THE ISOLATION OF CELL NUCLEI FROM RAT BRAIN

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ABSTRACT

A method for preparing highly pure cell nuclei from adult rat brain, using both differential and isopycnic centrifugation in sucrose media, is described. The morphology of these preparations was examined by both phase contrast and electron microscopy. The isolated nuclei retained many aspects of their *in situ* morphology; in particular, the nuclear envelope was double layered and interrupted by pore-like discontinuities, and the nucleoli consisted of irregular masses of densely packed granules. Analyses of these nuclear preparations for cytochrome oxidase and cholinesterase activity, as well as RNA/DNA ratio, indicated minimal contamination with mitochondria and microsomes. Problems involving the homogenization technique, choice of ionic conditions in the homogenization medium, and choice of optimal density of the sucrose solution used for the final purification of nuclei are discussed. Results of application of the technique to isolation of adult rat liver nuclei are also reported.

INTRODUCTION

Although there have been numerous applications of differential centrifugation (as developed by Hogeboom and Schneider, 1) to the preparation of subcellular fractions of brain (2), these methods do not yield nuclear fractions of sufficient purity for isolation of nuclear material uncontaminated by significant amounts of cytoplasm. In particular, brain nuclear fractions, prepared by differential centrifugation, followed by washing of the nuclear sediment, are likely to be contaminated by one or more of the following components: (*a*) myelin, (*b*) capillary fragments, (*c*) mitochondria, (*d*) microsomes, and (*e*) red blood cells.

There are several procedures (which involve techniques other than simple differential centrifugation and washing) published in the literature for isolation of rat liver nuclei, of purity adequate for isolation and characterization of nuclear components (3, 4). However, none of these techniques appear to be directly applicable to brain, for the following reasons: (*a*) nuclei from brain, particularly those from neurons, are more easily ruptured than those from liver, so that particular care must be given to the conditions of homogenization; (*b*) nuclei from brain, representing an extremely heterogeneous cell population of different types of neurons and glia, vary greatly both in size and density, and therefore do not behave uniformly during either differential or isopycnic centrifugation; and (*c*) the large amount of myelin in brain distinctly hinders attempts to wash brain nuclei free of contaminating particles.

If, however, rat brain is gently homogenized with the homogenizer described by Dounce *et al.* (5), then washed thoroughly by several cycles of differential centrifugation, and the crude nuclear pellet finally sedimented in hypertonic sucrose of a density adequate to float subcellular constituents other than nuclei (6, 7, 8), then small yields of

highly pure brain nuclei may be obtained. This communication describes the details and results of such a procedure, employing both light and electron microscopy as well as cytochemical procedures as control methods.

METHODS

The procedure as finally developed will be described below, and comments on the various aspects of the procedure will be made in the Discussion.

Preparation of Nuclei

Male albino rats, 70 to 100 days old, with a brain weight of 1.7 to 1.9 gm, were decapitated; their brains were rapidly removed, cleaned of meninges, and blotted with filter paper to remove surface blood. The remainder of the procedure was done at 0° to 4°C. For homogenization each brain was processed in halves in the following manner: each half brain (weighing 0.85 to 0.95 gm) was minced with scissors for two minutes in a small beaker and homogenized with six passes of a "loose" pestle in a "small" (10 ml) Dounce homogenizer (5) (Blaessig Glass Specialties, Rochester, New York), using 9 ml of the following homogenizing solution: 0.32 M sucrose, 0.001 M $MgCl_2$, 0.0004 M KH_2PO_4, 0.0004 M K_2HPO_4, pH 6.7 to 6.8. The homogenates from a single brain were combined and spun in a heavy duty 40 ml round bottom centrifuge tube for 10 minutes at 850 *g* (av.) in a horizontal rotor. The supernatant fluid was removed by aspiration and the crude nuclear sediment washed twice by vigorous resuspension with a glass stirring rod in 9 ml of homogenizing solution, followed by centrifugation for 10 minutes at 850 *g* (av.). If it was desired to prepare cytoplasmic fractions, the combined supernatants from the first three spins were saved. The crude nuclear sediment was then washed three more times (the last wash in a 40 ml graduated, conical centrifuge tube) with 18 ml of homogenizing solution used for each wash, followed by centrifugation for 8 minutes at 600 *g* (av.). After the final low-speed spin, the crude nuclear sediment was resuspended in homogenizing solution to a total volume of 4.5 ml, and a hypertonic sucrose solution (2.39 M sucrose, 0.001 M $MgCl_2$, 0.0035 M K_2HPO_4, 0.0007 M adenosine triphosphate disodium salt, pH 6.7 to 6.8) was then added to give a final volume of 32.5 to 33.0 ml, and a final sucrose concentration of 2.05 to 2.15 M. The suspension was then vigorously mixed and spun for 2 hours at 50,000 *g* (av.) in the SW 25.1 rotor of the Spinco model L ultracentrifuge. The purified nuclei were recovered as a small, clear gelatinous pellet at the bottom of the centrifuge tube, while the contaminants, which float on the hypertonic sucrose, were easily discarded.

Phase Contrast and Electron Microscopy

For phase contrast microscopy the isolated nuclei were resuspended in the original homogenizing medium, mounted on a glass slide, and covered with a coverslip. During the initial development of the isolation procedure the frequent use of the phase contrast microscope for checking the effect of each step of the procedure on nuclear morphology was found to be imperative. Phase contrast photomicrographs were taken with either a Zeiss or Officine Galileo photomicroscope.

Electron microscopic examinations were performed on five separate preparations of brain nuclei. For this purpose a 2 per cent solution of osmium tetroxide at pH 7.4 in either Veronal buffer (9) or a bicarbonate-buffered saline glucose medium containing 27 mM potassium (10) was added to an equal volume of resuspended nuclear sediment. After an initial fixation period of 5 to 15 minutes, the mixture was centrifuged for 10 to 15 minutes at low speed. The pellet thus obtained was rapidly dehydrated with increasing concentrations of ethanol and divided into small blocks. Either one of the following embedding media was used: (*a*) a 9:1 mixture of butyl and methyl methacrylate with 0.075 per cent uranyl nitrate (11) introduced as a filler, and with 0.25 per cent of the initiator, benzoyl peroxide, added prior to prepolymerization; or (*b*) a mixture of 2 parts Epon 812 and 5 parts dodecenyl succinic anhydride (DDSA) with 1.71 per cent of 2,4,6-tri(dimethylaminomethyl)phenol (DMP-30) added as an accelerator.[1]

Except for the plastic mixture used, the embedding with Epon generally followed the procedure outlined by Luft (13). Sections were cut with an LKB ultramicrotome fitted with a glass or diamond knife. The sectioning of the tissue blocks presented considerable difficulties because of contamination of the nuclear pellets with minute glass fragments, derived mainly from the homogenizer. Only with the diamond knife could the resulting sectioning artefacts be reduced to an acceptable minimum. The sections were placed on collodion carbon-filmed grids and examined with an RCA EMU-3C electron microscope at magnifications from 3,000 to 21,000 and

[1] After testing of several variants, this mixture was found to combine adequate preservation of tissue with good sectioning quality and with somewhat higher contrast in the final electron photomicrographs. Nadic® methyl anhydride (National Aniline Division, Allied Chemical & Dye Corp., New York City), which has been shown to add to the electron opacity of epoxy resins (12), was omitted; the increased contrast which was observed in the final electron photomicrographs was attributed to this omission.

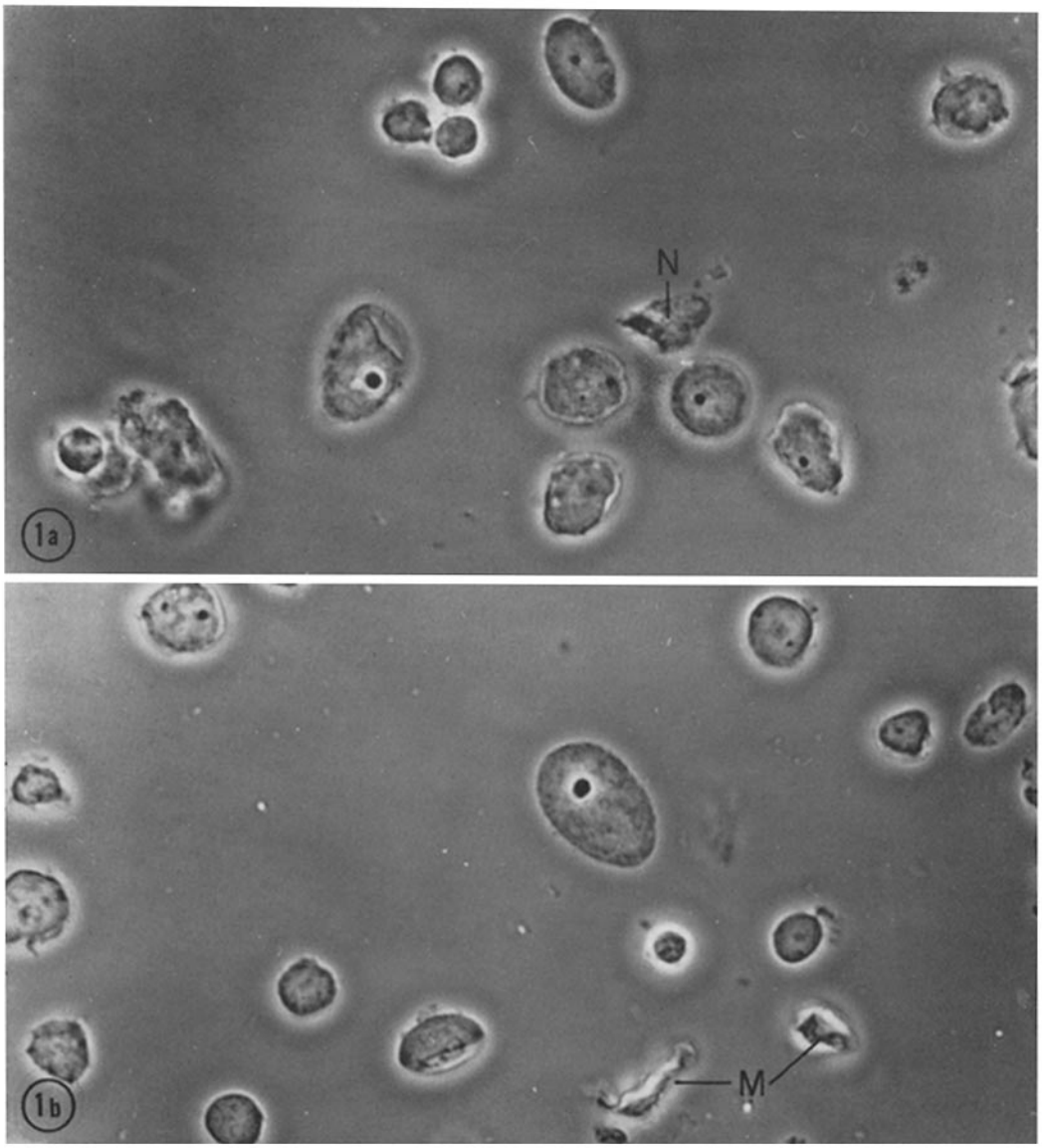


FIGURE 1

Two representative pictures of the resuspended final brain nuclear pellet as seen in the phase contrast microscope. The specimen is composed of round and ovoid nuclei of various sizes and shapes. Sometimes the nuclear contour appears distorted (*N*) and a few myelin fragments (*M*) are seen as contaminants. Phase contrast micrographs, × 1000.

photographically enlarged to between 2.5 and 4 times.

Chemical Analyses

RNA and DNA were determined by the procedure of Scott *et al.* (14). Cytochrome oxidase was measured by the procedure of Cooperstein and Lazarow (15). Assays were performed at a cytochrome *c* concentration of 1.7×10^{-5} M, with a final reaction volume of 3 ml. Cholinesterase was measured by titration of acid produced during hydrolysis of acetylcholine at constant pH. This assay was done in a pH-stat under

the following conditions: temperature 37°C; 0.004 M acetylcholine; 0.1 M NaCl; 0.01 M $MgCl_2$; pH 7.4.

RESULTS AND DISCUSSION

Phase Contrast Microscopy

The isolated brain nuclei were extremely fragile, as evidenced by their tendency to distort or rupture under slight mechanical pressure. They also had a disturbing tendency either to agglutinate or to adhere to glass surfaces. Because of these properties, it was difficult to achieve a uniform focal plane for phase photomicrography of a large number of nuclei. Similar problems in phase contrast microscopy of neuronal material have previously been noted by Deitch and Murray (16).

Individual nuclei varied greatly with respect to size, density of nucleoplasm, and number of nucleoli (Figs. 1, 2). Larger nuclei contained prominent single nucleoli, similar to those seen in neurons, while smaller ones had one, two, or more nucleoli, as observed in various glia cell types. Most of the nuclei were round or ovoid and clearly outlined, although a moderate percentage had irregular, disrupted contours. Contaminating, non-nuclear material was present in a minimal amount and consisted of fragmented myelin sheaths and capillaries, as well as granular material too small to be identified.

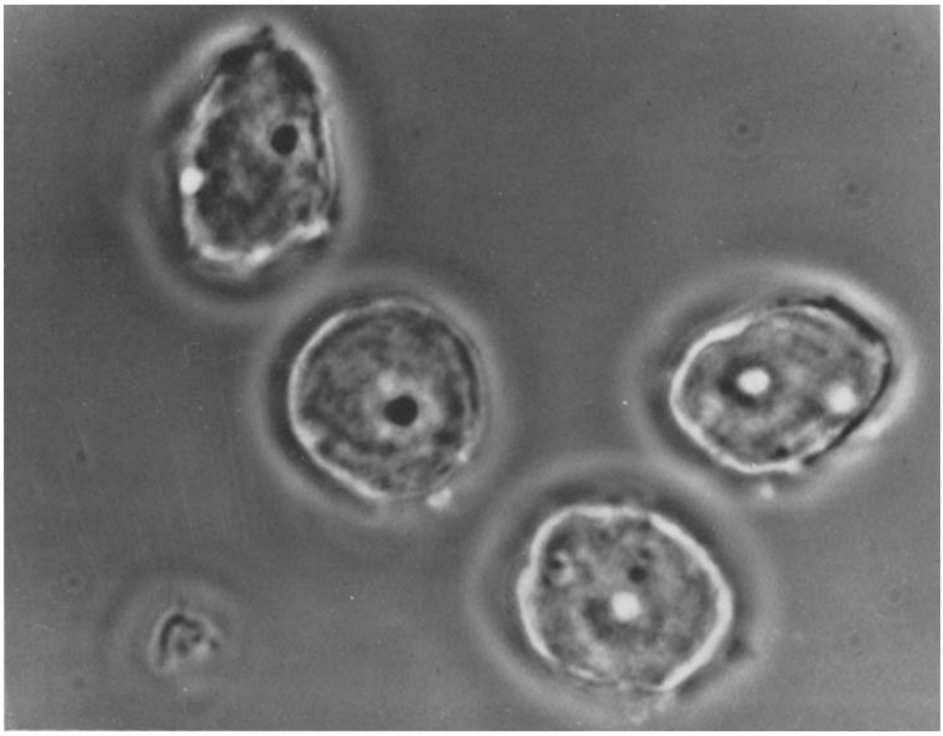


FIGURE 2

Four nuclei of the resuspended nuclear pellet at high magnification. The nuclei are well delineated and contain one or more nucleoli. The appearance of nucleoli as either dark or light intranuclear configurations is attributable to different focal planes. Phase contrast micrograph. × 2500.

Electron Microscopy

Electron microscopy corroborated the observations obtained by phase contrast microscopy. The over-all shape of the nuclei and the amount of space between them depended greatly on the degree of packing during the sedimentation which followed the addition of osmium fixative. It was desirable to pack the nuclei very gently, even if this necessitated a resedimentation during dehydration. Gentle packing gave better separation and morphology of the nuclei in the pellet, while tight

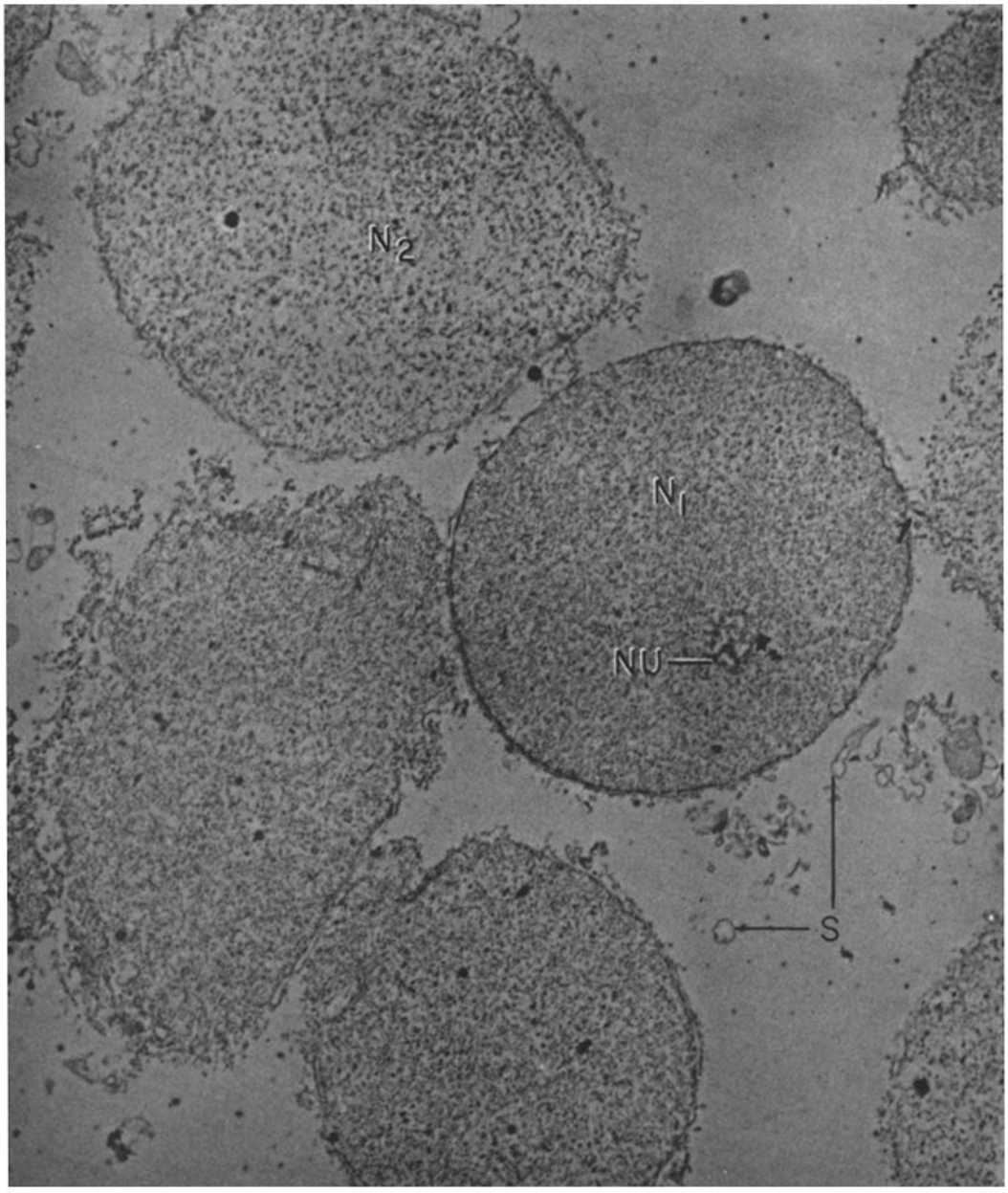


FIGURE 3

Electron micrograph of sectioned brain nuclear pellet. The nuclei vary with respect to their over-all opacity and the characteristics of their contours (*e.g.*, N_1 and N_2). A nucleolus is indicated at *NU*. Some contaminants, in the form of smooth membranes (*S*) and unidentifiable material, are seen interspersed between the nuclei. Epon 812 embedding. Approximately × 12,000.

packing yielded nuclei which frequently were distorted, sometimes to such a degree that tortuous nuclear contours fitted into each other in a mosaic-like pattern. In such instances, no satisfactory delineation of individual nuclei was obtained, and contaminants were not easily recognized.

With optimal packing conditions, the nuclear

profiles usually exhibited a round or oval shape outlined by a double layer of membranes (Fig. 3). Nucleoli were easily differentiated within the nuclear matrix as aggregates of opaque, small granules embedded in the nucleoplasm (Fig. 4). The nucleoplasm itself consisted mainly of fine, short, filamentous and granular profiles of relatively low density; the packing of these elements was less dense in isolated nuclei than in nuclei in intact tissue (Fig. 5). At low magnifications, the over-all opacity of individual nuclei varied. Some types of nuclei were more opaque, usually round, and appeared completely outlined by the nuclear membrane; other types were less opaque, ovoid, and frequently showed disruption of nuclear membranes. It was impossible, by electron microscopy, to make an unequivocal identification of nuclei in terms of their derivation from various types of cells present in the central nervous system. It could be inferred, however, that the less opaque, ovoid, and more fragile nuclei were derived from either neurons or (in case they displayed two nucleoli) from astrocytes. The more opaque and rounded nuclei resembled those of oligodendrocytes, but no criteria could be found to identify microglial nuclei.

The nuclear envelope was frequently seen as two, generally parallel, membranous layers which periodically became confluent and thus enclosed somewhat enlarged perinuclear cisterns (Fig. 5). At the gaps between these sites of juncture of the two membranes, the nucleoplasm was in continuity with the extranuclear space through pore-like formations. The outer nuclear membrane in general appeared smooth, although occasionally there were a few dense granules attached to its surface.

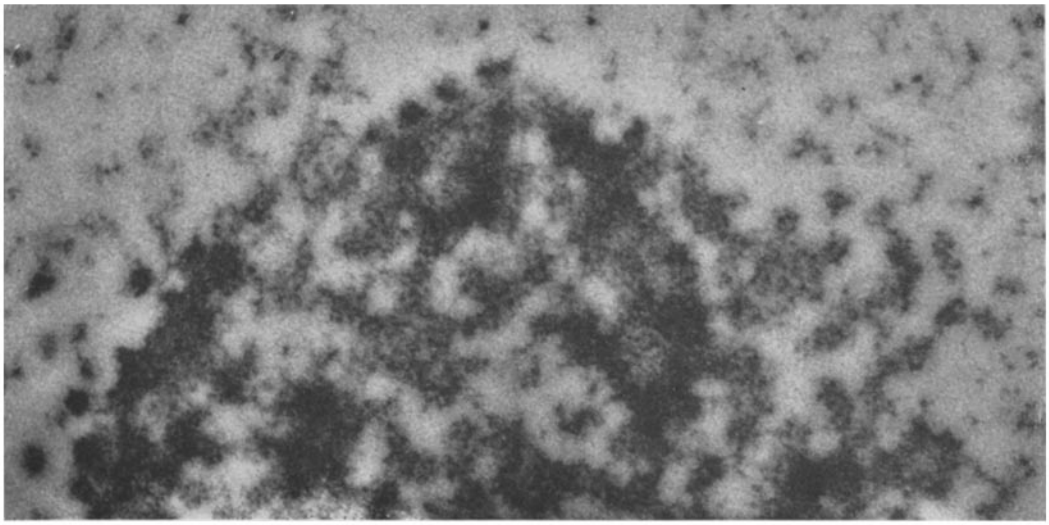


FIGURE 4

Section through a portion of a nucleolus contained in a nucleus of the isolated pellet. The micrograph exhibits the characteristic nucleolar composition in the form of aggregated, opaque, small granules. Epon 812 embedding. Approximately × 55,000.

Occasionally, small groups of non-nuclear tissue fragments were observed in the spaces between the nuclear profiles; these are described below in order of diminishing occurrence. The predominant non-nuclear elements were myelin sheath fragments (Fig. 6). The individual lamellae within these fragments usually were separated and distorted, in contrast to the regular, parallel, or concentric array observed in intact material. In some instances, where a concentric arrangement of myelin layers was retained, some intra-axonal constituents, such as mitochondria and a few dense granules, were seen. Mitochondria were a second contaminant; they usually appeared with a continuous boundary, a swollen matrix, and with their cristae displaced towards the periphery of the organelle (Fig. 7). Membranous profiles

were a third contaminant; they could be identified either as portions of endoplasmic reticulum as indicated by a few attached dense granules or, rarely, as cell boundaries if they enclosed small amounts of cytoplasmic material. Some small filamentous or granular structures of low opacity remained unidentified. These were sometimes aggregated like nucleoplasm, but the absence of a distinct nuclear membrane did not permit a positive identification. A few configurations could be identified as portions of capillaries when relatively intact cell processes enclosed a lumen and were adjoined by a basement membrane-like material on the outer surface. As a rule, such lumina appeared devoid of formed elements.

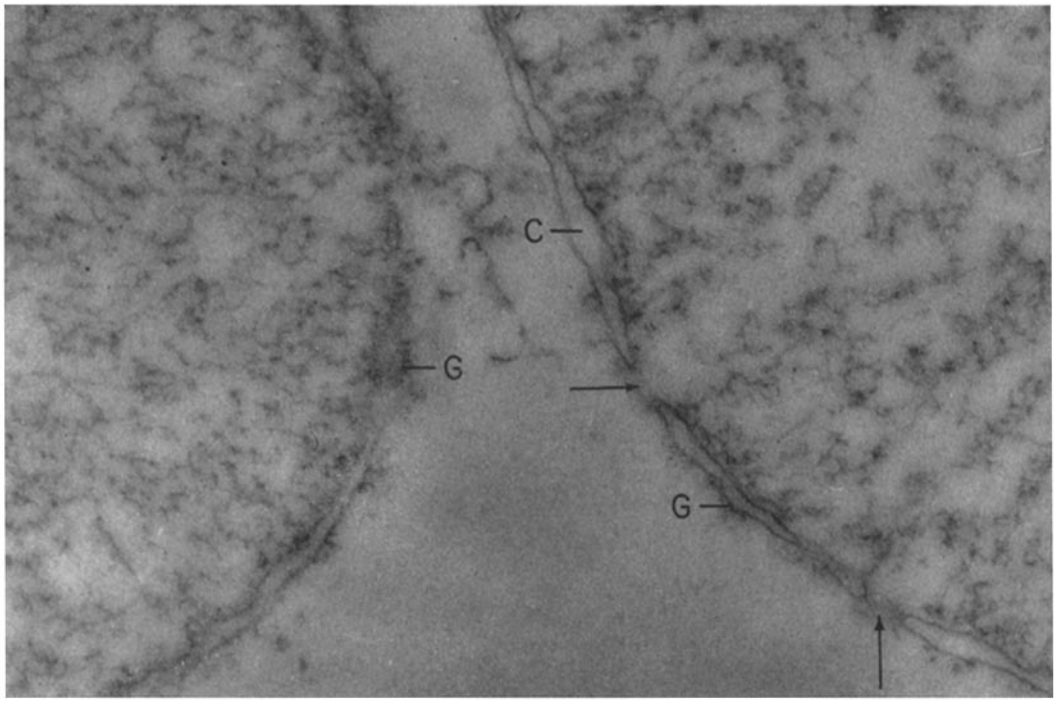


FIGURE 5

Section through peripheral portions of two nuclei of the pellet. The perinuclear cistern (*C*) appears somewhat enlarged within the double layer of membranes. Nuclear pores are indicated by arrows. Occasionally, a few opaque, small granules (*G*) are attached to the outer nuclear membrane. The nucleoplasm contains widely separated filamentous and small granular material as formed elements. Epon 812 embedding. Approximately × 80,000.

Yield of Nuclei

The yield of DNA in the final nuclear pellet was 280 (±10) μg per brain, which is approximately 11 per cent of the total of 2500 (±100) μg of DNA to be found in a 1.8 gm rat brain. Phase contrast microscopy and chemical determination of DNA showed that most of the nuclei in the crude nuclear sediment were floated by the hypertonic sucrose; these consisted of nuclei of capillary segments, as well as neuronal and glial nuclei of relatively low density. Inasmuch as an appreciable percentage of total brain nuclei are derived from capillaries (17) (and almost no capillaries are present in the final nuclear pellet), the true yield of non-capillary nuclei is greater than 11 per cent. The yield of neuronal and glial nuclei in the final nuclear pellet may be increased by lowering the density of the hypertonic sucrose, but this increased yield is accompanied by increasing contamination with myelin, capillaries, mitochondria, and microsomes.

Cytochemical Assay of Purity

In an attempt to quantitate the contamination by non-nuclear material, two separate brain

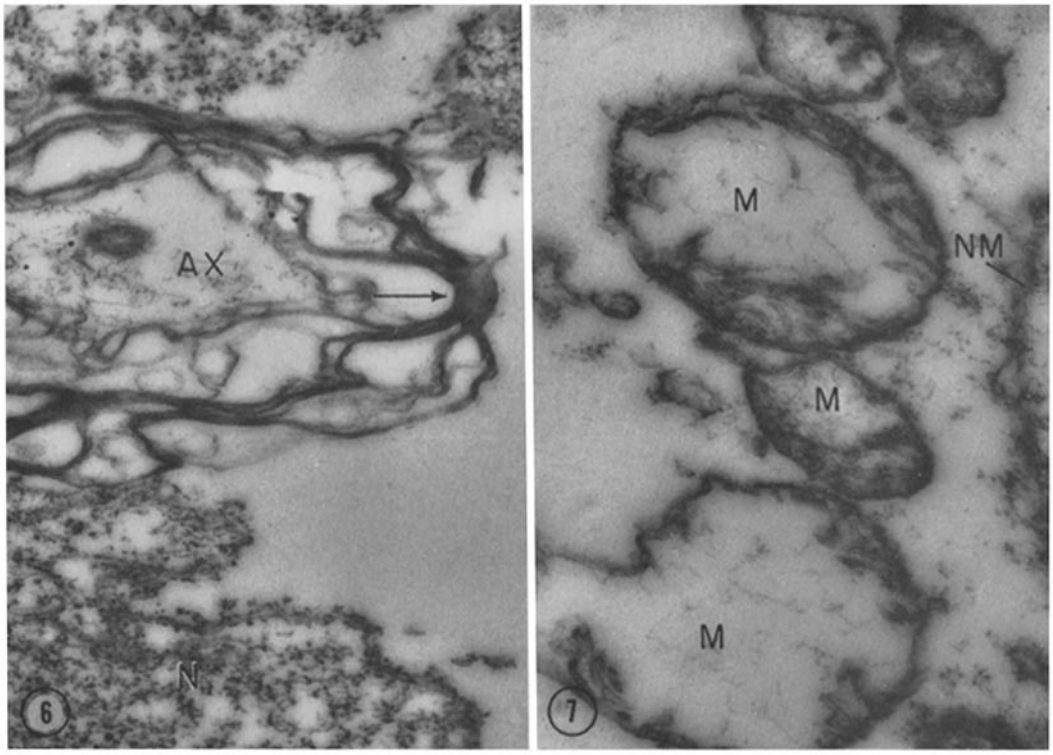


FIGURE 6

Contaminating myelin fragment in nuclear pellet. The individual myelin lamellae are separated and distorted except for the one area indicated by the arrow. Enclosed axoplasmic constituents are seen at *AX*. A portion of a nucleus is designated at *N*. Methacrylate-uranyl nitrate embedding. Approximately × 35,000.

FIGURE 7

Occasional mitochondria (*M*) in the nuclear pellet. They usually appear swollen with their cristae at the periphery. An adjacent nuclear membrane at *NM*. Methacrylate-uranyl nitrate embedding. Approximately × 50,000.

nuclear preparations were analyzed for cytochrome oxidase and cholinesterase. Cytochrome oxidase is known to be an exclusively mitochondrial enzyme (1), and therefore should be absent in a pure nuclear preparation. Neuronal nuclei are also known to be extremely low in cholinesterase (18), which is concentrated in the microsomal fraction (19, 20); hence low cholinesterase activity in a nuclear preparation indicates little microsomal contamination. Moreover, the presence of significant amounts of microsomes should increase the RNA/DNA ratio of the nuclear preparation, since microsomes contain large amounts of RNA but no DNA.

The results of the cytochrome oxidase and cholinesterase assays are reported in Table I. The total activities reported for the nuclear fraction are those for the nuclear pellet as isolated from one brain of wet weight 1.8 gm. It can be seen that there was little activity of either cytochrome oxidase or cholinesterase in the isolated nuclear fraction, and thus there did not appear to be significant mitochondrial or microsomal contamination. Both enzymes were found to be entirely stable to the hypertonic sucrose solution used for isolation of nuclei, so that the low amounts recovered in the nuclear fraction do not reflect inactivation of enzyme actually present.

The yield of RNA in the nuclear pellet was 62 (±3) μg per brain, which gives an RNA/DNA ratio of 22 per cent. If the density of hypertonic sucrose used for the final spin was lowered significantly, greater contamination of the nuclear pellet with cytoplasmic granules resulted (as could readily be noted under the phase microscope), and the RNA/DNA ratio rose to figures above 30 per cent.

Comments on the Isolation Procedure

1) IONIC COMPOSITION OF HOMOGENIZING SOLUTION: Omission of both K^+ and Mg^{++} ions led to swelling of nuclei and disappearance of nucleoli. Omission of Mg^{++} alone produced nuclei with poorly defined nucleoli. A pH of 6.7 to 6.8 was optimal; a more acid pH produced pyknotic, dense nuclei, while a more alkaline pH led to greater rupture of nuclear membranes and poorer definition of nucleoli. In the above respects rat brain nuclei are quite similar to liver nuclei (21, 22). Inclusion of chelating agents which bind divalent cations (23) is undesirable for preparation of nuclei with well preserved ribonucleoprotein structures, since it is known that divalent cations are essential for maintaining the integrity of ribonucleoproteins (24, 25).

2) HOMOGENIZATION: Brain nuclei were found to be much more easily disrupted than liver nuclei, even under gentle conditions. Vigorous homogenization led to almost total disappearance of large nuclei in the final pellet. The pestles of the small (10 ml) Dounce homogenizer, as supplied by Blaessig Glass, come in two degrees of tightness, "loose" and "tight." For preparation of brain nuclei, only loose pestles which allow the homogenizer tube containing 10 ml of water at 25°C to fall free of the pestle in less than 6 seconds should be used. If homogenization is done properly, nuclear breakage may be minimized, so that less than 7 per cent of the total brain DNA is found in the combined supernatants from the first three spins.

TABLE I

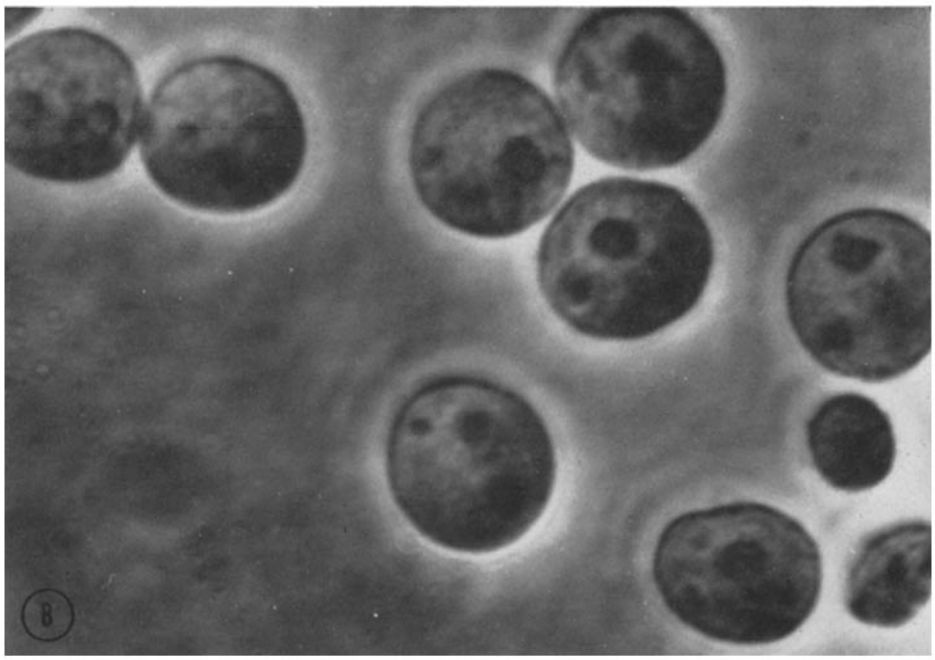
Enzymatic Assay of the Purity of Rat Brain Nuclei

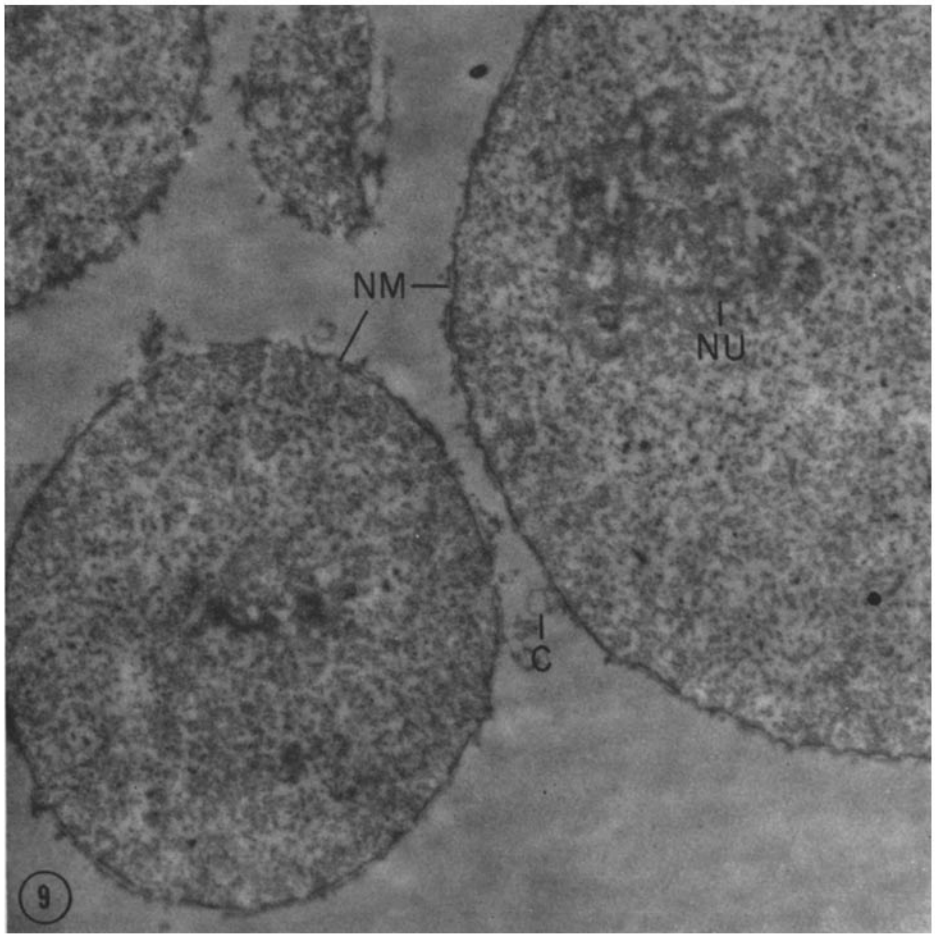
Enzyme	Fraction	Preparation	Total activity‡	Specific activity
			*Units**	*Units/mg DNA*
Cytochrome oxidase	Unfractionated brain	1	690	280
Cytochrome oxidase	Unfractionated brain	2	450	180
Cytochrome oxidase	Nuclei	1	0.44	1.6
Cytochrome oxidase	Nuclei	2	0.20	0.8
Cholinesterase	Unfractionated brain	1	26	10
Cholinesterase	Unfractionated brain	2	18	7
Cholinesterase	Nuclei	1	0.04	0.16
Cholinesterase	Nuclei	2	0.02	0.08

* A unit of cytochrome oxidase activity is defined as follows: if Δ log [ferrocytochrome *c*] per minute = 1 (under the conditions defined by Cooperstein and Lazarow, 15), then one unit of activity is present. A unit of cholinesterase activity corresponds to 1 μM of acetylcholine hydrolyzed per minute. All figures reported are the average of two or more determinations.

‡ Represents that derived from 1.8 gm wet weight of tissue.

3) INITIAL WASHES AND LOW SPEED CENTRIFUGATION: The pooled supernatants from the first three washes represent a convenient source of cytoplasmic material, relatively free of nuclei, if it is desired to prepare mitochondrial, microsomal, or supernatant fractions. The extensive washing and recentrifugation of the homogenate remove the greater part of contaminating myelin, mitochondria, and microsomes, with minimal loss of nuclei. However, in spite of the numerous washings, the crude nuclear sediment which remained after six centrifugations was still grossly contaminated with cytoplasmic granules, myelin, and capillaries, as was readily apparent under the phase microscope. Further





purification is thus required if a reasonably pure nuclear preparation is to be achieved.

4) HYPERTONIC SUCROSE SOLUTION: The inclusion of ATP was found to give better preservation of the morphology of nuclei, in conformity with an earlier report by Cole *et al.* (26) for mouse spleen. Brain nuclei are somewhat less dense than liver nuclei, since the proportion of brain nuclei which float in sucrose solution of density = 1.27 is much greater with brain than with liver and thus the yield is less for brain. Although it has been noted that nuclei prepared in hypertonic sucrose may be depleted of low molecular weight constituents (4), it appears that isolation in hypertonic sucrose is satisfactory if it is desired to retain high molecular weight constituents such as nucleoproteins.

Application of the Procedure to Tissues other than Adult Rat Brain

Nuclei have been isolated from adult rat liver using the identical procedure (except for the use of a "loose" pestle with a falling time of 9 to 10 sec., see HOMOGENIZATION above). Examination of liver nuclear preparations by phase microscopy (Fig. 8) revealed less heterogeneity of nuclear morphology, less nuclear breakage, and less contamination with other subcellular particles than was noted for nuclear preparations from brain. The liver nuclei also showed much less tendency to agglutinate and stick to glass surfaces, and a greater resistance to mechanical breakage. One of the liver nuclear pellets, examined with the electron microscope (Fig. 9), was characterized by the uniformity of nuclei observed. All nuclei appeared round and well delineated by a double membrane which was interrupted by pore-like discontinuities. Nucleoli were prominent, and the nucleoplasm generally was of higher opacity than that seen in isolated brain nuclei. The amount of non-nuclear constituents was extremely small and usually consisted of fragmented, smooth membranous profiles. The yield of DNA was 1 mg per gm wet weight of original tissue (compared to a total of 2 mg initially present), which is a significantly greater yield than that obtained from brain. The RNA content of these nuclear preparations was 180 μg per mg DNA, which gave an RNA/DNA ratio of 18 per cent. Assays done on one preparation of adult rat liver nuclei for cytochrome oxidase and cholinesterase are reported in Table II, and it can be seen that a high degree of purification of the nuclei has been achieved.

TABLE II

Enzymatic Assay of the Purity of Rat Liver Nuclei

Enzyme	Fraction	Total activity‡	Specific activity
		*Units**	*Units/mg DNA*
Cytochrome oxidase	Unfractionated liver	825	410
Cytochrome oxidase	Nuclei	0.67	0.67
Cholinesterase	Unfractionated liver	1.45	0.73
Cholinesterase	Nuclei	0.02	0.02

* See Table I.

‡ Represents that derived from 1 gm wet weight of tissue.

The procedure as outlined above has also been

FIGURE 8

Resuspended liver nuclear pellet. Representative field of round and well outlined nuclei with one or more nucleoli. Phase contrast micrograph. × 2200.

FIGURE 9

Electron micrograph of sectioned liver nuclear pellet. Nucleolar (*NU*) and nuclear membrane (*NM*) configurations appear well preserved. Membranous contaminant at *C*. Epon 812 embedding. Approximately × 15,000.

used for preparation of nuclei from guinea pig brain (27), and it has been shown that such guinea pig brain nuclei contain less than 1 per cent cholesterol (dry weight). Inasmuch as guinea pig myelin is approximately 20 per cent cholesterol (dry weight) (27), the myelin contamination of guinea pig brain nuclei prepared by the present method is not more than 5 per cent.

The preparation and characterization of native RNA from nuclei of brain and liver of the adult and newborn rat, and a comparison of both physical and functional properties of nuclear and cytoplasmic RNA's from the above sources, are reported elsewhere (28, 29).

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