ELECTRON MICROSCOPIC AND BIOCHEMICAL CHARACTERISTICS OF NUCLEI AND NUCLEOLI ISOLATED FROM RAT LIVER

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ABSTRACT

Rat liver nuclei were freed of cytoplasmic contamination by washing with Triton-X-100 and subsequent centrifugation through 2.2 M sucrose. Electron microscopic examination showed that the outer membranes of the nuclei had been removed, but that the nuclei otherwise resembled the nuclei of intact liver. Morphological studies, chemical estimations of DNA, RNA, and protein and the estimation of cytoplasmic "marker" enzymes suggested that contamination of nuclei by cytoplasmic components was limited. These nuclei were obtained in yields of about 70% and were suitable for the isolation of nucleoli. Nucleoli were isolated by the breaking of the nuclei by ultrasound and subsequent differential centrifugation. In ultrastructural appearance, the isolated nucleoli resembled nucleoli in intact tissue. However, at high magnifications the "granular" component of isolated nucleoli appeared to consist of tightly twisted fibers. The nucleoli could be obtained in yields of at least 30%, and the values for the chemical composition of the isolated nucleoli agreed with values previously reported.

INTRODUCTION

Early procedures for the isolation of nuclei in aqueous media (1–3) employed differential centrifugation in an attempt to separate the nuclei from contaminating particles on the basis of differences in sedimentation rate. However, such "nuclear fractions" remained contaminated with unbroken cells, blood cells, and cytoplasmic components, in spite of repeated washing and resedimentation of the nuclei. This contamination arose from the fact that in (light) isotonic media all particles in the centrifuge tube of a horizontal rotor sediment in the same direction. If the homogenate was layered over a denser medium (4–6), the latter acted as a barrier to retard the sedimentation of the lighter, more slowly sedimenting particles. However, the nuclei were considerably contaminated with cytoplasmic components (7).

Separation of the nucleus from contaminating particles is facilitated if the density of the medium is adjusted so that it is slightly less than that of the nuclei. Nuclei then sediment towards the bottom of the tube during centrifugation, but lighter components, such as cytoplasmic organelles, whole cells, erythrocytes, and nuclei with tabs of cytoplasm float to the surface. This principle was first applied in nonaqueous procedures (8–11) and was subsequently adapted to aqueous methods by the use of 2.2 M sucrose (12–20). These methods appeared to give satisfactory preparations of nuclei, although the yields were often low (16, 17) and the methods were difficult to adapt to the processing of large quantities of tissue. Yields could be substantially increased if the sucrose concentration of the homogenate was adjusted to

1.62 M (21) prior to centrifugation through 2.3 M sucrose.

In the present study, we wished to correlate the ultrastructure of isolated nuclei with the structure and function of isolated nuclear subfractions. Hence, we needed a method which would give morphologically intact nuclei. Furthermore, since fractionation of the nucleus and isolation of nucleoli were contemplated, a method capable of yielding fairly large quantities of nuclei was desirable. Finally, since we were particularly interested in isolating nuclear ribosomes, it was essential that contamination by cytoplasmic ribosomes be minimized.

The method of Maggio et al. (17) for the isolation of nuclei from guinea pig liver seemed to give nuclei which were well preserved. However, when we applied this method to rat liver, the yields of nuclei were extremely low, in agreement with the finding of Wilson and Hoagland (22). Yields were enhanced by inserting a layer of 1.5 M sucrose between the homogenate (in 0.88 M sucrose) and the 2.2 M sucrose (23). However, the method could still accommodate only limited amounts of tissue homogenate.

Detergents have recently been used to solubilize membranous components of the cytoplasm (24–31). Detergents remove the outer membrane of the nucleus while leaving the interior of the nucleus apparently intact. The detergents used were usually the nonionic type (24–47), although a mixture of a nonionic and an anionic detergent has been used (28, 29). In this case, the nuclei were thought to contain no mature ribosomes.

The nuclei obtained by the method of Hymer and Kuff (26) using Triton X-100 were found to be extensively contaminated with whole cells. It appeared that some cell membranes of rat liver cells were resistant to disruption by Triton-X-100. In the method finally devised, rat livers were homogenized in isotonic sucrose containing magnesium ions, and the crude nuclear pellet obtained after centrifugation was washed with Triton-X-100. The washed nuclei were purified by centrifugation through 2.2 M sucrose. The nuclei obtained by this method were found to be morphologically intact, when examined with the electron microscope, and contained little cytoplasmic contamination. The method was suitable for isolation of nuclei from several rat livers. In contrast to the findings of Penman (28), these nuclei were found to contain ribosomes (32).

Several procedures have been used to fragment nuclei prior to the isolation of nucleoli. These have included agitation of nuclei over glass beads (33), dispersion of nucleoplasm and nuclear membranes by stirring nuclei in a mixer (34), homogenization in 70% glycerol phosphate (35), and compression and decompression in a French pressure cell (36). The most commonly used device for breaking nuclei and liberating nucleoli is the sonic oscillator (37–43). The nucleolus is refractory to destruction by sonic vibrations, but the nucleoplasm is readily dispersed. Finally, Penman et al. (44) used a Tris buffer containing 0.5 M NaCl and DNase to lyse HeLa cell nuclei. In most methods, the nucleoli are isolated by differential centrifugation, but they have also been purified by sedimentation through 2.2 M sucrose (36, 40).

In the present study, isolated rat liver nuclei prepared according to the method previously outlined were disrupted by ultrasonication. The nucleoli were isolated from the sonicate by differential centrifugation (41).

MATERIALS AND METHODS

ANIMALS: Male Wistar rats weighing 175–225 g were obtained from Woodlyn Farms, Guelph, Ontario. These rats were specific pathogen free ("SPF"). Experiments with Wistar rats (non-SPF) from other suppliers gave widely variable results. The rats were fed ad libitum on Purina Laboratory Chow and water until 16–20 hr prior to sacrifice, when they were given water only.

REAGENTS: ATP,[1] bovine serum albumin, cytochrome *c*, glucose-6-phosphate, NAD, NMN, nicotinamide, Na L-lactate, *p*-nitrophenyl phosphate, yeast sRNA, and uric acid were obtained from Sigma Chemical Co., St. Louis, and yeast alcohol dehydrogenase was from Nutritional Biochemicals, Cleveland. Calf thymus DNA was the gift of Dr. M. Moscarello, Department of Biochemistry, University of Toronto, and Triton-X-100 was the gift of Rohm and Haas, Toronto. All other chemicals were analytical or reagent grade. Glass-distilled water was used throughout.

MEDIA: "Medium H" (homogenizing medium) contained 0.25 M sucrose and 0.005 M $MgSO_4$.

"Triton Medium" contained 0.25 M sucrose, 0.005 M $MgSO_4$, and 0.5% (v/v) Triton-X-100.

MORPHOLOGICAL STUDIES: Nuclei and nu-

[1] Abbreviations: ATP = adenosine triphosphate, NAD = nicotinamide adenine dinucleotide, NMN = nicotinamide mononucleotide, DNA = deoxyribonucleic acid, sRNA = soluble ribonucleic acid, TCA = trichloroacetic acid, OD = optical density.

cleoli were counted in a hemocytometer under a phase-contrast microscope.

For light and electron microscopy, suspensions of nuclei and nucleoli were mixed with an equal volume of the appropriate stock solution of fixative, and a pellet was formed by centrifugation in a conical centrifuge tube at 4000 g[2] for 10 min at 2°C in the swinging bucket rotors of Sorvall RC-2 or RC-3 centrifuges. The stock solutions of fixative used were 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.4, and 10% formalin in 0.18 M phosphate buffer, pH 7.4. The preparations were fixed in osmium tetroxide for 1 hr, or in formalin for 1 hr followed by osmium tetroxide for 30 min. Fixation was carried out at 4°. The pellets were cut into small cubes, dehydrated in increasing concentrations of ethanol, and embedded in Epon-Araldite. After fixation, nuclei and nucleoli were frequently left in 70% alcohol at 4° overnight with no apparent change in preservation. Sections 0.5–1.0 μ thick were cut with glass knives and were stained with Azure II-methylene blue (45). Ultrathin sections were cut with a DuPont diamond knife on an LKB ultramicrotome. They were mounted on 300-mesh grids and stained with saturated aqueous uranyl acetate for 1 hr, followed by lead hydroxide (46) for 20 min. Light micrographs were made with a Zeiss Ultraphot photomicroscope. Electron micrographs were made with a Philips EM 200 electron microscope with an accelerating voltage of 60 kv.

CHEMICAL ESTIMATIONS: Extraction of acid-soluble components and lipids was performed as described by Schneider (47). The residue was then hydrolyzed with 2 ml of 1 N potassium hydroxide for 1 hr at 20°C. The extract was acidified with an equal volume of cold, 2N perchloric acid, and the insoluble perchlorate, DNA and protein were allowed to precipitate at 4° for 1 hr. The supernatant obtained after centrifugation was assayed for RNA. The residue was then hydrolyzed in 5% TCA at 90°C for 30 min, conditions resulting in minimal destruction of the deoxyribose responsible for the color formed in the diphenylamine reaction (48). The extracts were cooled at 4° for 30 min and the supernatant obtained after centrifugation was assayed for DNA. The protein residue was dissolved in 2 ml of 1N NaOH by heating to 90°C for 10 min. The insoluble perchlorate was removed by centrifugation and the supernatant assayed for protein. RNA was measured by the orcinol reaction (49), DNA by the diphenylamine reaction (50), and protein by the method of Lowry et al. (51). Standards were yeast sRNA, calf thymus DNA, and bovine serum albumin.

ENZYME ASSAYS: NAD pyrophosphorylase was assayed by the method of Kornberg (52). Glucose-6-phosphatase was assayed by the procedure of Swanson (53), and inorganic phosphate by the method of Lowry and Lopez (54). Cytochrome oxidase was estimated by the method of Hogeboom and Schneider (55), uricase by the method of Schneider and Hogeboom (56), and lactic dehydrogenase by the method of Neilands (57). Acid phosphatase was estimated by measuring the increase of absorbance at 410 mμ after hydrolysis of p-nitrophenyl phosphate to p-nitrophenol and inorganic phosphate. The reaction mixtures contained 1.0 ml of each of the following: (i) 0.05 M acetate buffer, pH 5.0, (ii) 0.4% (v/v) Triton-X-100, (iii) 3.65×10^{-2} M p-nitrophenyl phosphate, (iv) a suitable dilution of homogenate or nuclear suspensions. Blanks were run in which either the enzyme or substrate was omitted.

ISOLATION OF NUCLEI: We fasted the rats overnight (16–20 hr) to deplete the liver of glycogen. They were stunned by a blow to the head, and the livers were quickly excised and placed in ice-cold homogenizing medium (Medium H). All subsequent operations were done in a cold room at 4°C, and all centrifuges were refrigerated to 2°C. The livers were then passed through a custom-made, stainless steel tissue press of the type described by Maggio et al. (17). This operation removed much of the connective tissue. The pressed liver was collected in about four volumes of medium H, and the suspension was homogenized by six up-and-down strokes of a Potter-Elvehjem type of homogenizer (Tri-R Instruments, New York). The pestle clearance was 0.007–0.009 inches and the speed of the motor was 1000 rpm. The homogenate was filtered through four layers of cheesecloth and centrifuged at 750 g for 10 min in the Sorvall HB-4 swinging bucket rotor. The supernatant was discarded, and the pellet was resuspended in a volume of Triton medium equal to about three times the original wet weight of liver. The suspension was gently homogenized with two up-and-down strokes of the homogenizer and centrifuged at 750 g for 10 min. The reddish pellet was resuspended in a volume of Triton medium about four times the original wet weight of liver. The suspension was layered over 0.88 M sucrose containing 1.5 mM $CaCl_2$ (17) and centrifuged at 4,000 g for 8 min. The upper layer was now very red in color, and the pellet was a light tan color. Usually, there was little material at the interface, although if the quantity of nuclei was large relative to the cross-sectional area of the centrifuge tube, many nuclei became trapped at the interface. This problem could be avoided by using 290-ml centrifuge bottles. The pellets were resuspended in 2.2 M sucrose containing 0.5 mM $CaCl_2$ (17) by one stroke of the homogenizer, and the suspension was centrifuged at 90,000 g for 1 hr in the SW 25.1 rotor of a Spinco Model L or L 2-65 preparative ultracentrifuge. After centrifugation, the tube contained a tightly packed brown pellicle at the top, a clear

[2] Gravitational forces refer to the maximum force exerted at the bottom of the centrifuge tube.

supernatant, and a tan-colored pellet at the bottom of the tube. The pellicle was removed with a spatula, and it contained whole cells, nuclei with cytoplasmic tabs, and other debris. The supernatant was decanted, and the sides of the tube were wiped dry with a cotton swab. The nuclei were then carefully resuspended in 0.25 M sucrose for isolation of nucleoli, or in Medium H for morphological or enzymatic studies.

ISOLATION OF NUCLEOLI: Nuclei were isolated from at least four rat livers according to the procedure described above. These nuclei were suspended in 40 ml of 0.25 M sucrose containing *no* divalent cation.[3] The suspension of nuclei was sonicated for 1 min at full power with a Blackstone Model BP-10 ultrasonic generator fitted with a 1½-in. probe. The nuclei were sonicated in a 100-ml beaker which was surrounded by crushed ice. The sonicate was centrifuged at 184 *g* for 5 min in the Sorvall HB-4 rotor for removal of unbroken nuclei and aggregated debris. (This centrifugation was found to remove many nucleoli. In agreement with Ro and Busch (58), it was subsequently found that the yield of nucleoli could be almost doubled without increasing the percentage contamination of whole nuclei, if this step was omitted). The supernatant was layered over 10 ml of 0.88 M sucrose and centrifuged at 2000 *g* for 10 min. The nucleoli sedimented to the bottom of the tube, whereas the chromatin and other nucleoplasmic components remained above the 0.88 M sucrose. The nucleolar pellets were resuspended in 0.25 M sucrose (30 ml). The suspension was again layered over 0.88 M sucrose and was centrifuged at 2000 *g* for 10 min. The nucleoli formed a white ring or film on the bottom of the tube, and they were resuspended in 0.25 M sucrose.

RESULTS

Morphological Observations

In light micrographs, most nuclei were well preserved and the nucleoli stained intensely (Fig. 1 *a*). A few nuclei were broken, and some fibrous structures, probably corresponding to collapsed nuclei, were present. As determined by direct counting in a hemocytometer under phase contrast, the percentage contamination of nuclei by whole cells was less than 0.09%.

After formol-OsO_4 fixation (Fig. 1 *b*), the nucleoplasm of isolated nuclei was homogeneous and the "network-like" pattern of the nucleolus was well preserved. The nuclear pores were present, and the outer membrane was removed to a large extent, although occasional short fragments remained. Mitochondria, lysosomes, and microbodies were never seen with the electron microscope in these fractions, although occasional smooth-surfaced vesicles of the endoplasmic reticulum and cell membranes were present. These appeared to resist disruption by Triton-X-100 and co-sedimented with the nuclei through the 2.2 M sucrose. (It was later found that this membranous contamination could be eliminated by using 5 mM of $CaCl_2$ in the 2.2 M sucrose rather than 0.5 mM of $CaCl_2$.). At high power (Fig. 2), the "fibrillar" and "granular" regions of the nucleolus were present, although the short fibrils 50 A in diameter, originally described as comprising the fibrillar regions (59), were not well resolved. With formol-OsO_4 fixation, the condensed areas of chromatin at the periphery of the nucleus and around the nucleolus ("nucleolus-associated chromatin") were not clearly demarcated from the interchromatinic areas. Narrow fibrils, as small as 20 A in diameter, were sometimes seen inside the vacuoles within the nucleolus. These may have represented intranucleolar chromatin. Perichromatin granules (60) were sometimes seen in the chromatin areas, but were not prominent. Sometimes electron-opaque granules, about 200 A in diameter, were present in the chromatin or interchromatin areas, but with formol-OsO_4 fixation it was difficult to distinguish these clearly from fibrillar components (100–200 A in diameter) of the nucleoplasm which may have been "kinked" or cut in cross-section.

With fixation in OsO_4 alone, the condensed regions of chromatin of isolated nuclei were prominent, outlining the nuclear pores and the nucleolus (Fig. 3 *a*). The perichromatin granules and nucleoplasmic granules were more prominent than in the case of formol-OsO_4 fixation. A high power micrograph of the nucleolus and nucleoplasm of an isolated nucleus is shown in Fig. 3 *b*. Perichromatin granules were quite well demarcated. The granular components of the nucleoplasm ("nucleoplasmic granules") were better visualized than with formol-OsO_4 fixation, although at times they appeared to be part of a fibrillar network in the nucleoplasm.

Light micrographs of isolated nucleoli showed that the fraction was homogeneous, although occa-

[3] In spite of the emphasis given to the importance of calcium ions in preventing the destruction of nucleoli during sonication (39, 41, 42), we found that, if divalent cations were present, it was impossible to separate the nucleoli from adherent chromatin by differential centrifugation.

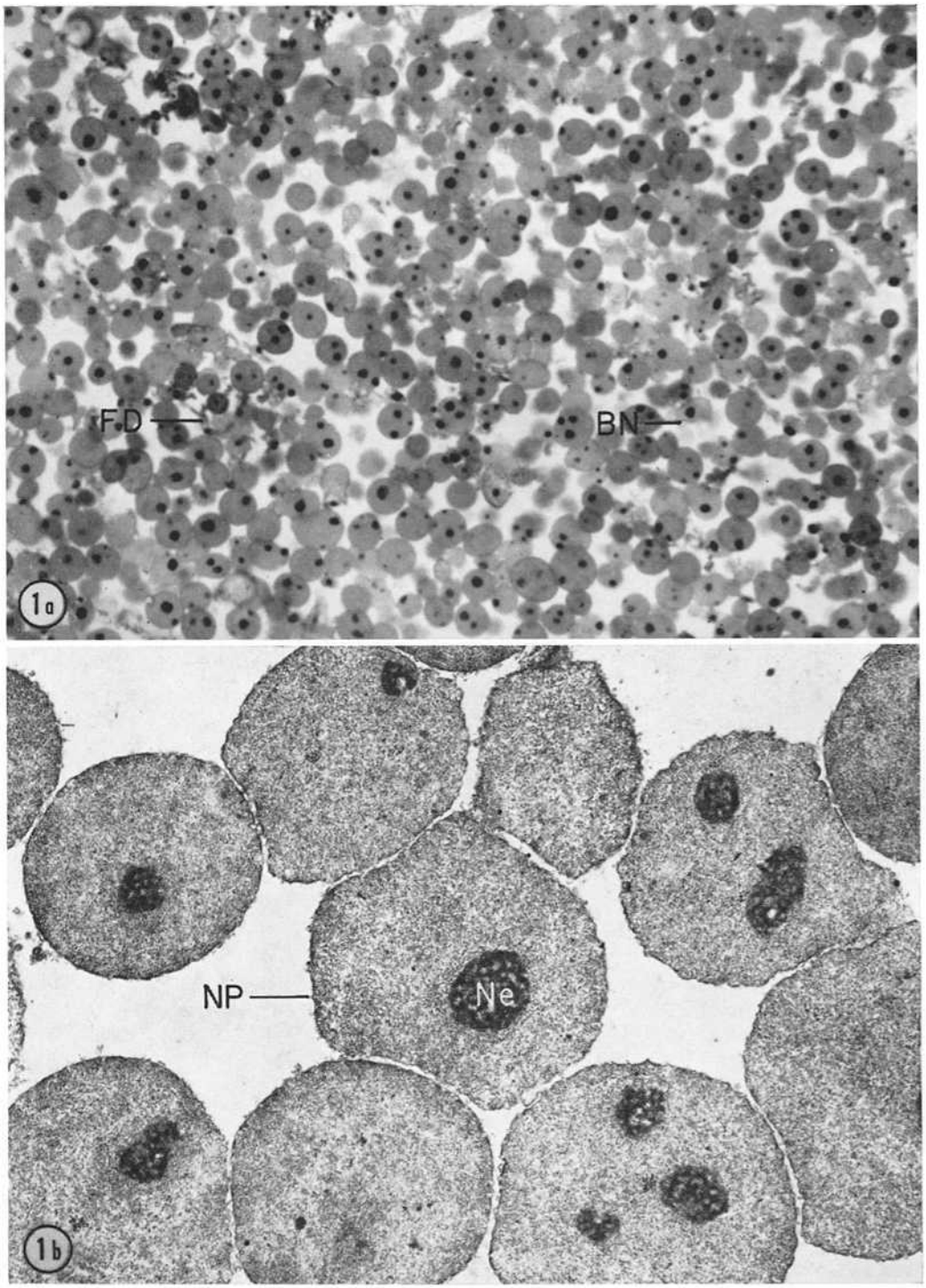

FIGURE 1 *a* Light micrograph of isolated nuclei. The majority of nuclei show rounded profiles and the nucleoli stain intensely. Some broken nuclei (*BN*) and fibrous debris (*FD*) are present. Formol-OsO_4 fixation; Azure II-methylene blue stain. × 720.

FIGURE 1 *b* Low-power electron micrograph of isolated nuclei. Nuclear profiles are round and the nucleoplasm is homogeneous. The outer membranes have been removed, but the nuclear pores (*NP*) remain intact. The network-like pattern of the nucleolus (*Ne*) is preserved. Formol-OsO_4 fixation; uranyl acetate –lead hydroxide stain. × 4160.

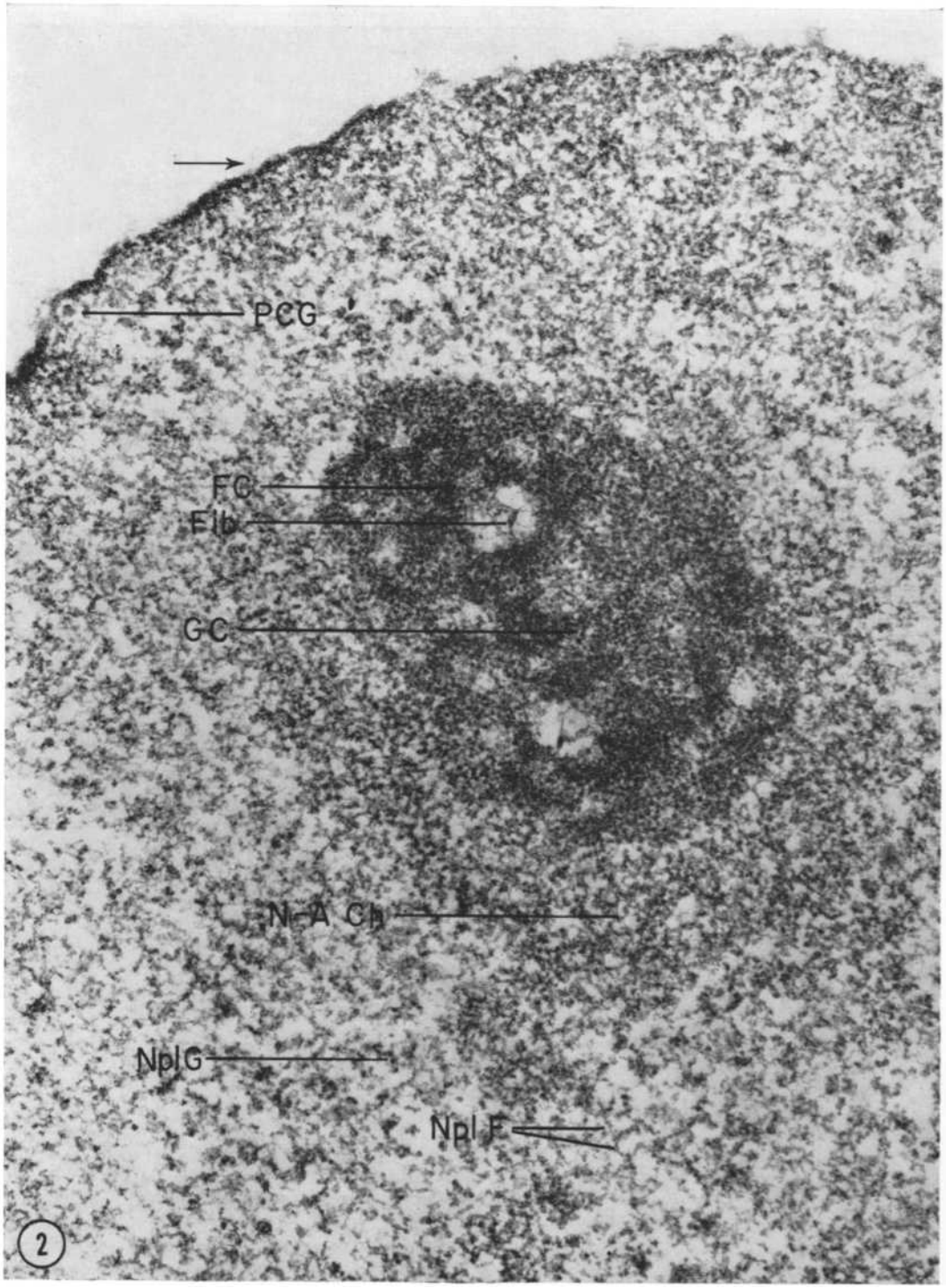

FIGURE 2 High-power electron micrograph of isolated nucleus. The outer membrane is absent (arrow) and the chromatin is not clearly demarcated. Occasional perichromatin granules (*PCG*) are visible. The nucleoplasm is homogeneous, although nucleoplasmic fibrils (*Npl F*) and nucleoplasmic granules (*Npl G*) can be distinguished. The fibrils measure about 100 A in diameter, and the granules 150–200 A in diameter. The nucleolus-associated chromatin (*N-A Ch*) is not prominent. The nucleolus is composed of fibrillar components (*FC*) and granular components (*GC*). Narrow fibrils (*Fib*) in the nucleolar vacuoles may represent intranucleolar chromatin. Formol-OsO_4 fixation; uranyl acetate–lead hydroxide stain. × 50,000.

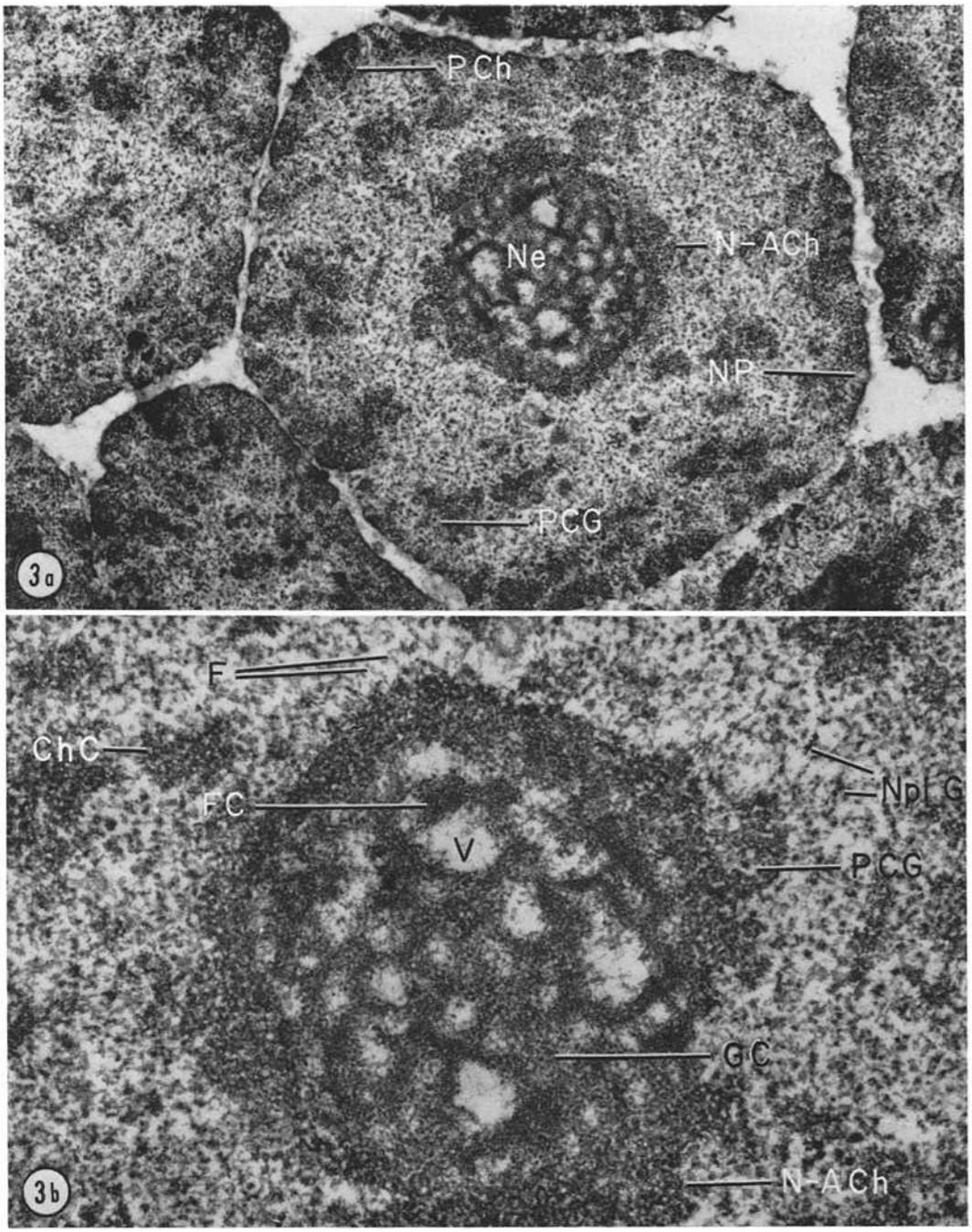

FIGURE 3 *a* Low-power electron micrograph of isolated nuclei. The peripheral chromatin (*PCh*) and the nucleolus-associated chromatin (*N-A Ch*) are prominent. The network-like structure of the nucleolus (*Ne*) is well preserved, as are the nuclear pores (*NP*). The nucleoplasm is granular and perichromatin granules (*PCG*) are well demarcated. The outer membrane is absent. OsO_4 fixation; uranyl acetate–lead hydroxide stain. × 13,500.

FIGURE 3 *b* High-power electron micrograph of nucleolus and nucleoplasm of isolated nucleus. The chromatin centres (*ChC*) and nucleolus-associated chromatin (*N-A Ch*) are condensed. The nucleoplasmic granules (*Npl G*) are well visualized, and fibrils (*F*) radiate from the nucleolus into the nucleoplasm. The nucleolus is composed of vacuoles (*V*), granular components (*GC*), and fibrillar components (*FC*), although the latter are too compact to reveal the 50 A fibrils clearly. *PCG*-perichromatin granule. OsO_4 fixation, uranyl acetate–lead hydroxide stain. × 47,500.

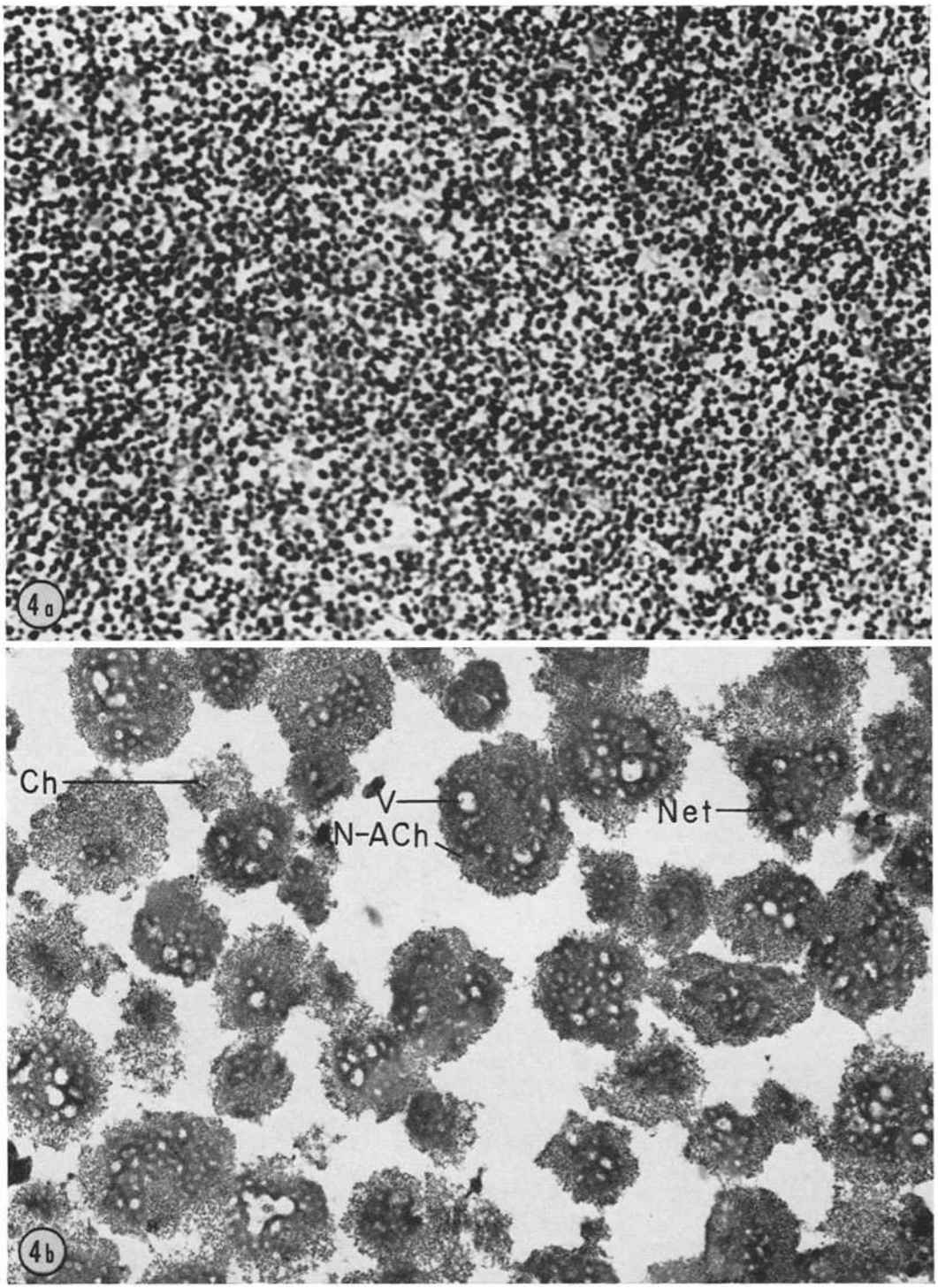

FIGURE 4 *a* Light micrograph of isolated nucleoli. The preparation consists predominantly of deeply staining nucleoli. Some contamination by nonnucleolar components is also present. OsO_4 fixation; Azure II-methylene blue stain. × 780.

FIGURE 4 *b* Low-power electron micrograph of isolated nucleoli. Most of the particles are nucleoli with a well preserved network-like structure (*Net*) enclosing nucleolar vacuoles (*V*). A thick cuff of nucleolus-associated chromatin (*N-A Ch*) surrounds the nucleoli. Some fragments of chromatin (*Ch*) are also present. OsO_4 fixation; uranyl acetate–lead hydroxide stain. × 9,500.

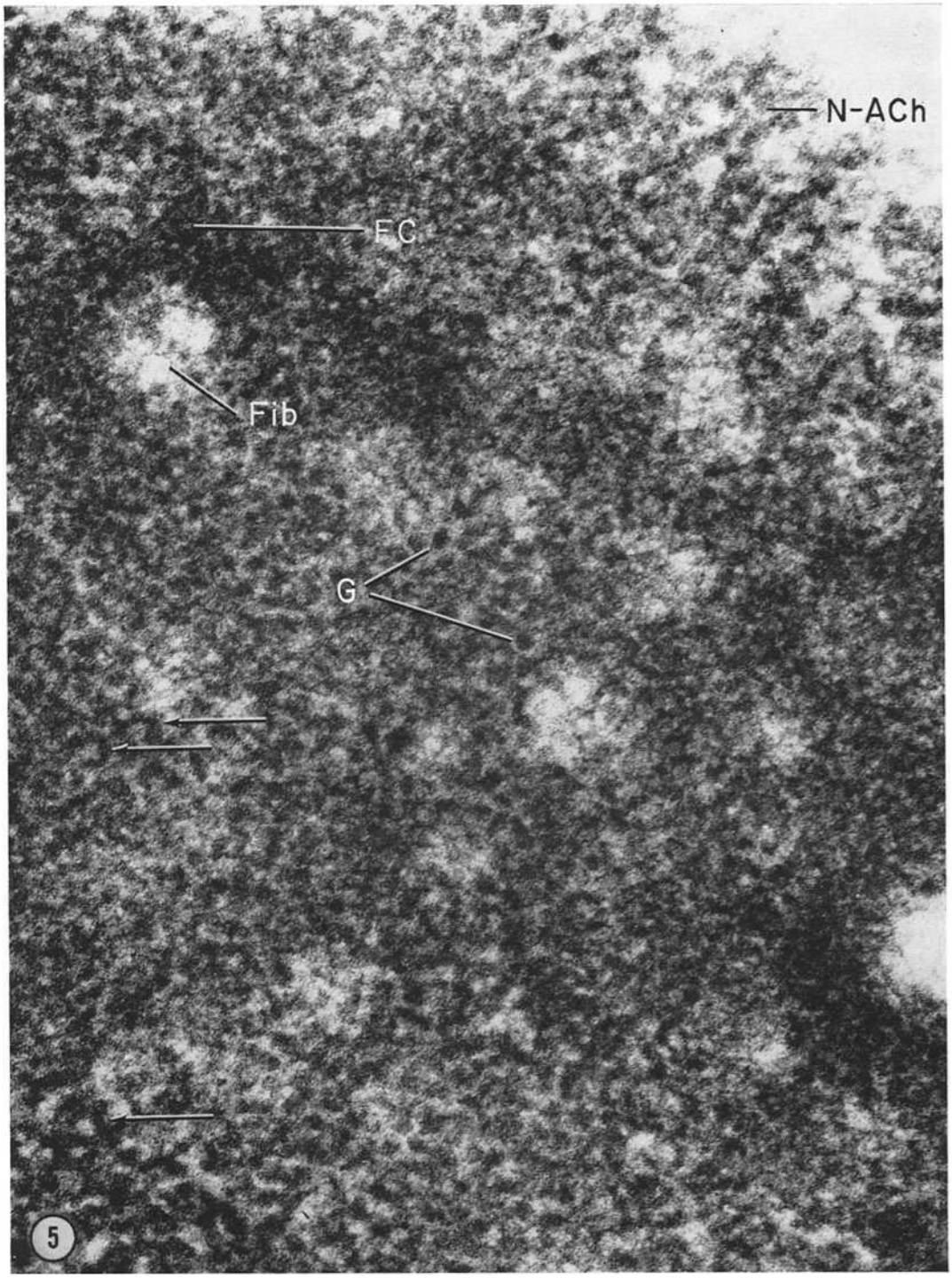

FIGURE 5 High-power electron micrograph of isolated nucleolus. The fibrillar component (*FC*) is present, and occasional narrow fibrils are visible in these areas. The vacuoles contain a fibrillar material (*Fib*) and the nucleolus-associated chromatin (*N-A Ch*) is present. Although some granules (*G*) are clearly defined, the "granular regions" are composed of twisted fibers about 150–200 A in diameter (unmarked arrows). OsO_4 fixation; uranyl acetate–lead hydroxide stain. × 140,000.

sional contamination by chromatin and fibrous material was present (Fig. 4 *a*). As determined by direct count, the percentage of intact nuclei in the preparation of nucleoli was less than 0.058. Isolated nuclei were found to contain an average of 1.6 nucleoli per nucleus. It was calculated that the yield of nucleoli from the original homogenate was 30%, and from the isolated nuclei the yield was 45.8%. The recovery of nucleoli closely approximated the value of 27% found by other authors (41). It has already been mentioned that the yield of nucleoli could be greatly enhanced by omitting the centrifugation at 184 *g*.

A low-power electron micrograph of isolated nucleoli (Fig. 4 *b*) shows that the substructure of the nucleoli was remarkably intact. The network-like appearance of the nucleolus was preserved, and the "granular" and "fibrillar" components were visible. The nucleolus-associated chromatin remained adherent to the nucleoli, and some free fragments of chromatin were also present. At high power (Fig. 5), the intranucleolar vacuoles were seen to contain a fine fibrillar material, possibly intranucleolar chromatin. The "granular component" was less distinct at this magnification. Rather, the granules seemed to be part of a network of tightly twisted fibers about 200 A in diameter. A similar observation was made previously by others (61).

Biochemical Observations

The chemical characteristics of isolated nuclei are summarized in Table I. The yield of nuclei from the homogenate as determined by hemocytometer counts was 73.7%, whereas the yield determined by estimation of DNA was 65.5%. This close correspondence could be better appreciated if the values for μg of DNA per 10^6 nuclei in the homogenate and nuclear preparation were compared. In the homogenate, there was an average of 12.6 μg of DNA per 10^6 nuclei, whereas in the nuclear fraction there were 11.5 μg of DNA per 10^6 nuclei. This finding apparently showed that each nucleus lost a slight amount of DNA during the isolation procedure, but the difference was not statistically significant ($P > 0.5$). This calculation assumes that all cellular DNA is found in the nucleus, an assumption which is substantially correct, although there is a small proportion of cellular DNA in mitochondria (62).

The chemical composition of isolated nuclei is expressed in terms of ratios and also in terms of

TABLE I

Chemical Composition of Nuclei

	Yield of nuclei from homogenate: Hemacytometer counts 73.3%; DNA estimation 65.5%			
	μg DNA/10^6 nuclei	Homogenate:* 12.6 ± 0.722 (difference not significant by t-test ($P > 0.5$))		Nuclei:* 11.5 ± 1.75
		Chemical composition: Ratios		
Ratio*	RNA/DNA	DNA/Protein	RNA/Protein	Method
Homogenate (18)§	2.18 ± 0.044 (2.5)‡ (2.22 ± 0.09)	0.017 ± 0.001 (0.016)	0.0365 ± 0.0007 (0.047)	Sadowski and Steiner (13) (21)
Nuclei (21)§	0.108 ± 0.004 (0.16) (0.108 ± 0.002)	0.661 ± 0.042 (0.21)	0.069 ± 0.003 (0.034)	Sadowski and Steiner (13) (21)
	Chemical composition:* μg/10^6 nuclei			
	μg DNA/10^6 nuclei	μg RNA/10^6 nuclei	μg protein/10^6 nuclei	
Nuclei (18)§	11.5 ± 1.75 (9.76)	1.20 ± 0.071 (2.95)	17.2 ± 0.77 (40.5)	Sadowski and Steiner (63)

* All values are means ± standard error of mean.

‡ Values of other authors are shown in brackets.

§ Number of experiments.

the amount of DNA, RNA, and protein per 10^6 nuclei (Table I). The ratios for the homogenate, as well as the values reported by other authors, are provided for comparison. Generally, the results were comparable with those obtained by other authors. The nuclei had less RNA than those isolated by the Chauveau procedure (13, 63) as shown by the low ratio of RNA/DNA and the low amount of RNA per 10^6 nuclei. The RNA/DNA ratio was identical to that obtained by Blobel and Potter (21). These nuclei appeared to contain less protein than those isolated by other methods, as shown by the high DNA/protein ratio and low amount of protein (17.35 μg) per 10^6 nuclei. On the basis of the RNA/DNA ratio of the homogenate, the μg of RNA and DNA per 10^6 nuclei, it was calculated that 4.55% of cellular RNA was located in the nucleus. A similar calculation for protein showed that 2.32% of cellular protein was located in the nucleus. Blobel and Potter (21) found that 4.7% of total cellular RNA was in the nucleus.

A summary of enzymatic studies of isolated nuclei is presented in Table II. Six enzymes localized to particular sites within the cell were measured. Specific activities of cytoplasmic enzymes were expressed relative to protein and DNA. Those expressed relative to DNA reflected the diminution of contamination by cytoplasm better than those expressed relative to protein, probably because DNA is virtually confined to the nucleus whereas protein is present in both the nucleus and the cytoplasm. Furthermore, soluble proteins may have escaped from the nuclei, whereas DNA appeared to remain constant. The contamination of the nuclei by cytoplasmic components was limited. With the exception of glucose-6-phosphatase (1.36%), well below 1% of activities of the cytoplasmic enzymes in the original homogenate was present in the nuclear fraction. NAD

TABLE II

Enzymatic Studies of Isolated Nuclei

Enzyme	Cellular localization	Specific activity: Units*/mg protein		Specific activity: Units*/mg DNA		% Original activity in nuclear fraction
		Homogenate	Nuclei	Homogenate	Nuclei	
Glucose-6-phosphatase (3)‡	Membranes of ER	1.78	1.058	90	1.8	1.36%
Cytochrome oxidase (2)	Mitochondria	1.405	0.195	82.5	0.292	0.18%
Acid phosphatase (2)	Lysosomes	0.4975	0.1299	25.4	0.032	0.18%
Uricase (2)	Microbodies	0.53	0.18	28.1	0.22	0.50%
Lactic dehydrogenase (1)	Cell sap	0.59	0.0026	34.9	0.00374	0.0077%
		Specific activity—Units/mg Protein				
NAD Pyrophosphorylase (2)	Nucleus	Nuclei	Nucleoli			
		96.2	502.9			

* *Units of activity*

Glucose-6-phosphatase—μMoles phosphate liberated in 15 min.

Cytochrome oxidase—$1/T_{100}$: T_{100} is the time which would be needed to oxidize fully the substrate (cytochrome *c*) if the reaction proceeded at a velocity equal to the initial velocity. (The reaction rate was linear for only 1 min). Full oxidation was achieved by adding a grain of potassium ferricyanide.

Acid phosphatase—increase in OD at 410 mμ per hour.

Uricase—decrease in OD at 292.5 mμ per hour.

Lactice dehydrogenase—μMoles of Na-L-lactate oxidized per min.

NAD pyrophosphorylase—mμMoles of NAD synthesized in 30 min.

‡ Number of experiments shown in brackets.

TABLE III

Chemical Composition of Isolated Nucleoli

Ratio (11 experiments)

Ratio	RNA/DNA*	DNA/Proteins*	RNA/Protein*	Method
	0.50 ± 0.06 (0.965)‡	0.53 ± 0.065 (0.118)	0.25 ± 0.031 (0.114)	Sadowski and Steiner (41)

μg per 10^6 Nucleoli (11 experiments)

μgRNA/10^6 nucleoli*	μgDNA/10^6 nucleoli*	μg Protein/10^6 nucleoli*	
0.175 ± 0.021 (0.224)	0.37 ± 0.066 (0.232)	0.59 ± 0.059 (1.97)	Sadowski and Steiner (41)

Percentage of RNA, DNA, protein (DNA + RNA + protein = 100%)

RNA	DNA	Protein
15.5%	32.5%	52.0%

* Values are means ± standard error of mean.

‡ Values of other workers shown in brackets.

pyrophosphorylase activity was present in isolated nuclei. As noted by other authors (64), this enzyme had a higher specific activity in the nucleolus than in the nucleus as a whole and it appeared to be activated following sonication.

The results of chemical analyses of isolated nucleoli are presented in Table III. Data of Desjardins et al. (41) are also provided. The results are expressed both as ratios and in terms of the amount of RNA, DNA, and protein per 10^6 nucleoli. It can be seen that the nucleoli contained more DNA, but less protein than the nucleoli analyzed by other authors (41). Isolated nuclei were found to contain an average of 1.6 nucleoli per nucleus[4] as determined by direct counts under phase contrast. It was calculated that nucleolar RNA constituted 23.4% of nuclear RNA, nucleolar DNA 5.17% of nuclear DNA, and nucleolar protein 5.49% of nuclear protein. If it is considered that the dry matter of the nucleolus consists only of RNA, DNA, and protein, the percentages of these components in isolated nucleoli amounted to 15.5, 32.5, and 52.0, respectively.

DISCUSSION

The method for the isolation of nuclei from rat liver described in this paper is based on principles already used by other workers. Membranous components of the cytoplasm were solubilized by the use of the nonionic detergent Triton-X-100 (26). Since some whole cells were resistant to lysis with this detergent, the nuclei were further purified by centrifugation through 2.2 M sucrose (12, 13). Divalent cations were present in the media throughout the procedure (17). The use of alkaline media buffered with Tris (21) was found by us to result in extreme fragmentation of the nuclei. Previously described methods had the disadvantage of providing low yields of nuclei (17), or being unsuitable for the isolation of nuclei from several grams of liver (17, 21). The present method permitted the recovery of about two-thirds of the nuclei, and could be adapted for the purpose of isolating the nuclei from several rat livers. These nuclei were subsequently used for the isolation of nucleoli.

The method for the isolation of nucleoli was similar to that proposed by other authors (39, 41). Nuclei were disrupted by ultrasound, and the nucleoli purified by differential centrifugation. A slight modification of this method was that divalent cations were not present in the media, since it was impossible to separate nucleoli from chromatin by differential centrifugation if divalent cations were present.

The ultrastructural appearance of isolated nuclei

[4] This value is somewhat lower than that obtained by others (39, 41, 65).

varied according to the method of fixation used. When fixed with formol-OsO_4, the nucleoplasm was so homogeneous that the areas of condensed chromatin were not clearly demarcated and the granular elements of the nucleoplasm were not prominent. When only OsO_4 was used as fixative, the areas of condensed chromatin were accentuated and the granular elements of the nucleoplasmic regions were more pronounced. This observation is the converse of that obtained with intact liver, for aldehyde fixation of intact liver makes the condensation of chromatin and the granular appearance of the nucleoplasm more pronounced than is seen with osmium fixation (66, 67). In isolated nuclei fixed with OsO_4, the nucleoplasm was found to contain electron-opaque granules about 200 A in diameter. These were designated "nucleoplasmic granules" and probably correspond to the ribonucleoprotein particles of the nuclear sap and nucleonemes (68) or to the interchromatin granules (69). The interchromatin granules have recently been claimed to contain both RNA and protein (70). The "intranuclear ribosomes," whose isolation is described in a subsequent paper (32), were thought to correspond to the nucleoplasmic granules.

The ultrastructural appearance of isolated nucleoli corresponded closely to the ultrastructure of nucleoli seen in the isolated nuclei and described by other authors (59, 69) in intact mammalian cells. However, the "granular" component of the nucleolus appeared to consist of a network of tightly coiled fibers when examined at high power. This may account for the failure to isolate ribonucleoprotein particles from nucleoli (32).

The nuclear fraction contained less RNA than that obtained with the Chauveau procedure (13, 63). This may have reflected relative freedom from contamination by cytoplasmic ribosomes and ribosomes on the outer nuclear membrane, since contamination by these components was minimal as determined by electron microscopy. Under the conditions employed, extraction of RNA from the nucleus is said to be limited (21). However, this low RNA/DNA ratio could also reflect breakdown of RNA by nuclear ribonuclease (71) since detergents have been shown to activate this enzyme (64). The isolated nuclei contained less protein than that found by other authors. This low amount of protein could have been due to absence of cytoplasmic contamination or to the loss of soluble proteins from within the nucleus. Unfortunately, it is difficult to distinguish between these alternatives, since measures to remove cytoplasmic contamination (in this case, washing with Triton-X-100), might also extract proteins from the nucleus. Finally, the isolated nuclei appeared to have retained all of their DNA.

The estimation of cytoplasmic marker enzymes confirmed morphological and chemical studies which suggested that contamination of the nuclei with cytoplasmic components was limited. While most enzymes measured were present in minute amounts in the nuclear fraction, 1.36% of the glucose-6-phosphatase activity present in the homogenate sedimented with the nuclear fraction. This may have been caused by the small amount of membranous contamination observed with the electron microscope. Another possibility is that glucose-6-phosphatase activity, which is present in the perinuclear space (72), remained adsorbed to the nucleus after removal of the outer nuclear membrane with Triton-X-100.

The isolated nucleoli contained about twice as much DNA as RNA, a fact which may, in part, have been due to the thick cuff of nucleolus-associated chromatin adherent to the nucleoli. In addition, there were undoubtedly some fragments of free chromatin present as well. The content of nucleolar proteins was somewhat lower than reported by other authors. This may have been caused by a loss of water-soluble proteins during the isolation of nuclei or nucleoli. Similarly, the content of RNA in the nucleoli was lower than found by other authors, perhaps due to hydrolysis of RNA by nuclear RNases activated by sonication of the nuclei (64). Density gradient analyses of nucleolar RNA will provide information on the quality of RNA in these nucleolar preparations.

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