# ISOLATION AND SOME CHARACTERISTICS OF CELL NUCLEI FROM BRAIN CORTEX OF ADULT CAT

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## ABSTRACT

A rapid detergent method for the isolation of nuclei from cat brain cortex is described. It involves the homogenization of the tissue in buffered 0.34 M sucrose with the addition of the non-ionic detergent Cemulsol NPT 12 and the subsequent low speed centrifugal sieving of the nuclei through two layers of sucrose (0.68 M and 1.0 M). The final purification is achieved by high speed centrifugation (40,000 *g*) of the nuclear suspension layered over 1.8 M sucrose. Observations by light microscopy indicate that highly purified and well preserved nuclei are obtained from neurons and glial cells. Electron microscopy reveals some microsomal contaminants adhering to the nuclear membrane. According to an analysis of the nuclear size distribution, a considerable loss of smaller nuclei (10 to 20$\mu^2$), mainly from glial cells, occurs during the purification procedure. The action of different detergents is compared, the best results being obtained with Cemulsol NPT 12 or Triton X-100. Chemical analyses of the purified nuclear fraction give the following content expressed in picograms per nucleus: DNA, 6.54; RNA, 2.94; cholesterol, 1.50; and protein, 97.5. The sucrose density gradient centrifugation of nuclei isolated from cat brain cortex shows that their density is equal to or higher than that of 2.2 M sucrose and is thus similar to the density of nuclei from other tissues. The observation of a varying influence of different suspending media on the density of brain cell nuclei resolves the conflicting data in the literature on the density of these nuclei.

## INTRODUCTION

In comparison with other cellular structures from nervous tissue, nuclei have been studied in the least detail (1). The classical cell fractionation techniques were applied to brain by Brody and Bain in 1952 (2), but little attention has been paid to the cytological characteristics of the nuclear fraction obtained by their method, and for this reason the chemical and biochemical pattern of this fraction (3) needs to be reevaluated. The main difficulties in the isolation of brain cell nuclei arise from the abundance of contaminating materials in the nuclear fraction, such as intact cells, myelin and capillary fragments, erythrocytes, nerve endings, mitochondria, microsomes, and other intracellular particles. A satisfactory elimination of these primarily lipoprotein structures is achieved by the method of Sporn *et al.* (4). This method, however, requires many repeated and prolonged

centrifugations and gives a rather low yield—factors limiting its application to metabolic studies. Because of the difficulties encountered in the isolation of cell nuclei from the brain of adult animals, some investigators (5) have worked with brain cell nuclei isolated from newborn animals, in which the extent of myelinization is low. Further, data in the literature concerning the density of brain cell nuclei are rather contradictory (4, 6–8), a situation which causes additional difficulties in the selection of appropriate hypertonic sucrose solutions for the isolation procedures.

In the last three years, different detergents have been successfully employed for the isolation of nuclei from liver and ascites tumor cells (9), HeLa cells (10), and nucleated erythrocytes (11). Hymer and Kuff (12) carried out a comparative study on the use of different detergents for the isolation of nuclei from liver, kidney, and various tumors. The abundance of lipoprotein structures in nerve tissue suggested the possible usefulness of detergents in techniques for the isolation of nuclei from this tissue.

In the present paper a procedure is described for the isolation of highly purified cell nuclei from the brain cortex of adult cats by the use of the non-ionic detergent Cemulsol NPT 12 combined with centrifugal sieving through hypertonic sucrose solutions. Results obtained during the development of this technique as well as some characteristics of the nuclear fraction are presented. A preliminary note about these results has been published (13).

## METHODS

The experiments were carried out with anesthesized adult cats of both sexes, sacrificed by bleeding. The brains were removed and used immediately. The final procedure for the preparation of nuclei was as follows.

### *Final Procedure*

After the removal of meninges, the brain cortex was rapidly but carefully dissected from white matter in the cold. All subsequent operations were performed at 4°C. The sucrose medium contained 0.005 M Tris-HCl, 0.002 M $MgCl_2$, and 0.001 M KCl, and the pH was adjusted, if necessary, to 6.5 (glass electrode). In order to minimize possible ribonuclease contamination in sucrose, the solutions were heated at 100°C for 5 minutes. The tissue was homogenized with 0.34 M sucrose in a Potter-Elvehjem glass homogenizer fitted with a Teflon pestle. The 10 per cent homogenate was filtered through 2 to 4 layers of cheesecloth, and a 2 per cent solution of Cemulsol NPT 12 was added to a final concentration of 0.1 per cent. The mixture was rehomogenized with 2 to 3 tractions of the pestle. The homogenate (10 to 15 ml) was layered carefully over two layers (30 to 40 ml each) of sucrose solutions (upper layer 0.68 M, and lower layer 1.0 M sucrose) in a centrifuge bottle with a flat bottom and large surface (78.5 $cm^2$) and centrifuged for 10 minutes at 1600 *g*. The sediment was resuspended by shaking it in 0.34 M sucrose and, after overlayering, centrifuged again under the same conditions. The yield at this stage of the purification procedure was 90 to 95 per cent according to DNA determinations. The nuclear fraction obtained was resuspended in 1.0 M sucrose, layered over an equal volume (10 to 15 ml) of 1.8 M sucrose, and centrifuged for 40 minutes at 40,000 *g* (average) in the preparative refrigerated ultracentrifuge MSE Superspeed 40. Nuclei of neurons and glial cells were obtained as a white pellet at the bottom of the centrifuge tube, with a yield ranging from 25 to 32 per cent. In contrast to this layering technique, direct suspension of the 1600 *g* nuclear fraction in 1.8 M sucrose results in a higher yield of nuclei with slightly lower purity.

### *Phase Contrast and Electron Microscopy*

The purity of the nuclear fraction obtained at different stages of the isolation procedure was controlled with the phase contrast microscope. The observations were performed on suspensions of nuclei in 0.34 M sucrose. The number of nuclei in a given suspension was determined by counting the unstained nuclei in a Burker chamber.

The electron microscope observations were carried out on samples of the nuclear fraction resuspended in 0.34 M sucrose and centrifuged down in small tubes (50 × 8 mm). The thin layer of nuclear material at the tube bottom was fixed *in situ* (at 4°), according to Maggio *et al.* (14), as follows: 7 per cent HCHO in 0.1 M phosphate buffer (pH, 7.2) for 3 hours and 1 per cent $OsO_4$ in the same buffer for 1 hour. After dehydration with ethanols and embedding in butyl: methyl methacrylate (4:1), sections were cut on the Citte ultramicrotome (Reichert). Observations were performed with the Tesla BS 242A electron microscope at 60 kv.

### *Determination of Nuclear Size*

The size of nuclei was determined on smears of the sediment fixed with 70 per cent ethanol and stained according to Giemsa-Romanovsky. In order to minimize tonicity effects of the suspension medium on nuclear size, a portion of the original (1600 *g*) nuclear fraction was kept under 1.8 M sucrose during the isolation of the more highly purified nuclei requiring also 1.8 M sucrose as described above. Nuclear

smears were photographed at a magnification of 400 times, the pictures were projected on a screen graduated with an object micrometer, and the two diameters of nuclei were determined. The surface of nuclei was calculated in square microns according to the formula for an elliptical surface.

### *Chemical Analyses*

Chemical analyses were made on suspensions of the nuclear fraction. Ribonucleic acid and deoxyribonucleic acid were determined by the two-wavelength method of Tsanev and Markov (15) as adapted for brain tissue by Santen and Agranoff (16). Cholesterol was determined by the method of Stadtman (17), and protein according to Lowry *et al.* (18) with a bovine serum albumin calibration curve.

### *Sucrose Density Gradient Centrifugation*

These analyses were performed by layering the suspension of nuclei over a discontinuous sucrose density gradient with sucrose concentrations ranging from 1.4 to 2.2 M, the difference between two adjacent sucrose solutions being 0.2 M. Centrifugation was carried out in the swing-out rotor of the refrigerated ultracentrifuge MSE Superspeed 40.

### *Materials*

Analytical grade reagents were used in all experiments. The detergents from the Cemulsol NPT series (nonyl-phenyl-polyoxyethylene having respectively 4, 6, 8, or 12 moles of ethylene oxide) are designated NPT 4, 6, 8, or 12 and were a product of Société des Produits Chimiques de Synthèse, Bézons, Seine-et-Oise, France.

## RESULTS AND DISCUSSION

### *Morphological Characteristics of the Nuclear Fraction*

The nuclear fraction obtained by the technique described consists of neuron and glial cell nuclei. In the phase contrast microscope, the nuclei of neurons can be seen to have a round or elliptic shape and generally one prominent and well shaped nucleolus (Fig. 1). In suspension they are well separated from one another and do not form clusters, although groups of 2 to 4 nuclei can be seen. At higher magnifications the well shaped nucleolus contrasts even more markedly with the light background of the nucleoplasm (Fig. 2). The nuclear membrane is well preserved and delineated in most nuclei, and no adhering cytoplasmic threads could be observed. These features are seen better with the anoptral contrast microscope (Fig. 3). Glial cell nuclei are markedly smaller, with varying shape and a denser nucleoplasm. In most of them several dark granules can be observed by phase contrast microscopy, but it is difficult to ascertain whether these represent nucleoli or chromatin material (Fig. 4). It deserves mention that neuron and glial cell nuclei are difficult to observe in the same focal plane in phase contrast micrography. This difficulty is probably due mainly to the different sizes of these two types of nuclei. The nuclear fraction obtained contains a minimal amount of contaminating material, which consists mainly of blood capillary fragments and filamentous formations with dark granules, the latter most probably originating from disrupted nuclei or myelin fragments (Fig. 5).

Electron microscopy confirms the picture observed by phase contrast and anoptral contrast microscopy. The nucleoli of neurons are densely packed and finely granulated, whereas the chromatin material is spread uniformly over the broad nucleoplasmic area (Fig. 6). The nuclear membrane is evidently unbroken but shows some irregularities in its contour. In most cases the membrane has a granular aspect, and filamentous or rounded assemblies of small granules are prominent over its surface. Most probably these figures represent the small ergastoplasmic contaminants which have actually been observed, even in highly purified nuclear fractions, by other investigators who have at their disposal better electron microscopy equipment (4, 14). Glial cell nuclei have the same characteristic size and general appearance as was observed with the phase contrast microscope. Electron microscopy reveals, in addition, that only one of the granules observed with the phase contrast microscope in these nuclei may be identified as a nucleolus (Fig. 7).

### *Nuclear Size Distribution*

Since the nuclei from brain cortex show a very high heterogeneity in size and shape as well as in density of organic material (19), it was of special interest to follow the distribution of the different nuclei during the isolation procedure. Determinations of nuclear size were carried out on nuclei from the first 1600 *g* nuclear fraction including the entire nuclear population from the brain cortex homogenate, as compared with the purified nuclear fraction in the final pellet. The results of these experiments are presented in Fig. 8. As can be seen, the purification procedure results in a

considerable loss of smaller nuclei (10 to 20 $\mu^2$) which most probably originate from oligodendroglial and microglial cells. Data on human brain cortex nuclei show that nuclei of astrocytes have a surface of 30 to 60 $\mu^2$, whereas nuclei of oligodendrocytes are markedly smaller, ranging from 10 to 20 $\mu^2$ (20). It has been reported (21) that in rat brain cortex 18.5 per cent of the total population of nuclei originate from neurons and the remaining 81.5 per cent from glial cells. If one assumes that nuclei with a surface area ranging from 10 to 30 $\mu^2$ are mainly glial cell nuclei, then in our experiments they represent 72 per cent of the whole population, the remaining 28 per cent being shared by neuronal nuclei and larger glial cell nuclei. An increase in the sucrose concentration from 1.8 M to 2.2 M as used by Sporn *et al.* (4) gives a greater loss of the 10 to 20 $\mu^2$ nuclei. Therefore, in order to obtain a better recovery of all nuclear types from brain cortex cells, we used a lower (1.8 M) sucrose concentration, the purity of the nuclear fraction being ensured by the inclusion of the Cemulsol NPT 12 treatment.

### *Comparison of the Action of Different Detergents*

Preliminary experiments with Tween 80 (10) showed that this detergent does not exert a significant action on brain lipoproteins if used for the short term treatment required by our procedure. In a series of experiments the actions of different detergents from the Cemulsol NPT group, as well as of Triton X-100, were compared. The detergent action was checked by observing the sediments with the phase contrast microscope and, as shown in Table I, by determining the ratios RNA/DNA, free cholesterol/DNA, and protein/DNA in these sediments after two centrifugations at 1600 *g* as described under Methods.

It can be seen that after detergent treatment, but without 40,000 *g* centrifugation, the purest nuclear fractions, free of cytoplasmic contaminants, are obtained by the use of Cemulsol NPT 12 and Triton X-100. The actions of Cemusol NPT 4 and Cemulsol NPT 8 are significantly poorer, and centrifugation of the homogenate without detergent, layered over the sucrose solutions, results in a strongly contaminated nuclear fraction. The nuclear fraction obtained at this stage of the procedure with Cemulsol NPT 12 treatment is morphologically and chemically of rather high purity and it may be used without further purification in experiments requiring a rapid isolation of brain nuclei.

TABLE I

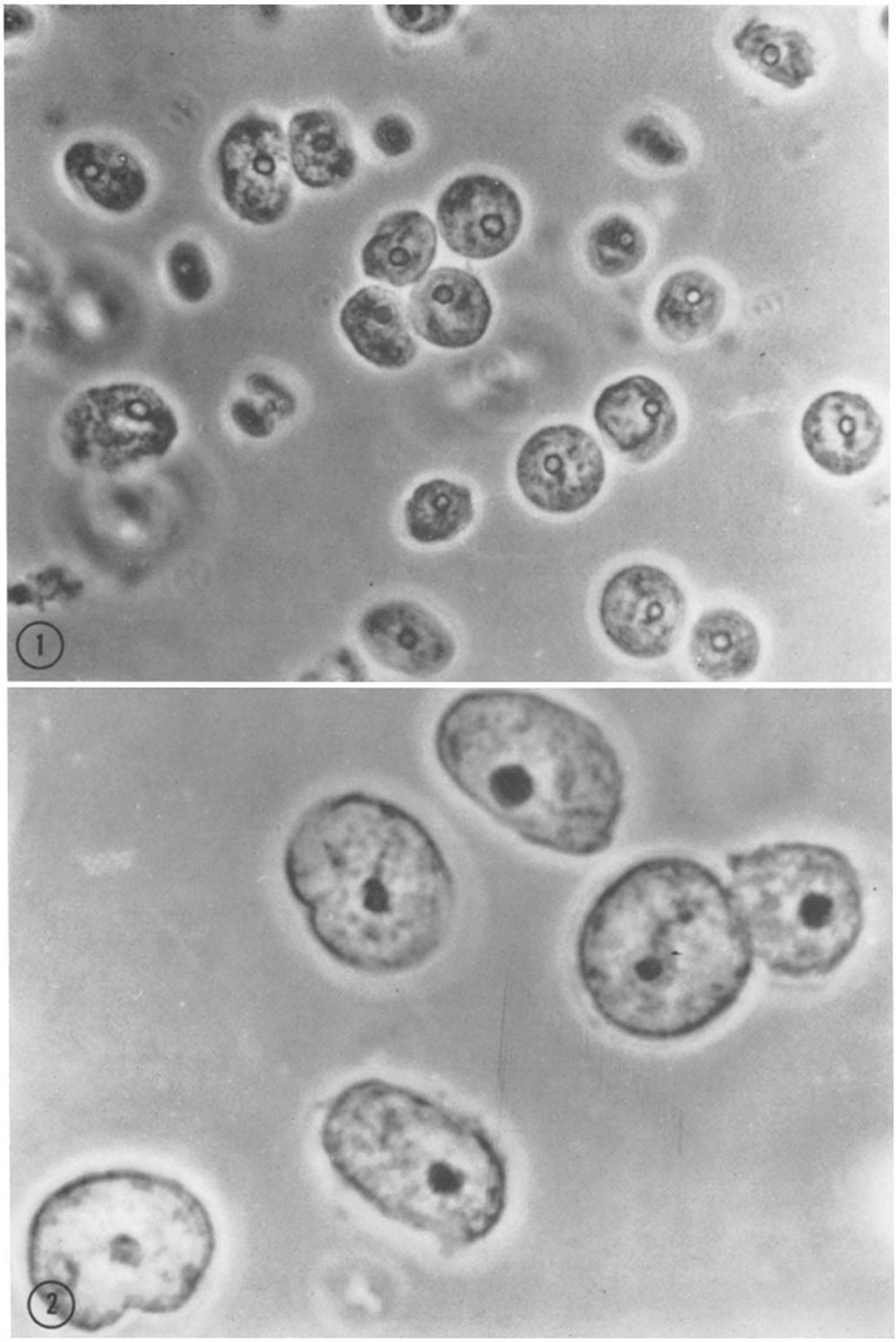
*Chemical Characteristics of the Nuclear Fraction of Cat Brain Cortex after the Action of Different Detergents*

| Treatment | Ribonucleic acid | Cholesterol | Protein |
|---|---|---|---|
| | $\mu g$ *RNA-P* / $\mu g$ *DNA-P* | *mg*/$\mu g$ *DNA-P* | *mg*/$\mu g$ *DNA-P* |
| Without detergent | 1.07 | 0.126 | 0.82 |
| + Cemulsol NPT 4 | 0.99 | 0.138 | 0.87 |
| + Cemulsol NPT 6 | 0.74 | 0.069 | 0.37 |
| + Cemulsol NPT 12 | 0.53 | 0.039 | 0.26 |
| + Triton X-100 | 0.50 | 0.036 | 0.37 |

The influence of the ionic composition and pH of the isolation medium was also investigated. The results on the role of $Mg^{2+}$ and $K^+$ are similar to those reported by other workers (4). Light microscopy indicated that a slightly lower pH (6.5) gives somewhat better preservation of nuclei than the pH of 6.7 to 6.8 used by Sporn *et al.* (4). If sucrose is omitted from the homogenizing medium (9), the nuclei are completely disrupted by the detergent treatment, although the smaller nuclei are somewhat more resistant than the larger ones. These experiments show that sucrose significantly protects nuclei against detergent action. On the other hand, when pure sucrose solutions (0.34 M) are used as the homogenizing medium, the effect of

FIGURE 1 Typical microscopic appearance of a suspension of purified cell nuclei from cat brain cortex, showing well preserved nuclei with prominent nucleoli, varying in size and shape. Neuronal nuclei are in the main focal plane. Phase contrast micrograph. × 360.

FIGURE 2 Neuronal nuclei from the purified nuclear fraction at higher magnification. Phase contrast micrograph. × 800.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Cemulsol NPT 12 is minimized. Therefore, appropriate ionic strength and pH of the sucrose medium are necessary in order to obtain the optimal effect of detergent treatment. The low temperature and short duration of Cemulsol NPT 12 action give additional advantages when metabolic studies are carried out.

### *Chemical Characteristics of Purified Nuclei*

In order to obtain information on the chemical composition of isolated nuclei from cat brain cortex we determined the DNA, RNA, cholesterol, and protein content of the final nuclear fraction, and the results were correlated with the nuclear count in the analyzed suspension (Table II).

TABLE II

*Chemical Composition of Purified Nuclei from Cat Brain Cortex*

(In picograms)

| | DNA | RNA | Cholesterol | Protein |
|---|---|---|---|---|
| Per picogram DNA | — | 0.45 | 0.23 | 14.9 |
| Per nucleus | 6.54 | 2.94 | 1.50 | 97.5 |

Mean values from two independent determinations are presented.

The content of DNA per nucleus in our preparations is in good correlation with the results of other authors (16, 22). The RNA content per nucleus is somewhat lower than that reported by Santen and Agranoff (16), but higher than that found by Sporn *et al.* (4); the RNA/DNA ratio in several experiments was never lower than 0.4. Adhering ribonucleoprotein particles might explain the higher RNA values of Santen and Agranoff (16), while some degradation or loss of nuclear RNA in the long term centrifugation technique of Sporn *et al.* (4) may account for a lower RNA content. A comparison of the data in Table II with the results presented in Table I shows that the main contaminants removed by high speed centrifugation in our procedure are lipoproteins (markedly decreased cholesterol/DNA and protein/DNA ratios), whereas only a limited removal of ribonucleoproteins occurs during this treatment (slightly decreased RNA/DNA ratio).

### *Distribution of Brain Cell Nuclei in Sucrose Density Gradient Centrifugation*

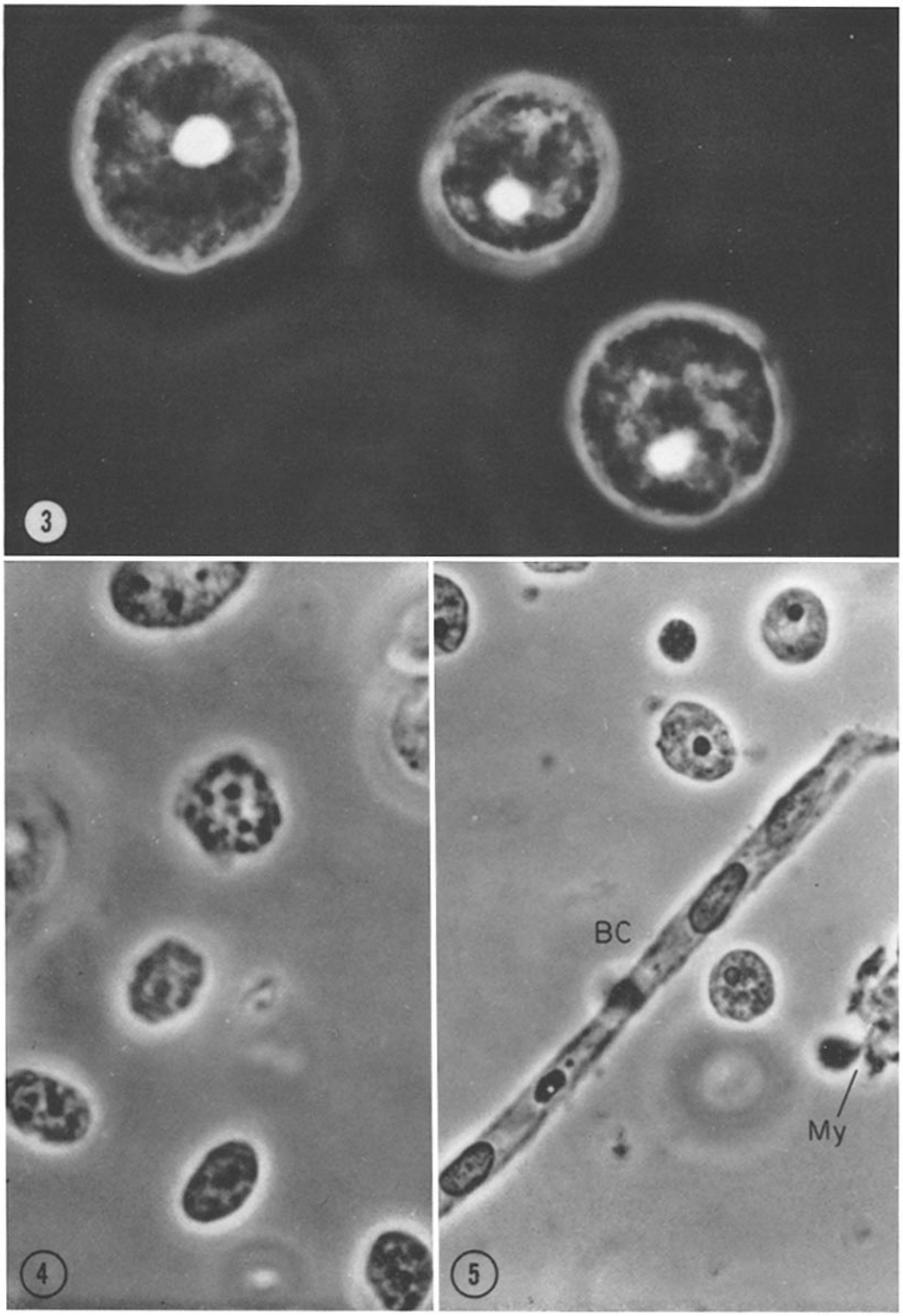
In preliminary experiments on the isolation of nuclei by the direct layering of a brain cortex homogenate over sucrose density gradients, we were unable to obtain reproducible results. In order to explain the variations we observed in the "density" of nuclei, sucrose density gradient centrifugation experiments were performed with the purified nuclear fraction (see Methods).

The nuclear pellet was suspended in buffered sucrose solutions of varying concentration from 0.34 M to 1.0 M and layered over a discontinuous sucrose gradient. Sedimentation of nuclei was achieved by centrifugation at 135,000 *g* for 30 minutes. The results of a typical experiment are shown in Fig. 9. The density level at which the band of the brain cortex nuclei appeared was directly dependent on the concentration of the sucrose solution used as a suspending medium. The nuclear suspension in 0.34 M sucrose gave a value for the density of nuclei lower than that of 1.8 M sucrose, while nuclei suspended in 1.0 M sucrose had a density equal to or greater than that of 2.2 M sucrose. Similar observations presumably led some authors (6 – 8) to the conclusion that brain cell nuclei have a markedly lower density than do

FIGURE 3 Neuronal nuclei from the purified nuclear fraction at higher magnification. The nuclear contour is well delineated and unbroken. One prominent nucleolus is to be observed. Anoptral contrast micrograph. × 800.

FIGURE 4 Glial cell nuclei from the purified nuclear fraction are situated in the main focal plane. Several dark granules are seen in the nucleoplasm. Phase contrast micrograph. × 800.

FIGURE 5 Main contaminants in the nuclear fraction from cat brain cortex. *BC*, blood capillary fragment; *My*, filamentous profile with several dark granules, most probably originating from myelin or nuclear fragments. Phase contrast micrograph. × 360.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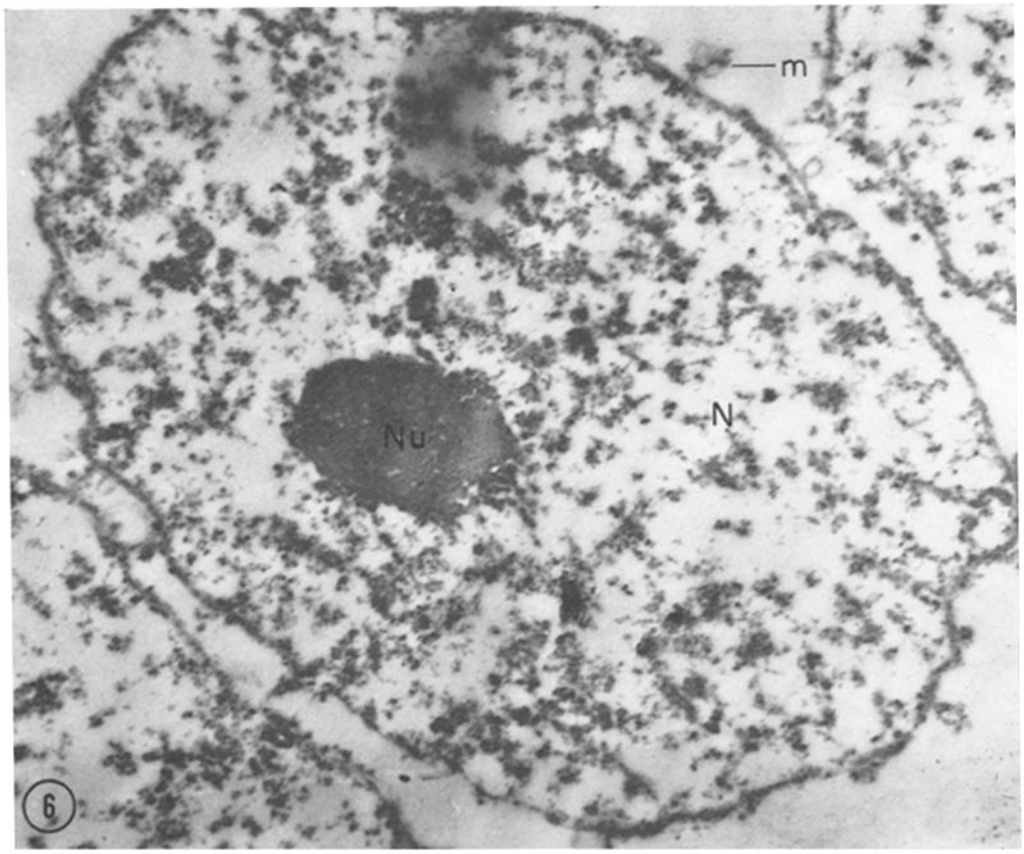
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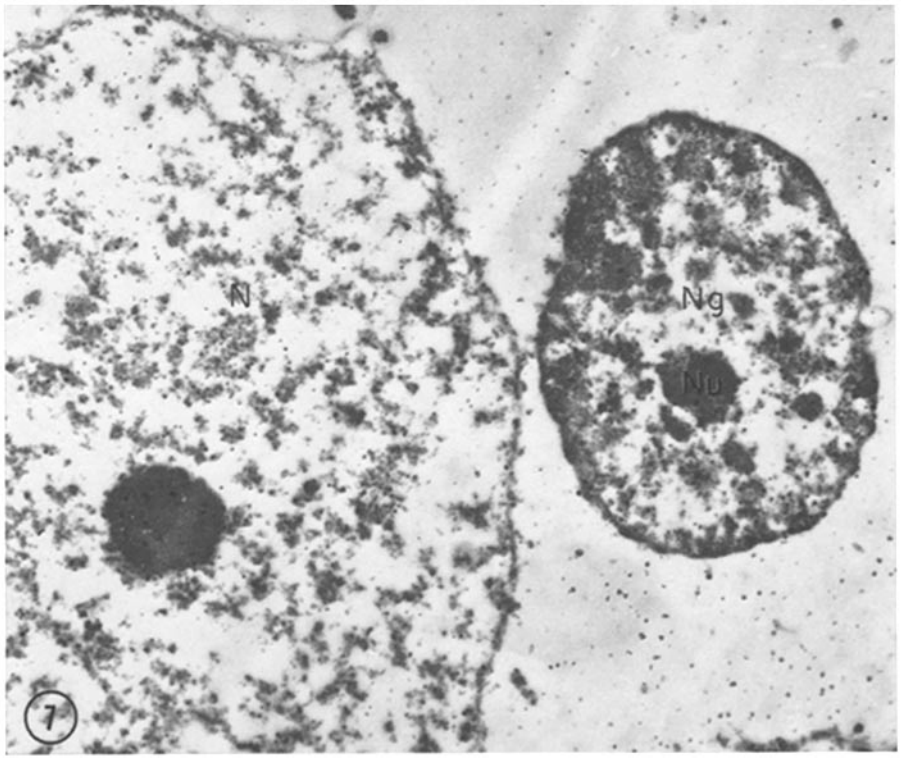
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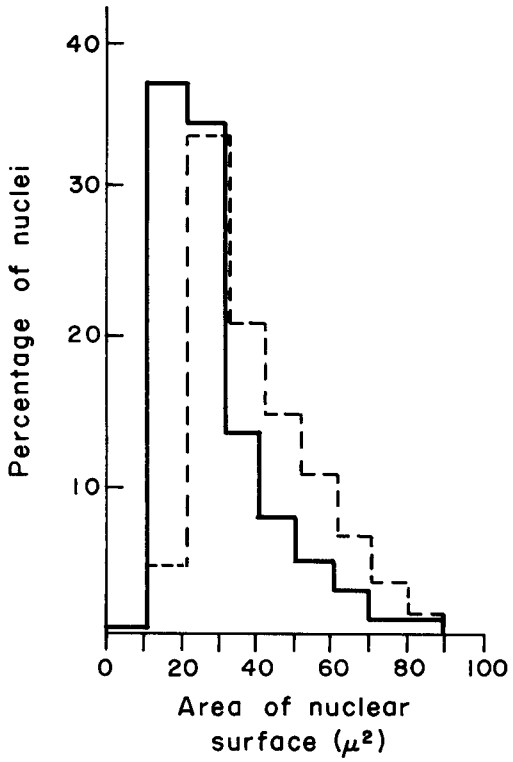


FIGURE 8 Nuclear size distribution of cat brain cortex cell nuclei from the first nuclear sediment (solid line) and the purified nuclear fraction (dashed line). The surface of 500 nuclei was determined for each of the two profiles.

FIGURE 6 Electron micrograph of the final nuclear pellet, showing one neuronal nucleus (*N*) and two adjacent nuclei. The nucleolus (*Nu*) is well delimited and consists of densely packed small granules. The nuclear membrane is unbroken, with granular appearance, and has several adhering filamentous or granular masses (*m*), most probably microsomal contaminants. × 18,500.

FIGURE 7 Electron micrograph showing one neuronal nucleus (*N*) and one glial cell nucleus (*Ng*). Only one of the granules in the glial cell nucleus may be identified as a nucleolus (*Nu*). × 18,500.

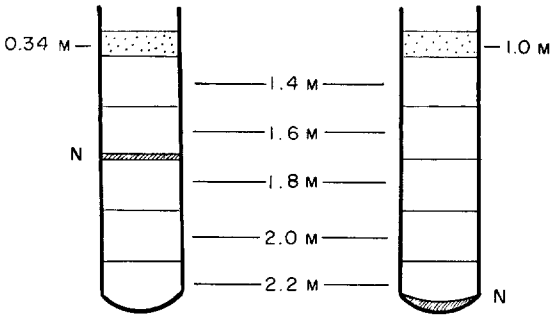


FIGURE 9 Representation of sucrose density gradient sedimentation of the purified nuclear fraction. Left, nuclei suspended in 0.34 M sucrose; right, nuclei suspended in 1.0 M sucrose. The nuclear suspensions were kept at 4°C for 60 to 90 minutes before centrifugation. The final position of the nuclear band (*N*) is schematically represented by cross-hatching. Centrifugation for 30 minutes at 135,000 *g*. For details see text.

the nuclei from other tissues. Thus Mandel *et al.* (8) reported a density for brain cell nuclei equal to or slightly higher than the density of 1.5 M sucrose. Our results, however, show that brain cell nuclei have a density similar or equal to that of nuclei from other tissues (see also reference 4). According to Chauveau *et al.* (23), the density of liver nuclei is higher than the density of 2.2 M sucrose, *i.e.*, 1.28. Further, our results indicate that brain nuclei equilibrate rapidly with the ambient medium and as a result their density varies in a manner similar to that reported for liver cell mitochondria (24). Such results have not been reported for liver nuclei, which leaves open the possibility of a higher permeability of brain cell nuclei and a more rapid equilibration of the latter with the medium. Another possibility is that equilibration of nuclei with the suspending medium is a general characteristic of nuclei which must be taken into consideration in studies employing a layering of the homogenate over hypertonic sucrose solutions.

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