ISOLATION OF CELL NUCLEI FROM THE MAMMALIAN CEREBRAL CORTEX AND THEIR ASSORTMENT ON A MORPHOLOGICAL BASIS

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ABSTRACT

An aqueous method is described for the isolation of highly purified nuclei from the cerebral cortex of adult guinea pigs. Erythrocytes were removed by a short-time perfusion of the brain, myelin fragments by a rapid mechanical method, and blood capillaries by a centrifugal sieving through dense sucrose solutions. The nuclear preparation retained the activity of ATP:NMN adenylyltransferase. Recoveries of DNA in the P4I, P4II, P_L and P_S preparations were 30, 43, 8, and 7%, respectively. Microscopy and phase contrast microscopy showed a satisfactory removal of erythrocytes, myelin fragments, capillaries, and cytoplasmic elements. Biochemical purity of samples was verified by the absence of several cytoplasmic enzyme activities. In the electron microscope, the majority of nuclei showed well-preserved nuclear membranes, with nuclear pores, and were provided with a finely textured nucleoplasm. Occasional contaminants were elements of endoplasmic reticulum and of the endothelium. Assortment of nuclei on a morphological basis showed that 55–65% and 47–53% of nuclei in the P4I and P4II preparations, respectively, consisted of neuronal nuclei. In the P_L preparation, the population of neuronal nuclei ranged between 72 and 83%, while 94–99% of the nuclei in the P_S preparation consisted of smaller nuclei, most likely of oligodendroglial origin.

INTRODUCTION

The isolation of pure nuclei from the nervous tissue is attended by various difficulties. In addition to blood cells, broken capillaries, and mitochondria, myelin and axonal fragments are major contaminants peculiar to the cerebral tissue. Further difficulty has been the differential isolation of nuclei of various origins, i.e., neuronal, glial, and endothelial nuclei.

In order to minimize the contaminating nonnuclear material, Sporn et al. (23) repeatedly washed their crude nuclear sediment before the final use of isopycnic centrifugation in sucrose media. This nuclear fraction, however, represents only 11% of the total DNA. Rappoport et al. (20) succeeded in isolating clean nuclei from the neonatal rat brain with a Triton X-100-sucrose medium; attempts to isolate nuclei from young and mature rat brains by the same procedure, however, yielded intact nuclei heavily contaminated with myelin and fat droplets. Recently, Hadjiolov et al. (5) have developed a procedure for the isolation of highly purified cell nuclei from the brain cortex of adult cats by means of nonionic detergent Cemulsol NPT 12 (Société des Produits chimiques de Synthèse, Bezon, Seine- et Visi, France) combined with centrifugal sieving through hypertonic sucrose solutions. Optimal detergent action required maintenance of fairly rigid parameters,

such as appropriate ionic strength and pH of the sucrose medium, and length of exposure to detergent–sucrose media.

The present paper offers a useful set of methods for the isolation of clean nuclei without using detergents. Erythrocytes were removed by a short-time perfusion of the brain in situ, myelin fragments by a rapid mechanical method, and blood capillaries by a centrifugal sieving through dense sucrose solutions. Differential isolation of neuronal and glial nuclei has also been attempted with considerable success. A preliminary account of this work has been given (12).

MATERIALS AND METHODS

Reagents

The following chemicals were obtained from the sources indicated: acetylthiocholine iodide (AthCh), *n*-butyrylthiocholine iodide (ButhCh), 5,5′-dithiobis[2-nitrobenzoic acid] (DTNB), and 3,5-diaminobenzoic acid (DABA) were from Tokyo Kasei Kogyo Co., Tokyo, Japan; ATP (disodium salt), NMN, DNA (from calf thymus, Type I), and RNA (from yeast, Type XI) were from Sigma Chemical Co., St. Louis, Mo.; Ficoll was from Pharmacia, Uppsala, Sweden; diisopropylfluorophosphate (DFP) was from Sumitomo Kagaku Co., Tokyo, Japan; Triton X-100 was from Rohm and Haas Co., Philadelphia, Pa. Whenever possible, other reagents were of the highest grade of purity commercially available. Disodium ATP was converted into the tris salt as described previously (15). Ficoll was dialyzed against distilled water for 10–15 hr before use.

Initial Tissue Dispersions

Adult guinea pigs of both sexes weighing 300–350 g were used in all experiments. Each animal was anesthetized for 45 sec in a bottle filled with ether vapor; the body subsequently was cooled in an ice bath for 60 sec; the heart was exposed; and then the head was perfused with 0.32 M sucrose at 38°C through an arterial cannula inserted into the left ventricle. The right auricle was cut and the perfusate drained, while the descending aorta was pressed to the spine by a fingertip. Perfusion for 60–90 sec was found sufficient to remove nearly all the erythrocytes from the brain. The entire, subsequent procedure was carried out at 0–4°C. Cerebral gray matter, usually 1.5 g per animal, was placed, within 4–5 min after the administration of anesthesia, into an ice-cold humid chamber, and tissues from six animals (approximately 10 g) were used in each run. Batches (5 g) were ground in 5 volumes of 0.32 M sucrose containing 1.5 mM $CaCl_2$ in a Dounce-type homogenizer with a plastic pestle (clearance about 150 μ); grinding involved eight strokes of the pestle, rotating at 1,500 rpm, for 2 min. The suspension was filtered through eight layers of gauze, and finally made up to 5% (w/v) by adding appropriate volumes of 0.32 M sucrose–1.5 mM $CaCl_2$.

Final Procedure for the Isolation of Nuclei (Fig. 1)

STEP 1: Four 50.0 ml portions of the homogenate were centrifuged at 2,800 rpm (1,000 g_{av}) for 15 min. Supernatants were decanted, and the sediments were combined and resuspended in 12.5 ml of the sucrose-$CaCl_2$ medium in a glass tube provided with a loose fitting ball-ended plastic pestle. This produced the preparation P1.

STEP 2: A 4.0 ml portion of the P1 suspension was layered on 1 ml of 2.3 M sucrose in a lusteroid tube fitting the swing-out head RPS 40 (Hitachi), and three equivalent tubes were centrifuged at 25,000 rpm (51,000 g_{av}) for 10 min (Hitachi model 40P ultracentrifuge). This produced a tissue block compressed right above the boundary between the 0.32 and the 2.3 M sucrose layers. Generally, no pellet was observed at the bottom of the tube; aging the homogenate or of the P1 suspension, however, produced a small pellet, consisting mainly of shrunken or ruptured nuclei. The 0.32 M sucrose layer exclusive of the tissue block, which was only slightly hazy, was removed with a Pasteur pipette and with suction. Part of the tube above the surface of the tissue block was cut off with scissors, the tissue was taken out upside down, and its surface was washed thoroughly with 0.32 M sucrose to remove the adhering, dense sucrose. The tissue block was much like a pudding in shape and will be designated so below. One-third of the upper part of the pudding appeared light brown, and the remaining lower part cream-colored. The upper portion appeared pink with the incompletely perfused brain, and deep red with the unperfused brain. The light brown portion was cut off manually with two razor blades operated from both sides. Three equivalent portions were resuspended, unless otherwise specified, in 6.5 ml of 0.32 M sucrose–1.5 mM $CaCl_2$ by gentle hand homogenization in a Dounce-type plastic pestle homogenizer. This produced the P2 preparation. In the microscope, the light brown portion of the pudding was seen to consist mainly of nuclei of various sizes. It contained a definite number of myelin sheath fragments and broken capillaries. The cream-colored portion consisted almost exclusively of myelin fragments and some contaminating mitochondria (Janus green stain). Only a few nuclei were present. The cream-colored portion was discarded during the preparation.

STEP 3: (*a*) A 2.0 ml portion of the P2 suspension was layered on the top of the Ficoll density

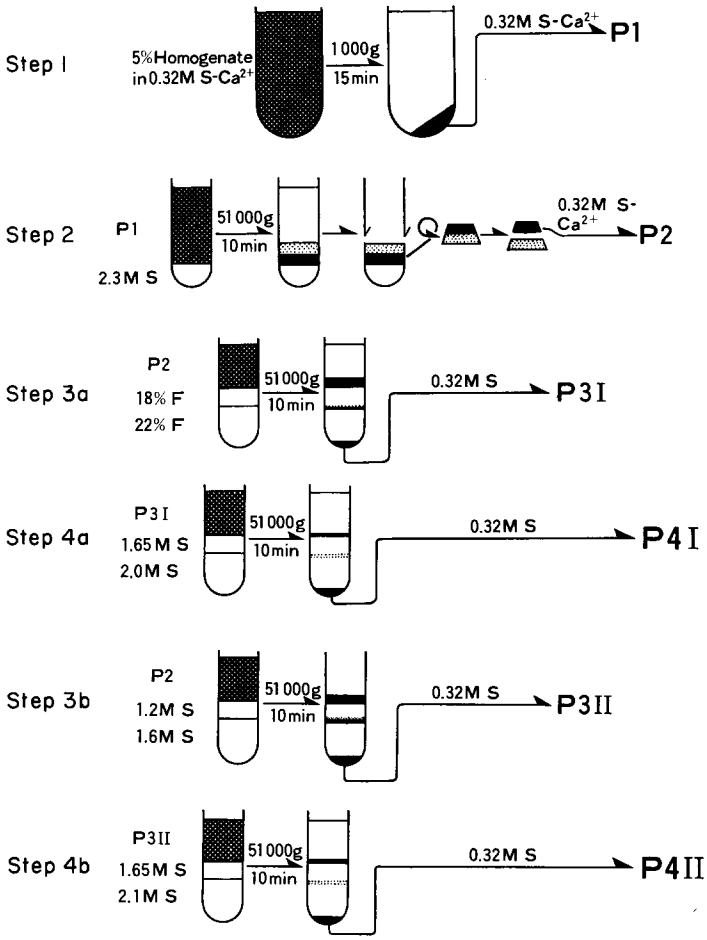


FIGURE 1 Schematic representation of the isolation procedures for cerebral nuclear preparations, P4I and P4II. P1, P2, P3I, and P3II are intermediate preparations. *S* denotes sucrose solution, and *F* Ficoll solution. The 0.32 M *S*-Ca^{2+} denotes 0.32 M sucrose containing 1.5 mM $CaCl_2$. Centrifugal forces are expressed as g_{av}; values calculated to the centres of the tubes.

gradient of 2 ml of 22% and 1 ml of 18% (w/v) Ficoll dissolved in 0.32 M sucrose. Three equivalent tubes were centrifuged at 25,000 rpm for 10 min in the RPS 40 swing-out head. This produced the following three layers: a thick, cream-colored band near the boundary between the 0.32 M sucrose and the 18% Ficoll layers, consisting mainly of myelin sheath fragments; a thin gray-white band at the interface between the 18% and 22% Ficoll layers, consisting of myelin sheath fragments, broken capillaries, and some small nuclei; a white pellet at the bottom of the tube, consisting mainly of nuclei and also some broken capillaries and a small number of myelin sheath fragments. The upper layers were removed by decantation, and three pellets were combined and resuspended in 6.5 ml of 0.32 M sucrose (P3I preparation).

(*b*) As an alternative to the Ficoll gradient, a discontinuous density gradient of 2 ml of 1.6 M sucrose and 1 ml of 1.2 M sucrose was used; this produced the P3II preparation, which was suspended, unless otherwise indicated, in 6.5 ml of 0.32 M sucrose.

STEP 4: (*a*) A 2.0 ml portion of P3I preparation was layered on the top of the discontinuous density gradient consisting of 2 ml of 2.0 M sucrose and 1 ml of 1.65 M sucrose in a tube fitting the RPS 40 head, and three equivalent tubes were centrifuged at 25,000 rpm for 10 min. A white band at the interface between the 0.32 and the 1.65 M sucrose layers consisted almost entirely of myelin components (Fig. 3). In a white band between the 1.65 and the 2.0 M sucrose layers were found concentrated broken capillaries (Fig. 4). The white pellet at the bottom of the tube consisted of pure nuclei virtually free of myelin

components and capillaries. Pellets from three tubes were combined and resuspended in an appropriate volume of 0.32 M sucrose. This produced the P4I preparation (Fig. 7), usually 3.0 ml.

(*b*) With the P3II preparation, the 2.1 M/1.65 M sucrose density gradient was used in order to ensure a satisfactory removal of blood capillaries. This produced the P4II preparation. Still, the removal of broken capillaries in the P4II preparation was not so complete as in the P4I preparation. Approximately 150–160 min were required to obtain the P4I or the P4II preparations, from the administration of anesthesia to the first animal.

Other Steps for Nuclear Fractionation (Fig. 2)

In order to obtain nuclear fractions rich in either the large nuclei (9–18 μ in diameter, in samples stained with aceto-orcein-fast green), or the small nuclei (6–9 μ in diameter), the following preparations were made.

A. PREPARATION RICH IN THE LARGE NUCLEI: A 1.0 ml portion of the P3II preparation, suspended in 3.5 ml of 0.32 M sucrose, was layered on the top of a discontinuous density gradient consisting of 2 ml of 2.4, 1 ml of 2.3 and 0.5 ml each of 2.0 and 1.65 M sucrose in a tube fitting the RPS40 head. Three equivalent tubes were centrifuged at 35,000 rpm (100,000 g_{av}) for 15 min. A white band at the interface between the 2.3 and the 2.4 M sucrose layers was collected using a tube slicer. Three equivalent bands were combined and diluted to 5 ml with 0.32 M sucrose. Dense sucrose was removed by centrifuging the samples at 15,000 rpm (18,400 g_{av}) for 5 min, and resuspending the pellets in 1.0 ml of 0.32 M sucrose. This produced the P_L preparation (Fig. 8).

B. PREPARATION RICH IN THE SMALL NUCLEI: A 4.0 ml portion of the P2 preparation, suspended in 4.5 ml of 0.32 M sucrose–1.5 mM $CaCl_2$, was layered on 1 ml of 2.3 M sucrose in a RPS40 tube, and centrifuged at 25,000 rpm for 10 min. This produced the second pudding which had a pink head portion and which was slightly more fragile than the first pudding. The pink portion of the second pudding was cut off, as described in Step 2. It was dispersed throughout 0.4 ml of 0.32 M sucrose with a plastic rod, was made up to 9 ml with 2.6 M sucrose, and was mixed thoroughly with the rod, special care being taken to minimize foaming. A 3 ml portion of this dense suspension was placed in a RPS40 tube, and three equivalent tubes were balanced with appropriate volumes of 0.32 M sucrose. These were centrifuged at 35,000 rpm for 15 min. After decantation, three pellets were washed thoroughly with 0.32 M sucrose, drained, and resuspended in an appropriate volume of 0.32 M sucrose (P_S preparation, generally 1.0 ml; Figs. 9 and 10).

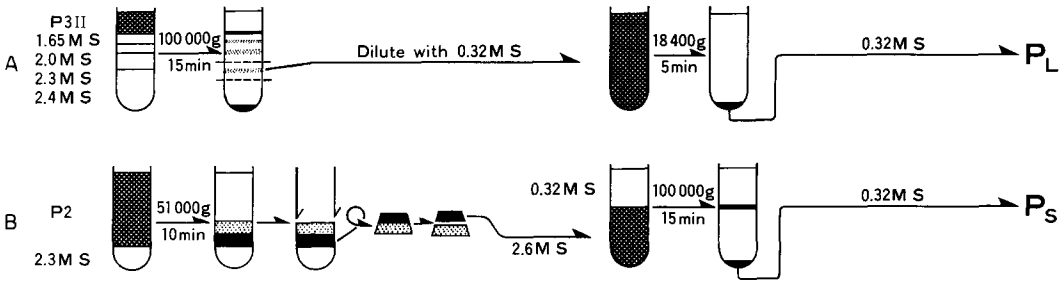


FIGURE 2 Differential isolation of large (P_L) and small (P_S) nuclear preparations. Abbreviations as in Fig. 1.

Microscopy

Each nuclear preparation stained with aceto-orcein–fast green, and occasionally with methyl green-pyronine, was examined in a Nikon SUR-Ke microscope. Phase contrast microscopy was carried out on samples dispersed in 0.32 M sucrose. Nuclei of neuronal, glial, or endothelial origins were differentiated in samples stained with aceto-orcein–fast green; criteria for this differentiation are detailed in Results and Discussion.

Electron Microscopy

Pellets of nuclear preparations were fixed for 2–3 hr at 0–4° with 0.33 M formaldehyde dissolved in 0.05 M Na-phosphate buffer, pH 7.6, or with 0.24 M glutaraldehyde dissolved in the same buffer (see reference 17). These were fixed further at 0–4° for 1 hr with 1% (w/v) OsO_4 dissolved in the same buffer. Tissues were dehydrated with several changes of acetone solutions of increasing concentration, and imbedded in Epon. Ultrathin sections were stained with uranyl acetate and examined with an HS-7 microscope (Hitachi) or with an HU-11B microscope (Hitachi).

Chemical Determinations

Protein was determined according to the method of Lowry et al. (16), after the prior removal of lipid materials with ethanol-ether (11:9, v/v). Bovine serum albumin was used as standard.

Nucleic acids were separated from the tissue according to the method of Schmidt and Thannhauser (21), as modified by Hutchison and Munro (9). In case of brain homogenate and crude nuclear preparations, DNA-containing precipitate was not completely dissolved in 0.1 N NaOH, and a small residue remained even after three repeated washings with 0.1 N NaOH. The content of DNA was determined in the DNA fraction dissolved in 0.1 N NaOH, together with three combined washings, using the indole reaction (1). DNA also was determined by fluorometry (13). Calf thymus DNA (Sigma, Type I) was used as standard. RNA was determined spectrophotometrically at 260 mμ, and also by the orcinol reaction (2), after the 0.3 N KOH-extracted samples were acidified by adding perchloric acid to a final concentration of 6%. Yeast RNA (Sigma, Type XI) was used as standard.

Succinate dehydrogenase (EC 1.3.99.1) was determined spectrophotometrically as previously described (15), and the activity was expressed in terms of ΔE_{550}/min/g of original tissue at 24°C.

Glutamate dehydrogenase (EC 1.4.1.2) was determined spectrophotometrically as described by Hogeboom and Schneider (6). In order to obtain constant activity, the tissue suspension was diluted with water, and treated further with 0.1% (v/v) Triton X-100 at 0–4° for 30–60 min. The final concentration of Triton X-100 in the assay mixture was less than 0.03%, which did not interfere with the assay. The dehydrogenase activity was expressed in terms of ΔE_{340}/min/g of original tissue at 24°C.

Acetylcholinesterase and cholinesterase (EC 3.1.1.7 and EC 3.1.1.8) were determined according to the method of Ellman et al. (3), with minor modifications given below. Reaction mixtures (3.0 ml) containing (final concentrations) Na_2HPO_4-KH_2PO_4 buffer, pH 8.0 (47 mM), 5,5′-dithiobis[2-nitrobenzoic acid] (DTNB; 0.33 mM) and the tissue suspension were shaken at 37.5° for 10 min, and the reaction was initiated by the addition of AthCh (0.1 ml) or ButhCh (0.1 ml) to give a final concentration of 0.5 or 1.0 mM, respectively. Shaking continued for another 10 min and the reaction was stopped by the addition of an excessive amount of physostigmine sulphate (1.0 ml; 0.1 mM). The tissue was spun down, and the extinction of yellow product was read at 412 mμ in samples from the supernatants. Nonspecific destruction of DTNB by the tissue suspension was controlled by a blank which did not contain AthCh or ButhCh. AthCh-hydrolyzing activity or ButhCh-hydrolyzing activity of the tissue was inhibited by 80% on addition of 0.1 mM or 1 μM DFP, respectively (11). The activity that remained after the addition of 0.1 mM DFP was no longer inhibited by 0.1 mM physostigmine sulfate, and was taken to represent the activity of nonspecific thioesterases. AChE plus ChE activity was expressed in terms of E_{412} [AthCh minus AthCh-0.1 mM DFP]/min/g of original tissue (37.5° C), and ChE activity in terms of E_{412} [ButhCh minus ButhCh-1 μM DFP]/min/g of original tissue (37.5°C).

Adenosine triphosphatases were determined as described by Kurokawa et al. (15). Combinations of univalent and bivalent cations used are given in the legend of Table V. The enzyme activity refers to μmoles of orthophosphate liberated/15 min/g of original tissue at 37.5° C.

ATP:NMN adenylyltransferase (EC 2.7.7.1) was determined as described by Kornberg (14), with the following modifications. Reaction mixture (1.0 ml) contained (final concentrations) glycylglycine buffer, pH 7.4 (50 mM), NMN (2.5 mM), ATP (5 mM), $MgCl_2$ (15 mM), nicotinamide (200 mM) and appropriate amounts of tissue suspension. In the case of homogenate, NaF (10 mM) was added in order to secure the linearity of the reaction. Reaction tubes were incubated for 20 min at 37.5°C, and 0.2 ml of ice-cold 30% (w/v) trichloroacetic acid was then added. After the precipitated protein was spun down, the pH of a 1.0 ml portion of the supernatant was adjusted to 8 by 1 N NaOH with bromthymol blue as an internal indicator, and the amount of NAD^+ was determined under conditions specified by Kornberg (14). The enzyme activity was expressed in terms of μmoles NAD^+ formed/20 min/g of original tissue, mg of protein, or mg of DNA (37.5°C). There was a linear relationship between the enzyme activity and the tissue concentration within the range of 1–6 mg of protein in the case of homogenate, and of 0.3–0.9 mg of protein in the case of nuclear preparations.

RESULTS AND DISCUSSION

Morphology of Isolated Nuclei

In the phase contrast microscope, the large majority of isolated nuclei appeared either round or elliptical, and differed markedly in size. Larger nuclei generally had one well-shaped nucleolus which sharply contrasted with the light nucleoplasmic area. In smaller nuclei, the nucleoplasm appeared darker. The nucleolus-like granules were contrasted less clearly, and were distributed over a wide range of nucleoplasmic area. The frequency of damaged nuclei was relatively small; less than 5% of the nuclei showed partially ruptured nuclear contour and herniation of the nucleoplasm.

With phase contrast microscopy as well as

microscopy of nuclear samples stained with aceto-orcein-fast green, a sort of "segmentation" of nucleoli was observed in some of the large nuclei (see Fig. 8). It was a common observation that nucleoli which apparently were segmented at one focus level were united roundly at another focus level. In some nucleoli, however, the segmentation actually seemed to have occurred. Whether this sort of segmentation of nucleolus represents an artefactual change from fractionation procedure, will be questioned.

Electron microscopy of the P4I preparation and the P4II preparation showed that contamination by blood cells, cell debris and mitochondria was virtually absent. Elements of the endoplasmic reticulum, however, were encountered occasionally. The majority of the sedimented nuclei showed the well-preserved nuclear membranes, in some instances with evident nuclear pores. These findings contrast those observed in nuclei isolated in sucrose–detergent media, which reportedly showed the removal or fragmentation of outer membrane and the disappearance of nuclear pores (8, 10, 25). Nuclei isolated by the present method generally had a finely textured nucleoplasm and, in typical cases, a well-defined nucleolus, nucleolus-associated chromatin, and ribosome-like, granular structures (Figs. 11 and 12). In some nuclei, however, portions of nucleoplasmic material tended to fade to varying extents.

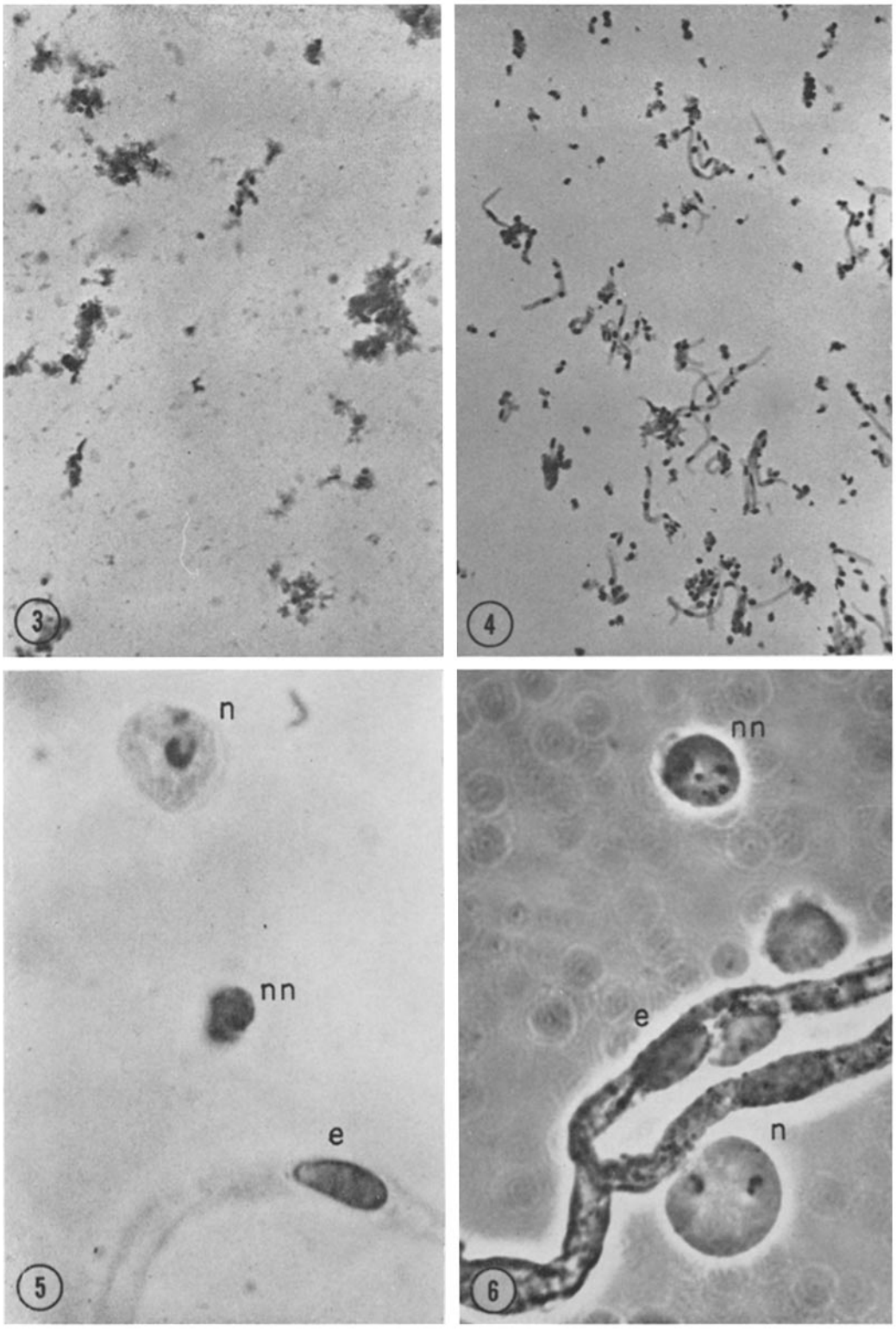
An attempt to differentiate the origin of isolated nuclei was made with preparations stained by aceto-orcein–fast green. Generally, it is dangerous to presume the origin of nuclei in isolated preparations. Particular difficulties were encountered in the differentiation of the astroglial nucleus and the neuronal nucleus of a smaller size, when the latter failed to show a well-defined nucleolus, although the staining of nucleoplasm apparently tended to be denser in the astroglial than in the neuronal nucleus. The identification of microglial nuclei was next to impossible. In our tentative attempt, the large nucleus, usually having one distinct nucleolus and showing light areas of nucleoplasm, was taken to be of neuronal origin; the smaller nucleus, showing several nucleolus-like granules over the nucleoplasmic area or in some cases without these granules, and showing denser nucleoplasm, was taken as oligodendroglial (see reference 4). Isolated nuclei were grouped into two classes, *neuronal* and *nonneuronal*. The *neuronal* consisted solely of neuronal nuclei differentiated as described above. The *nonneuronal* consisted (*a*) mainly of oligodendroglial and astroglial nuclei as differentiated on the above basis, (*b*) of nuclei that were not properly identified, and possibly of either neuronal or astroglial origin, and (*c*) of a negligible number of endothelial nuclei (Figs. 5 and 6; less than 0.5% of the total nuclei in the P4I preparation, and 2–3% in the P4II preparation). The population of neuronal nuclei ranged from 55 to 65% in the P4I preparation (Fig. 7) and from 47 to 53% in the P4II preparation. In the preparation P_L (Fig. 8), the population of neuronal nuclei ranged from 72 to 83%. In the pellet, which was obtained from placing the P3II preparation on the sucrose gradient (Fig. 2 *A*), the population of neuronal nuclei was consistently lower than that in the preparation P_L, smaller (possibly oligodendroglial) nuclei being increased in number. In contrast, 94–99% of the nuclei in the P_S preparation (Figs. 9 and 10) consisted of nonneuronal nuclei, and the large majority of these were most likely of oligodendroglial origin.

Nurnberger (19) has calculated that the nuclei in the rat cerebral cortex number 9.6×10^7 nuclei/g of fresh tissue and that the neuronal nuclei number 2.3×10^7 nuclei/g of fresh tissue, i.e., 24% of the total. When there is no serious

FIGURE 3 Micrograph of myelin components recovered at the interface between the 0.32 M and the 1.65 M sucrose layers in Step 4 a, Fig. 1. The sample was stained with aceto-orcein–fast green. Magnification, × 250.

FIGURE 4 Micrograph of the fragmented blood capillaries and some smaller nuclei recovered at the interface between the 1.65 and the 2.0 M sucrose layers in Step 4 a, Fig. 1. Stained with aceto-orcein–fast green. Magnification, × 250.

FIGURES 5 and 6 Appearances of neuronal (*n*), non-neuronal (*nn*), and endothelial (*e*) nuclei of the sample stained with aceto-orcein–fast green in the microscope (Fig. 5) and in the phase contrast microscope (Fig. 6). P3I preparation. Magnification, × 1,500.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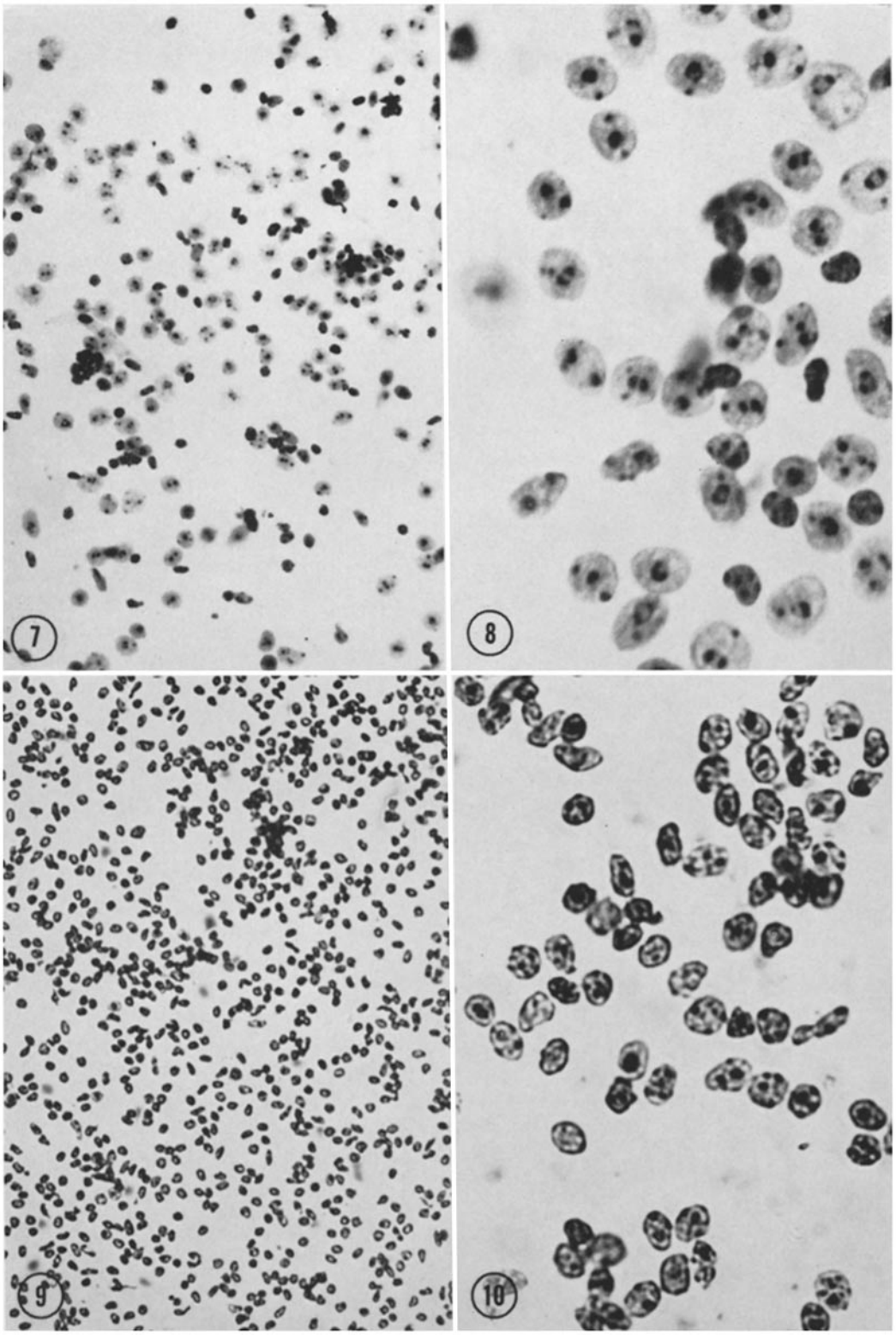
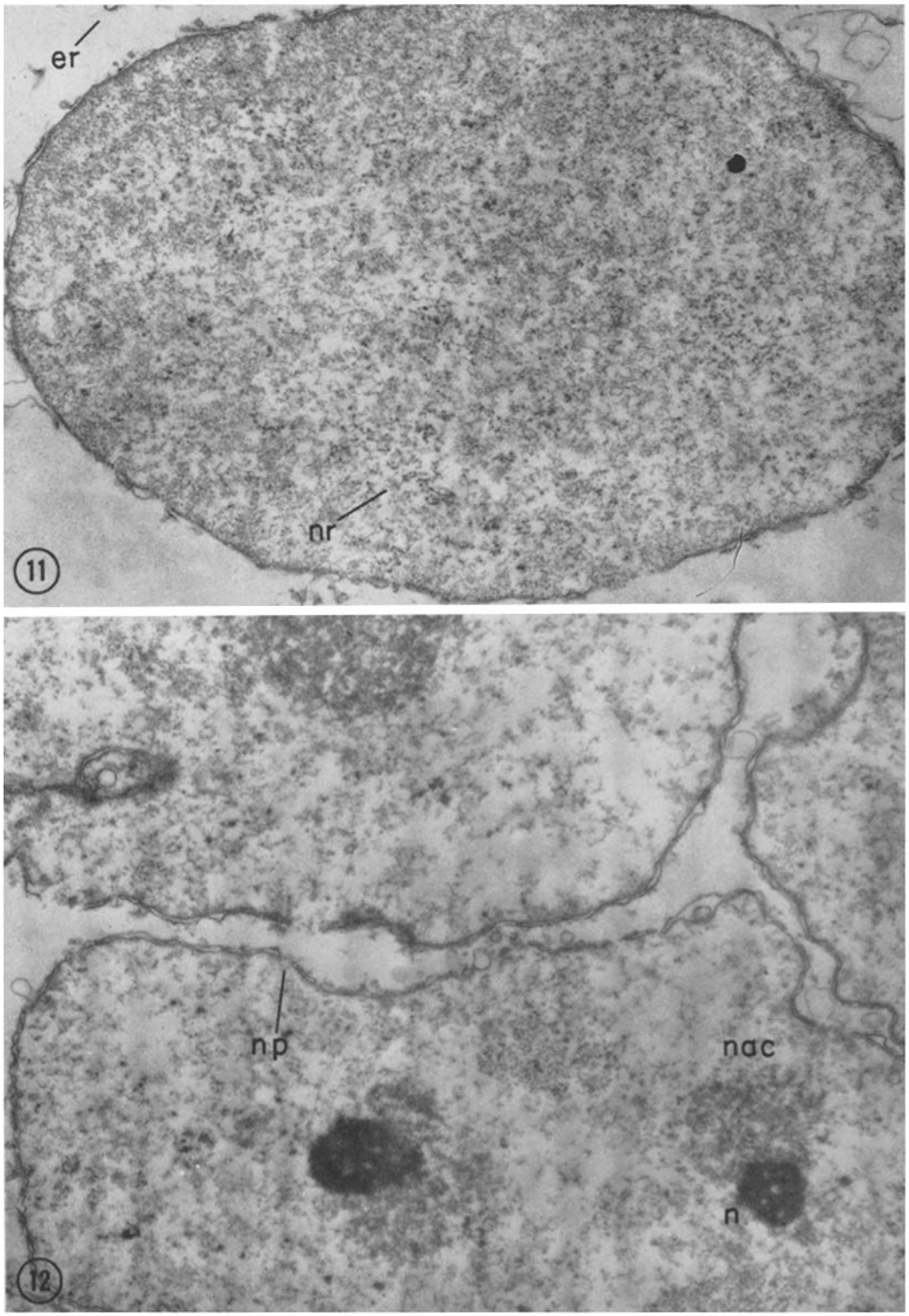


FIGURES 7–10 Micrographs of the P4I (Fig. 7), P_L (Fig. 8) and P_S (Figs. 9 and 10), each stained with aceto-orcein-fast green. For denotation of these cerebral nuclear preparations, see Figs. 1 and 2 and the text. Magnification, Figs. 7 and 9, × 250; Figs. 8 and 10, × 1,000.



FIGURES 11 and 12 Electron micrographs of isolated nuclei (P4II preparation), including nucleolus (*n*), nucleolus-associated chromatin (*nac*), nuclear pores (*np*) and nuclear ribosomes (*nr*). Occasional elements of endoplasmic reticulum (*er*) are also present. Magnification, Fig. 11, × 15,000; Fig. 12, × 12,000.

species difference in the ratios of cell types in the rat and the guinea pig, the greater number of smaller nuclei were lost during the ordinary isolation procedure (Fig. 1). This is indicated by the increase of the large (neuronal) nuclei in the preparations P4I and P4II.

Results in Preliminary Experiments

Red blood cells were found to sediment along with the cerebral nuclei, and attempts to remove them from the nuclear fraction by means of differential or density gradient centrifugation were unsuccessful. In this respect, the average density of nuclei from the cerebral cortex seems to be lower than that of hepatic nuclei (see references 17 and 18), but mention should be made of the fractionation method when the absolute density of the nucleus is discussed (see below). If the brain was not perfused in situ prior to the preparation, the upper portion of the first and second puddings turned deep red. A thin layer of erythrocytes which formed on the surface of the pudding was peeled off during the repeated washing of the surface with sucrose, but this did not remove the erythrocytes from the final nuclear fraction, the majority of which mingled with nuclei in the head portion of the pudding.

The large number of myelin sheath fragments in the crude nuclear preparation (P1) barred the free sedimentation of nuclei in the subsequent density gradient centrifugation. Simple differential centrifugation combined with repeated washing did not remove the myelin components satisfactorily. Moreover, this caused a more frequent rupture of nuclei, much loss of soluble components, and a change in sedimentation properties of the nuclei (see below). Triton X-100, although useful for the elimination of erythrocytes, failed to remove myelin fragments from the nuclear fraction when the myelinated adult brain was used (20). Hadjiolov et al. (5) employed a centrifuge tube with a flat bottom and a large surface (78.5 cm^2) to isolate nuclei; this reduced the barrier action of the dense myelin band at the interface of the sucrose gradient. In the present "pudding" method, the great majority of myelin fragments were removed while the nuclei were still under the isotonic condition.

In order to remove mechanically the broken capillaries from preparation P3I or preparation P3II, the suspension was strained through a mem-

TABLE I

Contents of Nucleic Acids and Protein in Various Nuclear Preparations
For the denotation of each preparation, see Fig. 1 and the text

	DNA		RNA		Protein
	a	*b*	*a*	*b*	
Homogenate	984 ± 15 (6)	841 (3)	1862 ± 70 (6)	2353 ± 124 (6)	87.8 ± 3.2 (6)
P4I	293 ± 38 (6)	247 (3)	106 ± 13 (6)	124 ± 25 (6)	0.85 ± 0.14 (6)
Recovery, %	29.8	29.4	5.7	5.3	0.97
Homogenate	918 (3)	—	—	—	82.9 (3)
P4II	387 (3)	—	—	—	1.09 (3)
Recovery, %	42.1	—	—	—	1.32
Homogenate	894 (3)	—	—	—	79.9 (3)
P_L	66 (3)	—	—	—	0.17 (3)
Recovery, %	7.5	—	—	—	0.21
Homogenate	920 (3)	—	—	—	84.0 (3)
P_S	66 (3)	—	—	—	0.09 (3)
Recovery, %	7.2	—	—	—	0.11

Values are means ± SD of the numbers of experiments given in parentheses.
DNA, μg/g of fresh tissue. (*a*) fluorometry. (*b*) indole reaction.
RNA, μg/g of fresh tissue. (*a*) UV absorption. (*b*) orcinol reaction.
Protein, mg/g of fresh tissue.

brane filter (pore size, 10–20 μ), nylon or silk bolting cloth, in which the openings ranged from 50 to 200 μ, and a bed (2–20 mm in thickness) of glass beads (50–200 μ in diameter). Some of these procedures were found effective in removing the capillaries. Low reproducibility and a considerable rupture of nuclei, however, made them inappropriate for routine use.

It was noted that the sedimentation properties of the pure nuclei changed depending upon various conditions under which the crude nuclear preparation had been treated. These included the kind, concentration, ionic strength, and pH of the suspending media, and also the intensity and duration of the homogenization and fractionation procedures. Generally, intense grinding, aging, or repeated washing of the tissue caused a greater density equal to or greater than that of nuclei suspended in 2.2 M sucrose. All of these observations indicate the importance of careful consideration of the fractionation conditions when discussing the 'density' of the nucleus; this will be relevant to the contradictory data reported in the literature concerning the density of brain cell nuclei (see reference 5).

Nucleic Acid and Protein Contents

The amounts of DNA, RNA, and protein found in the present nuclear preparations are given in Table I. Approximately 30% of DNA originally present in the filtered homogenate is recovered in the P4I preparation; recovery of DNA in the P4II is consistently higher than that in the P4I, the value being about 42%. These recoveries are

TABLE II

Nucleic Acid and Protein Contents in Nuclear Preparations at Various Stages of Purification

DNA was determined by fluorometry, and RNA by the orcinol reaction. Means ± SD of 6 experiments are given. For denotation of preparations, see Fig. 1 and the text. Values of RNA and protein in the original homogenate are given in Table I.

Preparations	DNA		RNA	Protein	RNA/DNA	Protein/DNA
	Fresh tissue					
	$\mu g/g$	%	%	%		
Homogenate	984 ± 15	100	100	100	2.39	89.2
P1	707 ± 50	72.0	15.7	19.9	0.52	24.6
P2	538 ± 104	54.7	10.8	6.9	0.47	11.3
P3I	351 ± 44	36.2	6.9	1.6	0.46	4.0
P4I	293 ± 38	29.8	5.3	0.97	0.42	2.9

amount of tissue to be sedimented through the suspending medium of the same concentration. When the pH of the P1 suspension was lowered to 4.8 with 10 mM citric acid, the fewer nuclei passed through the 20% (w/v) Ficoll layer; in contrast, raising the pH of the P1 suspension to 7.4 with 50 mM tris-HCl buffer caused the nuclei to pass through even the 26% (w/v) Ficoll layer. In the microscope, shifting of the pH to either side caused a distinct shrinkage or clumping of nuclei. Ca^{2+} could be replaced by Mg^{2+} without apparent changes in morphology of nuclei. Perfusion of the brain with isotonic NaCl or KCl caused a shrinkage of nuclei. Hadjiolov et al. (5) have reported that the value for the density of nuclei in a nuclear suspension in 0.34 M sucrose was lower than that of nuclei in a nuclear suspension in 1.8 M sucrose, while nuclei suspended in 1.0 M sucrose had a much higher than those in the cerebral nuclei isolated by Sporn et al. (23), and comparable with those reported in hepatic nuclei (17). Recoveries of DNA in the P_L and P_S are much less than in the P4I and P4II, the values being 8 and 7%, respectively.

In Table II are shown the recoveries of DNA, RNA, and protein at four successive steps of purification. The relatively large variation in the amount of DNA per gram of fresh tissue in the P2 fraction may reflect the minute difference between the methods used to cut the head portions of the puddings. This variation, however, is overcome during the subsequent procedure involving density gradient centrifugation, as is shown by relatively small standard deviations in the amount of DNA observed in the P3I and P4I fractions. The RNA/DNA ratio in the P4I preparation

TABLE III

Succinate Dehydrogenase (SDH) and Glutamate Dehydrogenase (GDH) Activities of Nuclear Preparations at Various Stages of Purification

	Preparations	
	SDH unit	GDH unit
Homogenate	13.8 ± 0.5 (4)	0.60 ± 0.07 (4)
P1	2.0	—
P2	0.8	—
P3I	0	—
P4I	0	0

Values are means ± SD of the numbers of experiments given in parentheses. Unit refers to ΔE_{550}/min/g of original tissue (24°) for SDH, and to ΔE_{340}/min/g of original tissue (24°) for GDH.

TABLE IV

Acetylcholinesterase (AChE) and Cholinesterase (ChE) Activities of Nuclear Preparations at Various Stages of Purification

	AChE + ChE unit	ChE unit
Homogenate	33.03 (2)	3.60 (2)
P1	6.59	1.22
P2	1.37	0.16
P3I	0.14	0.03
P4I	0.02	0.001

Mean values are given, with the number of experiments in parentheses. Unit refers to ΔE_{412}/min/g of fresh tissue at 37.5°.

agrees with that reported by Hadjiolov et al. (5), despite differences in isolation procedures.

Cytoplasmic Enzyme Activities

Table III shows the activities of succinate dehydrogenase and glutamate dehydrogenase in the original brain homogenate and in various nuclear preparations. The absence of estimated activities of these enzymes in the purified nuclear preparations is in accord with microscopic observation showing the nearly complete removal of mitochondria.

The activities, on a wet weight basis, of acetylcholinesterase and cholinesterase in the purified nuclear preparation (P4I) were found to be less than 0.1% of the value exhibited by the filtered homogenate (Table IV). Also, the activity of the sodium-plus-potassium–stimulated adenosinetriphosphatase system was virtually absent in the P4I preparation (Table V). These observations were taken to indicate that the present nuclear preparations were essentially free of contaminants from membrane fragments of microsomal nature and of nerve ending particles; the distribution of activities of acetylcholinesterase and of the adenosinetriphosphatase system in brain microsomes and in nerve ending particles has been demonstrated (7, 15, 22, 24).

The RNA amount in purified nuclear preparation represents the net results of two processes, the removal of extranuclear RNA and the leakage of intranuclear RNA. The activities of acetylcholinesterase and cholinesterase (Table IV) and of the sodium-plus-potassium–stimulated adenosinetri-

TABLE V

Activities of Adenosine Triphosphatases in Various Nuclear Preparations

Media	*a*	*b*	*c*	*d*	*e*	*f*	*g*	*h*	(*g*) − (*e*)
Homogenate	120.1	295.5	268.5	171.0	360.0	437.5	590.0	378.5	290.0
P1	27.4	63.4	51.1	34.9	70.5	97.5	132.0	81.5	61.5
P2	6.9	13.7	13.4	11.0	18.7	24.1	37.5	22.0	18.8
P3I	1.60	3.27	2.62	1.42	3.20	3.45	4.45	3.02	1.25
P4I	1.17	1.53	1.26	1.00	1.50	1.48	1.56	1.37	0.06

Unit of activity refers to μmoles Pi liberated/15 min/g of fresh tissue at 37.5°C. Mean values of two experiments are given. Ionic compositions of reaction mixtures are (*a*) None; (*b*) 6 mM $MgCl_2$; (*c*) 6 mM $CaCl_2$; (*d*) 100 mM NaCl; (*e*) 6 mM $MgCl_2$–20 mM KCl; (*f*) 6 mM $MgCl_2$–100 mM NaCl; (*g*) 6 mM $MgCl_2$–20 mM KCl–100 mM NaCl; and (*h*) (*g*) plus 100 μM ouabain. Each medium was buffered with 30 mM Tris-HCl at pH 7.4. Values of (*g*) − (*e*) represent the activity of the sodium-plus-potassium–stimulated adenosine triphosphatase system.

TABLE VI

ATP:NMN Adenylyltransferase Activity in Cerebral Nuclear Preparation

	ATP:NMN adenylytransferase activity		
	Fresh tissue	Protein	DNA
	unit/g	*unit/mg*	*unit/mg*
Homogenate	792 ± 54	9 ± 1	584 ± 40
P4I	181 ± 13	213 ± 15	615 ± 42

Unit refers to mμmoles NAD^+ formed/20 min at 37.5°C. Mean values ± SD of four experiments are given.

phosphatase system (Table V) are decreased to one thousandth during the purification steps from P1 to P4I, while the decrease in the RNA amount is only one-third, and the RNA/DNA ratio is decreased by only 20% (Table II). It thus appears that the RNA/DNA ratio, though often used as a criterion of purity of isolated nuclei (5, 23), is not necessarily an appropriate index to the extent of cytoplasmic contamination in the purified nuclear preparations.

Nuclear Enzyme Activity

The present nuclear preparation retained the activity of ATP:NMN adenylyltransferase (Table VI), indicating no serious loss of activity in this nuclear enzyme during the preparation. In preliminary experiments, it has been shown that the DNA-dependent RNA-polymerase system is also active in the P4I and P4II preparations.

After the present paper had been submitted for publication, a relevant article came to our attention. Løvtrup-Rein and McEwen (26) have succeeded in the fractionation of neuronal, astrocytic, and glial nuclei from adult rat brain, with the yield amounting to 28–35% of the DNA of the original homogenate. Their "nuclear suspension" contained some cytoplasmic debris, myelin fragments, and capillaries which were recovered in band 1 and band 2 separated in the subsequent density gradient centrifugation. The absence of data concerning the cytoplasmic enzyme activities in the paper of Løvtrup-Rein and McEwen (26) makes impossible a comparison of purity between their nuclear preparation and ours. The RNA/DNA ratio in their nuclear suspension is of the same order as that obtained by Hadjiolov et al. (5) from cat brain cortex and that obtained by us (Table II) from guinea pig cerebral cortex. In our results, neuronal nuclei appear lighter than 2.6 M sucrose, but in the sample prepared by Løvtrup-Rein and McEwen (26), neuronal nuclei are slightly heavier than 2.6 M sucrose. This discrepancy may be explained by the observation of Hadjiolov et al. (5) already cited in the last paragraph of Results in Preliminary Experiments in the text.

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REFERENCES

1. CERIOTTI, G. 1952. *J. Biol. Chem.* **198**:297.
2. CERIOTTI, G. 1955. *J. Biol. Chem.* **214**:59.
3. ELLMAN, G. L., K. D. COURTNEY, V. ANDRES, and R. M. FEATHERSTONE. 1961. *Biochem. Pharmacol.* **7**:88.
4. GLEES, P. 1955. Neuroglia. Morphology and Function. Blackwell Scientific Publications Ltd., Oxford.
5. HADJIOLOV, A. A., Z. S. TENCHEVA, and A. G. BOJADJIEVA-MIKHAILOVA. 1965. *J. Cell Biol.* **26**:383.
6. HOGEBOOM, G. H., and W. C. SCHNEIDER. 1953. *J. Biol. Chem.* **204**:233.
7. HOSIE, R. J. A. 1965. *Biochem. J.* **96**:404.
8. HUBERT, M.-T., P. FAVARD, N. CARASSO, R ROZENCWAJG, et J.-P. ZALTA. 1962. *J Microscopie.* **1**:435.
9. HUTCHISON, W. C., and H. N. MUNRO. 1961. *Analyst.* **86**:768.
10. HYMER, W. C., and E. L. KUFF, 1964. *J. Histochem. Cytochem.* **12**:359.
11. KATO, T., K. HIRAYAMA, and M. KUROKAWA. 1965. *Recent Advan. Res. Nervous System.* **9**:529.
12. KATO, T., and M. KUROKAWA. 1966. *Proc. Japan Acad.* **42**:527.
13. KISSANE, J. M., and E. ROBINS. 1958. *J. Biol. Chem.* **233**:184.
14. KORNBERG, A. 1950. *J. Biol. Chem.* **182**:779.

15. KUROKAWA, M., T. SAKAMOTO, and M. KATO. 1965. *Biochem. J.* **97**:833.
16. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. *J. Biol. Chem.* **193**: 265.
17. MAGGIO, R., P. SIEKEVITZ, and G. E. PALADE, 1963. *J. Cell Biol.* **18**:267.
18. MANDEL, P., T. BROKOWSKI, S. HARTH, and R. MANDEL. 1961. *J. Neurochem.* **8**:126.
19. NURNBERGER, J. I. 1958. *In* Biology of Neuroglia. W. F. Windle, editor. Charles C Thomas, Publisher, Springfield, Illinois. 193.
20. RAPPOPORT, D. A., R. R. FRITZ, and A. MORACZEWSKI. 1963. *Biochim. Biophys. Acta.* **74**: 42.
21. SCHMIDT, G., and S. J. THANNHAUSER. 1945. *J. Biol. Chem.* **161**:83.
22. SCHWARTZ, A., H. S. BACHELARD, and H. MCILWAIN. 1962. *Biochem. J.* **84**:626.
23. SPORN, M. B., T. WANKO, and W. DINGMAN. 1962. *J. Cell Biol.* **15**:109.
24. TOSCHI, G. 1959. *Exptl. Cell Res.* **16**:232.
25. ZALTA, J. P., R. ROZENCWAJG, N. CARASSO, et P. FAVARD. 1962. *Compt. Rend.* **255**:412.
26. LØVTRUP-REIN, H., and B. S. MCEWEN. 1966. *J. Cell Biol.* **30**:405.