedimentation profile. Each value was estimated with a planimeter for the area of a given peak. The results are average percentages from 2 to 3 experiments.

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<th>55 S</th>
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</tr>
</thead>
<tbody>
<tr>
<td>a-RNA</td>
<td>6.9</td>
<td>4.7</td>
<td>12.9</td>
<td>2.3</td>
<td>2.4</td>
<td>1.9</td>
<td>31.1</td>
</tr>
<tr>
<td>i-RNA</td>
<td>6.1</td>
<td>15.4</td>
<td>21.5</td>
<td>10.6</td>
<td>5.9</td>
<td>9.4</td>
<td>68.9</td>
</tr>
<tr>
<td>Total</td>
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<td>20.1</td>
<td>34.4</td>
<td>12.9</td>
<td>8.3</td>
<td>11.3</td>
<td>100.0</td>
</tr>
</tbody>
</table>

Fractionation of RNA with Sucrose Density Gradient Sedimentation

The sucrose density gradient sedimentation profile of the total RNA extracted from tumor nuclei is shown in Chart 1. The RNA was extracted from the nuclei with the SDS-phenoI procedure described in "Materials and Methods." Sharp peaks of 6, 18, and 28 S RNA were obtained, and shoulders of 35, 45, and 55 S RNA were noted. By comparison with the results obtained with the sucrose-calcium procedure, the amounts of 18 S and 28 S RNA were significantly decreased and the amounts of 45 S and 35 S RNA were proportionately increased.

SUBFRACTIONATION OF NUCLEAR RNA. Using modifications of the fractionation of RNA with phenol (17, 21, 22) attempts were made to improve the fractionation of a-RNA and i-RNA (Chart 2). When the pH was 5.5-6.2 in the initial mixture of the phenol and 0.02 M phosphate buffer, containing 0.18 M NaCl, 50% of the total RNA remained in the i-RNA fraction (Chart 2, a, b). However, the degree of resolution of the RNA on sucrose density gradient sedimentation was less satisfactory than that obtained when the fractions were separated at pH 4.7 (Chart 2, c, d). With the system buffered at pH 5.5, the a-RNA contained 6, 18, and 28 S peaks, and 45 S RNA was a shoulder. The i-RNA contained 18 S and 28 S peaks, but there was little resolution of the higher molecular weight components.

When the system was buffered at pH 4.7-5.1, the resolution of the RNA in both the a-RNA and i-RNA fractions was improved. The i-RNA accounted for approximately 75% of the total extracted; the pattern for the i-RNA contained an 18 and 28 S peak of RNA, a shoulder of 35 S RNA, and little 6 S RNA. The a-RNA contained a sharp peak of 6 S RNA, little 18 S RNA, a 28 S peak, and a small 45 S peak.

Table 3: Mass Isolation of Nuclear RNA from the Walker Tumor

<table>
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<tr>
<th></th>
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<tr>
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DISTRIBUTION OF RADIOACTIVITY IN HIGH MOLECULAR WEIGHT RNA. As noted previously, the RNA in the Walker tumor labeled initially with orthophosphate-32P is in the rapidly sedimenting fraction (24). At 20 min after injection of the isotope, the radioactivity was largely found in the i-RNA. The peak of the radioactivity was in the 35 S and 45 S regions. These results conform to earlier findings for whole nuclear RNA and suggest that the a-RNA has a much lower rate of uptake of isotope than the fractions of the i-RNA (21).

Discussion

In order to carry out further studies on subfractionation and isolation of individual molecular species of the rapidly sedimenting nuclear RNA of tumors, it is necessary to start with as pure a RNA preparation as possible. Although the sucrose-calcium procedure developed by Chauveau et al. (11) provides satisfactory nuclei from normal rat liver and some tumors (8), transplantable tumors such as the Walker tumor retain a substantial amount of cytoplasmic RNA when isolated by this technic.

Since there are significant losses of nuclear protein following treatment of nuclei with the citric acid procedure (13), this technic has been abandoned for studies on the nuclear proteins. In the present study, it was found that the citric acid procedure permits isolation of nuclei which retain high molecular weight RNA. Although there are considerable changes in the nuclear ultrastructure, the nucleolus is readily seen by phase microscopy, and the RNA/DNA ratio is unchanged.

In previous experiments, it was found that on separation of the a-RNA and i-RNA of the Walker tumor nuclear preparations obtained by the sucrose-calcium procedure, the i-RNA fraction contained radioactive peaks in the 10-16 S region, despite the fact that the whole nuclear RNA contained virtually all of the radioactivity in the 35 S, 45 S, and 55 S RNA following pulse labeling with orthophosphate-32P (24). With the citric acid procedure, the i-RNA contained radioactivity in the rapidly sedimenting RNA fractions.

Since 1 of the problems involved in isolation of high molecular weight RNA is the hydrolysis of this RNA by RNase (30), it appears that citric acid prevents the hydrolysis of high molecular weight RNA by extraction of the enzyme, chelation of divalent ions, or reduction of the pH to the point where the enzyme is inactive.

One of the questions that has existed about high molecular weight RNA is whether it is a naturally occurring intracellular product or an aggregate which is formed in the presence of divalent ions used for isolation of nuclei. The present study shows that high molecular weight RNA can be isolated under conditions in which divalent ions are not only absent from the homogenization media but are chelated by citric acid.

The zonal ultracentrifuge is of particular value in studies on large amounts of macromolecules. The capacity of the rotor used...
in the present studies is 60 times that of 1 tube in the commonly used SW-25.1 swinging bucket rotor. With the zonal ultracentrifuge, an improved separation of the various sedimentation peaks was obtained and localization of the early-labeled RNA in the 35 S, 45 S, and 55 S RNA was confirmed (24). With the availability of substantial amounts of the RNA of these sedimentation groups, it seems feasible now to carry out subfractionation studies, studies on stimulation of amino acid incorporation into proteins in vitro, and degradative analyses on RNA of transplantable tumors.

Acknowledgment

The authors wish to express their appreciation to Dr. William J. Steele and Dr. Masami Muramatsu for their many helpful suggestions, to Daniel A. Busch and Joe P. Arendell for their assistance, and to Mr. Charles Taylor for supplying the transplanted tumors.

References

FIG. 1. Nuclei of Walker 256 carcinosarcoma cells isolated in citric acid. Phase contrast, × 1100.

FIG. 2. Isolated nuclei of Walker 256 carcinosarcoma cells stained for ribonucleic acid with toluidine blue at pH 4.9. × 2000. The measured line in the figures represents 1 μ.
Mass Isolation of Nuclear RNA from Walker 256 Carcinosarcoma

**Fig. 3.** Electron micrograph of Walker tumor nuclei isolated in citric acid. Post-stained with uranyl acetate. N, nucleolus. × 8700.

**Fig. 4.** Higher magnification of a citric acid-isolated Walker tumor nucleus. Post-stained with uranyl acetate. N, nucleolus. × 15,000.
Fig. 5. A liver cell nucleus isolated in sucrose-calcium media. Post-stained with uranyl acetate. N, nucleolus. × 21,000.

Fig. 6. A liver cell nucleus isolated in sucrose-calcium media and subsequently treated with citric acid. Post-stained with uranyl acetate. N, nucleolus. × 21,000.
Utilization of the Citric Acid Procedure and Zonal Ultracentrifugation for Mass Isolation of Nuclear RNA from Walker 256 Carcinosarcoma


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