# ISOLATION AND FRACTIONATION OF RAT BRAIN NUCLEI

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ABSTRACT

A method for isolating pure and unaltered nuclei from rat brain by means of differential centrifugation is described. The isolated nuclei are further separated into discrete fractions of neuronal, astrocytic, and glial nuclei, with a yield amounting to 20 to 25% of the DNA of the original homogenate. Both the morphology and size of the nuclei remained unchanged. Problems concerning the composition of the isolation media, the use of detergents, as well as those raised by density gradient centrifugation in sucrose, Ficoll, and Dextran are discussed. Some values for the density of each type of brain nuclei are suggested.

## INTRODUCTION

The brain is an heterogeneous organ composed of a number of different cell types classed into two general categories: neurons and glia. Recent advances in the study of brain chemistry have revealed that cells in these classes have different and sometimes complementary functional roles in the brain (11, 14, 16). Such is apparently the case with RNA synthesis (15), a function almost exclusively localized to the cell nucleus. Although many of the results in this connection were obtained with the elegant microtechnique of Edström (3), a method for fractionating brain nuclei on a somewhat larger scale would be very useful for studying DNA-dependent RNA-polymerase activity and many other nuclear properties.

In recent years, numerous methods have been developed for isolating highly purified nuclei from liver and other tissues. Most of these techniques are based on differential centrifugation (9, 10, 20, 28, 29). In some cases considerable purification has been obtained by the addition of various detergents to the isolation medium (5, 10, 12, 13, 17, 25, 30). All these procedures provide nuclei that present very little visible cytoplasmic contamination and that usually have preserved their morphological characteristics. In some cases, these nuclei still incorporate amino acids and perform some nucleic acid synthesis in vitro. Slight modifications of such procedures have allowed various investigators to obtain satisfactory nuclear preparations from brain tissue (10, 25, 26, 28), despite the extreme fragility of the neuronal nuclei and the large amount of myelin present in this organ.

This paper describes the conditions required to obtain a nuclear preparation representative of the cellular population in vivo and presents a fractionation procedure employing sucrose density gradient centrifugation that yields separate nuclear fractions from neurons and from astrocytes and other glial cell types.

## MATERIAL AND METHODS

The experiments were carried out on Sprague-Dawley rats, of both sexes, weighing 170 to 200 g. The animals were sacrificed by decapitation after slight ether anaesthesia or stunning. The brains were removed immediately, rinsed in ice cold NaCl 0.9%, cleaned from meninges, and blotted with filter paper.

### *Solutions*

Homogenization solution: 0.32 M sucrose, 0.001 M $MgCl_2$, and 0.001 M potassium phosphate, adjusted to pH 6.5.

Purification solution: 2.39 M sucrose, 0.001 M $MgCl_2$, and 0.001 M potassium phosphate, pH 6.5.

Triton X-100: 10% (w/v) solution.

### *Preparation of Nuclear Suspension*

All operations were performed at 0–4°C. The brain tissue was carefully minced with scissors and homogenized by hand in a Potter homogenizer provided with a loosely fitting Teflon pestle (clearance 200 $\mu$) in 15 to 20 strokes. Fifteen ml of homogenizing solution containing 0.25% Triton were used per 1 g of brain. The homogenate was filtered through cheesecloth and centrifuged for 10 min at 850 *g* (average). After the supernatant was discarded, the pellet was resuspended in the same volume of homogenization medium without Triton and centrifuged for 10 min at 850 *g* (average). The sediment was washed once more in the same way and centrifuged for 8 min at 600 *g* (average). The crude nuclear pellet was resuspended in 5 ml of homogenization solution without Triton, mixed with 26 ml of purification medium, and centrifuged for 45 min in an Sw 25 rotor of the Spinco Model L preparative centrifuge at 25,000 RPM (53,500 *g*, average). The nuclei were obtained as a transparent sediment at the bottom, and the cytoplasmic debris gathered at the top. The pellet was resuspended in 1 ml of 2.0 M sucrose; the expression "nuclear suspension" used later in the text will refer to this suspension. This preparation was layered over a discontinuous gradient of hypertonic sucrose, 1 ml of each of these concentrations: 2.8, 2.6, 2.4, 2.2 M (bottom to top). The gradient was allowed to stand for 3 hr at 0°C before use.

Fractionation of the nuclei was achieved by centrifugation in an Sw 29 rotor for 30 min at 30,000 RPM (75,000 *g*, average). Such a separation is shown in Fig. 3. The bands were collected with a syringe provided with a large bent needle, and the hypertonic sucrose was washed off by dilution with 0.32 M sucrose. The nuclei were gathered by centrifugation at 1000 *g* for 10 min and resuspended in a small drop of remaining fluid. The whole isolation plus fractionation procedure was carried through in 2½ to 3 hr.

### *Chemical Analysis*

The DNA was measured by the microbiological method of Hoff-Jørgensen, as modified by Løvtrup and Roos (18). The RNA was determined by the orcinol reaction and the proteins, according to the method of Lowry et al. (19).

### *Microscopy*

The nuclear suspension and the isolated fractions were controlled in a Zeiss photomicroscope in phase contrast and with normal illumination. In the latter case the smears were stained with 0.5% crystal violet.

### *Nuclear Size Measurements*

The nuclei were dispersed in isotonic sucrose in the presence of 0.5% crystal violet, and their greatest diameter was determined under immersion in a light microscope fitted with a micrometric ocular, at a magnification of 1500.

## RESULTS AND DISCUSSION

### *Homogenization*

Because of their extreme fragility, greater than that of liver nuclei (28), brain nuclei, mainly those from neurons and astrocytes, are easily disrupted during the homogenization procedure. The use of a motor gave rise to damage even at a moderate speed. But an even more important factor is the clearance of the homogenizer. A clearance of at least 200 $\mu$ was found to be necessary (Maggio et al. (20) suggested a value of 0.009 in. for liver nuclei which are much smaller than nuclei from neurons or astrocytes) and a gentle homogenization by hand was achieved with a few up and down movements. Nuclear counts after such treatment showed more free intact nuclei with a diameter of over 10 $\mu$ than after homogenization with a tightly fitting pestle.

### *Homogenization Medium*

The influence of the concentration of each constituent, i.e. sucrose and ion content, and of the pH was studied. The higher the sucrose concentration, the purer the nuclear suspension. Yet, a molarity of over 0.4 is not recommended during the low speed centrifugation, since losses of free nuclei increase also.

The optimum concentration of $MgCl_2$, with respect to purity and separation of the various types of nuclei in the nuclear suspension, was found to be 0.001 M. Maggio et al. (20) tested $Ca^{++}$ ions for the isolation of liver nuclei and noticed that a 0.003 M solution brought about precipitation of the nucleoplasm, while concentrations of 0.0015 M or less gave satisfactory results. Phosphate and $Mg^{++}$ ion contents higher than 0.0015 M caused the same damage to brain tissue and caused a clear decrease in purity of the fractions after su-

crose density gradient centrifugation. This consequence was also stressed by Maggio et al. (20) who noted an increase of the RNA to DNA ratio, suggesting higher cytoplasmic contamination.

The pH turned out to be another factor of importance, especially in the clumping phenomenon. In the pH range 5.2–6.2, notable contamination of the nuclear suspension was observed. At pH 6.8–8.0, the gelation noticeably increased. The purest and best separated nuclei seem to obtain at pH 6.5. Sporn et al. (28) noticed also a greater tendency of the nuclear membranes to rupture at pH values over 6.8. Some authors (9, 25) claim an optimum pH of around 6. This difference might be due to the fact that their medium contained acetic or citric acid, but these substances are not to be recommended since they are thought to precipitate nucleoplasm and to extract important constituents (1, 20). However, Gill (9) also noted that slight acidity is more efficient in preventing agglutination than an increased ion content.

### *Detergents*

Isolations were performed with and without detergents. It was found that the purity as well as the yield was clearly improved by the use of such substances. Detergents enhance the lysis of erythrocytes and the rupture of mitochondria and other cytoplasmic constituents. Different kinds of detergents have been used for nuclear isolation (5, 10, 12, 13, 17, 25, 30), among which the main ones have been Cemulsol NPT 12 and Triton X-100. The former was shown by Zalta et al. (30) to provide pure nuclear suspensions, without cytoplasmic contamination, in which a single nuclear membrane was preserved, although nuclear pores disappeared.

For reasons of availability, Triton X-100 was chosen, as this substance has as good an effect as Cemulsol on brain nuclei (10). Triton-treated nuclei have at least one single intact nuclear membrane, as they can still swell in water (25). Fragments of the outer membrane have been observed after detergent treatment, but the degree of deterioration of this envelope is related to the length of time the nuclei were exposed to it (13).

The sensitivity to Triton is not the same for all types of nuclei. The neuronal nuclei were the most easily affected and their number decreased as the time of action of the detergent increased (Fig. 1) Therefore, efforts were made not to exceed a duration of contact of 20 min; at this time, less than 5% of the nuclei with a main diameter of over 10 $\mu$ had disappeared. After 60 min, their number fell from 38 to 14% of the total (Fig. 1).

The use of a detergent affected also the chemical composition of the nuclear suspension (Table I).

### *Nuclear Yield*

Since the presence of cytoplasmic contamination increases the RNA content of the nuclear preparation, we can consider only DNA determinations as a good criterion for the estimation of the nuclear yield, assuming that all the DNA is confined to the nucleus.

Nuclear yield, as indicated by the recovery of DNA, was dependent upon the presence of a detergent: it decreased from 28 to 35% to 11 to 14% in its absence. The total recovery in the

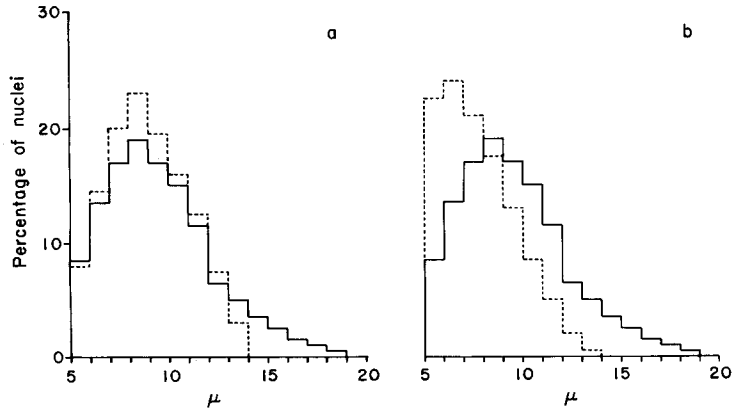


FIGURE 1 Nuclear size distribution of rat brain nuclei isolated in presence of Triton X-100 after a duration of contact of (*a*) 30 min and (*b*) 60 min (···). Control (—).

TABLE I

*Nucleic Acid and Protein Contents of Purified Nuclei from Rat Brain*

| Treatment | RNA | DNA | Proteins | RNA/ DNA | Proteins/ DNA |
|---|---|---|---|---|---|
| Without detergent | 108 | 243 | 3510 | 0.46 | 14.4 |
| + Triton X-100 15 to 20 min | 84 | 225 | 2050 | 0.37 | 9.1 |

Values given in μg per 1 g wet weight as represented in the filtered homogenate.
Mean values from 4 experiments.

nuclear suspension and the collected supernatants was 95 to 98% of the DNA of the filtered homogenate. The contaminants floating on the hypertonic sucrose contain many free intact nuclei whose migration was prevented by extensive crowding at the interfacial zone. This fraction can be resuspended and centrifuged again in purification solution. One obtains a new pellet of free, intact nuclei representing another 10 to 12%, so that the total yield can be brought up to 40 to 45%. Lengthening of the centrifugation time does not improve these values; even centrifuge runs of 30 min instead of 45 min led to the same results. Lowering the sucrose concentration increases the yield, but it also increases the contamination. A lower amount of purer nuclei was preferred.

### *Chemical Composition*

DNA, RNA (orcinol value corrected for DNA which weight for weight gives 11% of the reaction of RNA), and protein contents were determined in the nuclear suspension (Table I) and in the isolated fractions. The degree of purity was estimated by the ratios RNA to DNA and protein to DNA. It must be remembered that such values represent the net result of two processes: purification from cytoplasmic RNA and proteins, and extraction of nuclear material (1).

After Triton X-100 treatment, the protein content markedly decreased and the RNA to DNA ratio showed a parallel change. Since less cytoplasmic debris was visible in the light microscope, the results suggest that a considerable purification had occurred. Extraction of nuclear RNA and proteins is likely, however. The RNA to DNA ratio is of the same order as that obtained by Hadjiolov et al. (10) for cat brain cortex and notably higher than that found by Sporn et al. (28) for rat brain and by Siebert (27) for beef brain. This is probably due to the reduction of the number of preparative steps and the shortening of the centrifugation time which may diminish the loss of intranuclear components.

Analysis of each fraction showed that no appreciable purification or loss of constituents seems to occur during the sucrose density gradient centrifugation.

### *Density Gradient Centrifugation*

Several authors (4, 6, 7) have fractionated liver nuclei into classes with different DNA contents. As the intensity of DNA-staining varies from one cell type to another, one might devise a separation of the brain cell nuclei based on their differences in density. The work of Fisher et al. (6) served as a basis for such an attempt: a gradient of 2.7, 2.4, 2.2 M sucrose was made up, and 1 ml of nuclear suspension was layered on top. A centrifugation of 30 min at 30,000 RPM yielded a fine band of impurities at the interphase of 2.0 and 2.2 M sucrose, one band of nuclei between 2.4 and 2.7 M sucrose, and a nuclear sediment at the bottom. The first nuclear band could be called an "enriched neuronal fraction," as it contained more than 50% neuronal nuclei, but was still heavily contaminated by glial nuclei (the criteria used to define each group are indicated below). The pellet consisted of a mixture of both types. No clear separation was obtained. The density of many neuronal nuclei was greater than that of 2.7 M sucrose. Consequently, a new gradient of 2.8, 2.6, 2.4, and 2.2 M sucrose (1 ml of each) was used in the next attempts. This provided 4 bands (Fig. 3), located at each interphase, and a pellet. The morphology of these fractions will be described in the next section.

In order to avoid the crowding phenomenon occurring in sharp interfacial zones between the adjoining concentrations, we tried a continuous gradient ranging from 2.8 to 2.2 M sucrose, but no sharp banding resulted. Instead, the nuclei spread out in large zones and were very difficult to separate from each other. A compromise between these two kinds of gradients was achieved by allowing a discontinuous gradient to stand for 3 hr in order to permit diffusion at the boundaries.

As the pellet was composed of a mixture of oligodendroglial and microglial nuclei, an extra step of 3.0 M sucrose was added, but no clear

separation occurred. Nuclei of all sizes stayed at the boundaries of 2.8 and 3.0 M sucrose or went through the 3.0 M layer to the bottom. The density of the nuclei seems to depend upon the sucrose concentration of the medium. Falzone et al. (4) determined the density of hepatic nuclei as a function of the density of the medium and noted that the nuclear density varies with the density of the medium and always exceeds the latter. The same seems to hold for brain nuclei, and all of them would probably migrate to the bottom if centrifuged long enough. After 1 hr centrifugation through a gradient with highest sucrose molarity of 2.8, all nuclei did collect as a pellet at the bottom of the tube. The fractionation procedure reported in this study is, then, not simply an isopycnic one, but also takes into account the differences in migration velocities of the nuclear types and the viscosity of the fractionating medium. In view of the time-dependent nature of these factors, centrifuge runs did not exceed 30 min' duration.

In the standard conditions described under Methods, the neuronal nuclei stay above 2.8 M sucrose, which means that their density lies between 1.33 (the density of 2.6 M sucrose) and 1.35, while glial nuclei are heavier than 2.8 M sucrose (d = 1.352). Even if these values might be slightly higher than those in vivo, they are close to those of liver nuclei which have a density of 1.4 according to Chauveau et al. (2). Hadjiolov et al. (10) came to the same conclusion for nuclei from cat brain cortex.

It should be noted that measurements of nuclei showed no obvious difference in size before and after density gradient centrifugation. Presumably, there is very little osmotic shrinkage, if any, but the variations in densities might well result from nuclear permeability to sucrose, leading to an equilibrium between the sucrose in the medium and the intranuclear fluid.

We tried, therefore, to make up a gradient with a substance of much higher molecular weight and which consequently would pass the nuclear membrane at a much lower rate, if at all. Ficoll, a nonionic high polymer of sucrose, and Dextran (molecular weight about 80,000) (both obtained from Pharmacia, Uppsala, Sweden) were tested. Ficoll was used previously to obtain purified, metabolically active nuclei from calf thymus (8, 21). With Ficoll, no real fractionation occurred. The nuclei showed a tendency to settle to the bottom in a short time. The highest concentration of Ficoll which could be prepared was 75%, a solution which has a density equal to that of 2.1 M sucrose (d = 1.265) but a higher viscosity than the corresponding sucrose solution.

Slightly better results were obtained with Dextran (8) where two bands could be collected, but the population of each band was far from being homogeneous, and most of the nuclei migrated to the bottom. The same problem as with Ficoll arose here: the highest possible concentration of Dextran was 50% with a density of around 1.29. One conclusion which can be drawn from these experiments is that the density of the brain nuclei is higher than 1.29. The density changes occurring in hypertonic sucrose are thus only relatively small, and it was therefore decided to standardize the conditions for sucrose density gradients.

### *Morphology*

The criteria used to define the types of nuclei are those described by Nurnberger (22) and Nurnberger and Gordon (23) for rat brain and by Rabinowicz for human brain (24). Nuclei from neurons display a pale chromatin network with a well shaped single nucleolus, often centrally located, contrasting with the light background of the nucleoplasm. Those from astrocytes resemble very much the neuronal nuclei, except that they are often larger, their nucleoplasm is very faint, and they have two or more nucleoli. The glial nuclei can be divided into two groups: those from oligodendrocytes are smaller than neuronal nuclei, ovoid, dense, with a heavily staining chromatin and lightly staining peripheral nucleoli; those from microglia are the smallest and the darkest, without apparent nucleoli.

All these different types can be seen in Fig. 2 which shows a nuclear suspension before density gradient centrifugation. The bands obtained by fractionation, as they appear in Fig. 3, are numbered from top to bottom, *1*, *2*, *3*, *4*, and the pellet, 5. Band *1* is composed mainly of cytoplasmic debris and a few nuclei, and band *2* consists of altered nuclei, mostly damaged giant astrocytes, and fragments of myelin and capillaries.

Nuclei from the third fraction (Fig. 4) are large and pale, with two or three nucleoli and many small chromatin granules. These characteristics appear both under phase contrast and with ordinary illumination after crystal violet staining. These nuclei stay on top of 2.6 M sucrose (d =

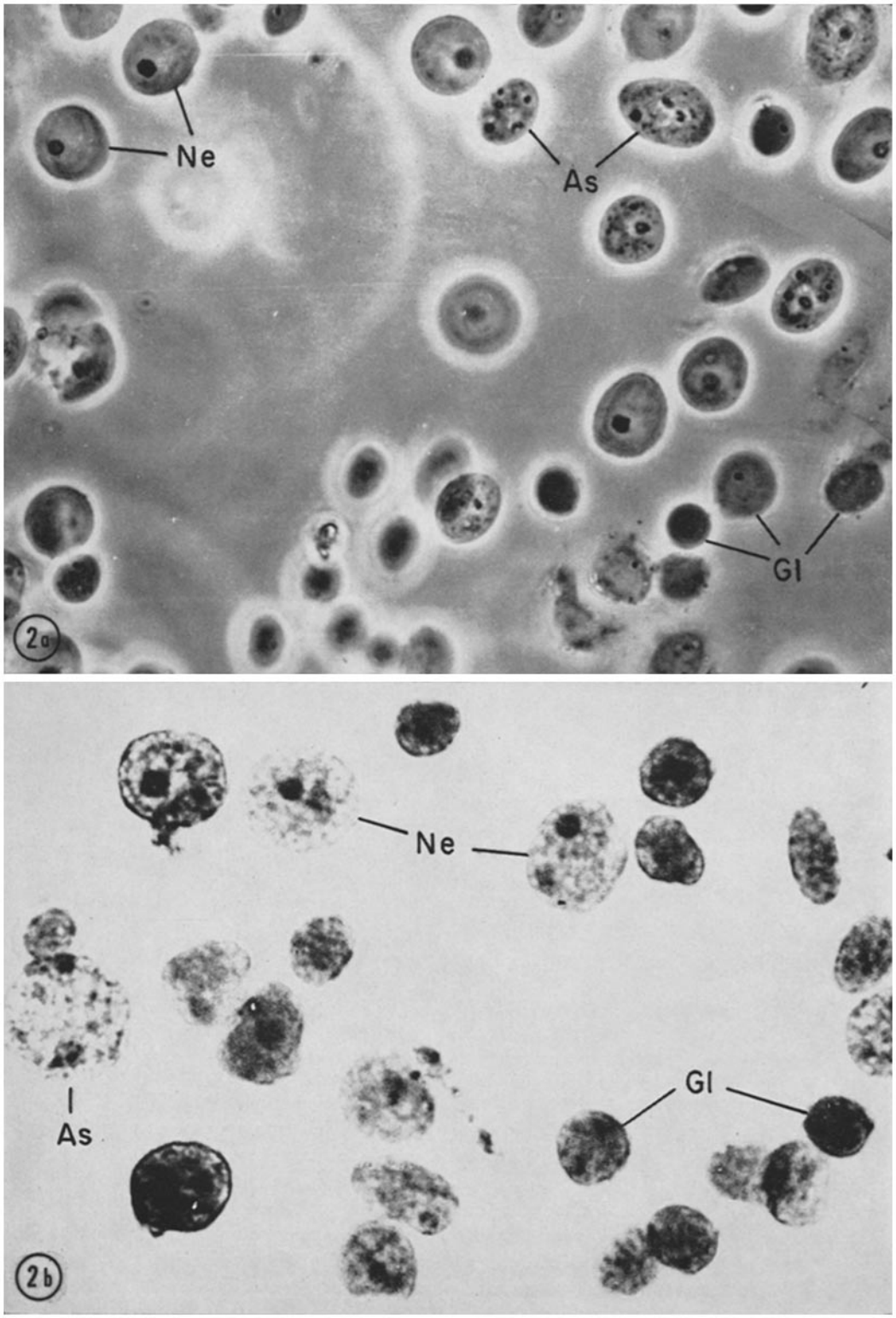


FIGURE 2 Micrographs of a suspension of purified nuclei from rat brain, showing neuronal (*Ne*), astrocytic (*As*), oligodendroglial and microglial nuclei (*Gl*). Fig 2 *a* phase contrast. × 600. Fig. 2 *b* normal illumination after crystal violet staining. × 1000.

1.328) and can be considered to derive from astrocytes. Over the 2.8 M sucrose layer (d = 1.352) gather large nuclei with light background which have, most often, only one very distinct nucleolus (Fig. 5). These are undoubtedly neuronal nuclei. The pellet or band 5 (Fig. 6) contains a mixture of oligodendroglial and microglial nuclei. Under phase contrast, the former are granulated, with light nucleoli, whereas the latter appear as very dark spots, without apparent structure. Their density is higher than 1.352.

The contamination of any one fraction with

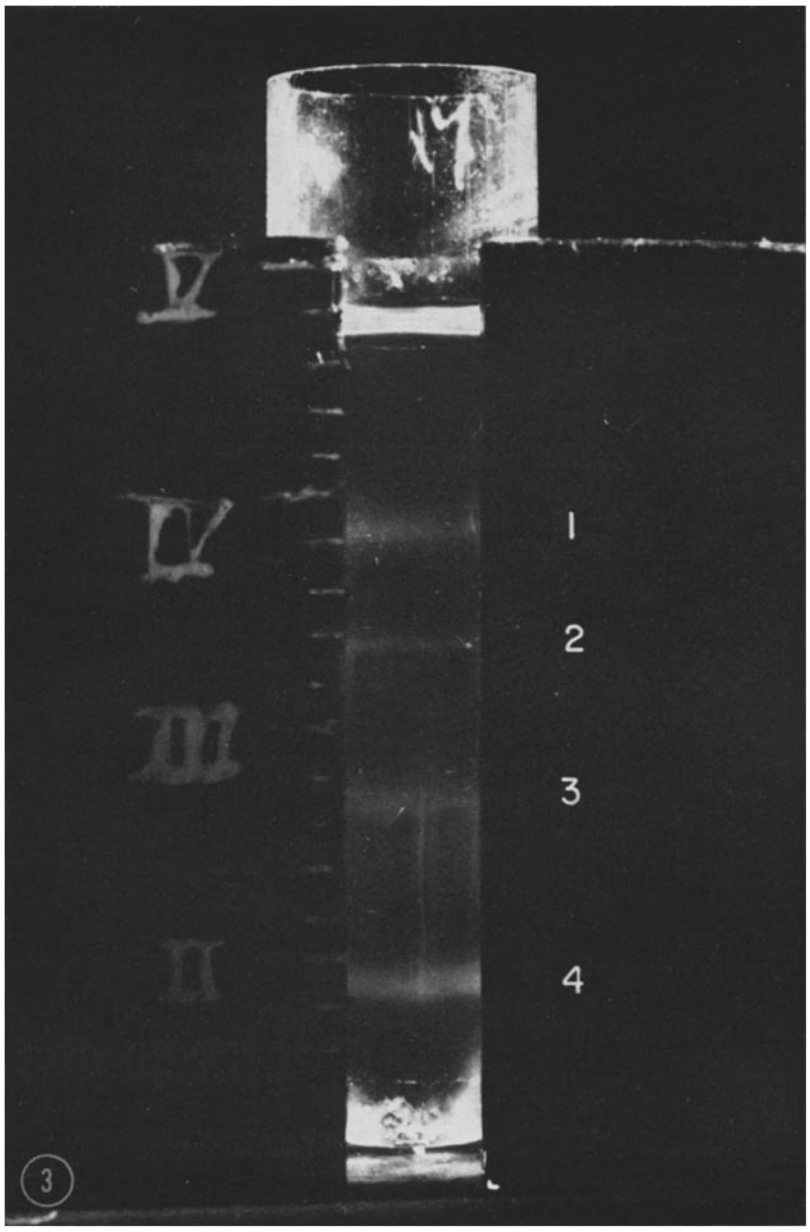


FIGURE 3 Fractionation of a nuclear suspension from rat brain after sucrose density gradient centrifugation. The figures at the right represent the numbers of the different bands. *1* and *2*, cytoplasmic debris; *3*, astrocytic nuclei; *4*, neuronal nuclei.

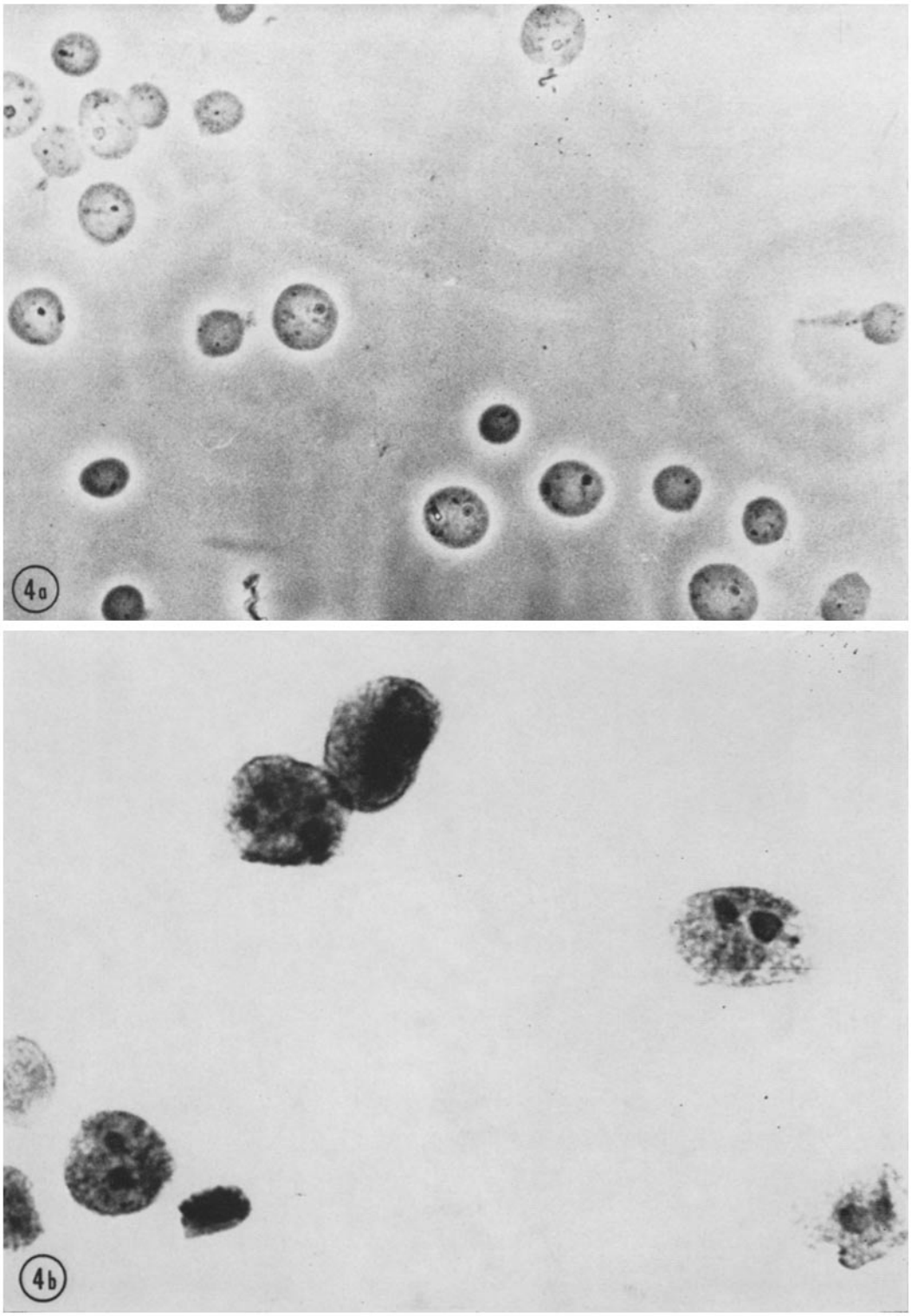


FIGURE 4 Astrocytic nuclei as obtained from band *3* after sucrose density gradient centrifugation. Two or more nucleoli are to be observed. Fig. 4 *a* phase contrast. × 400. Fig. 4 *b* crystal violet staining. × 1000.

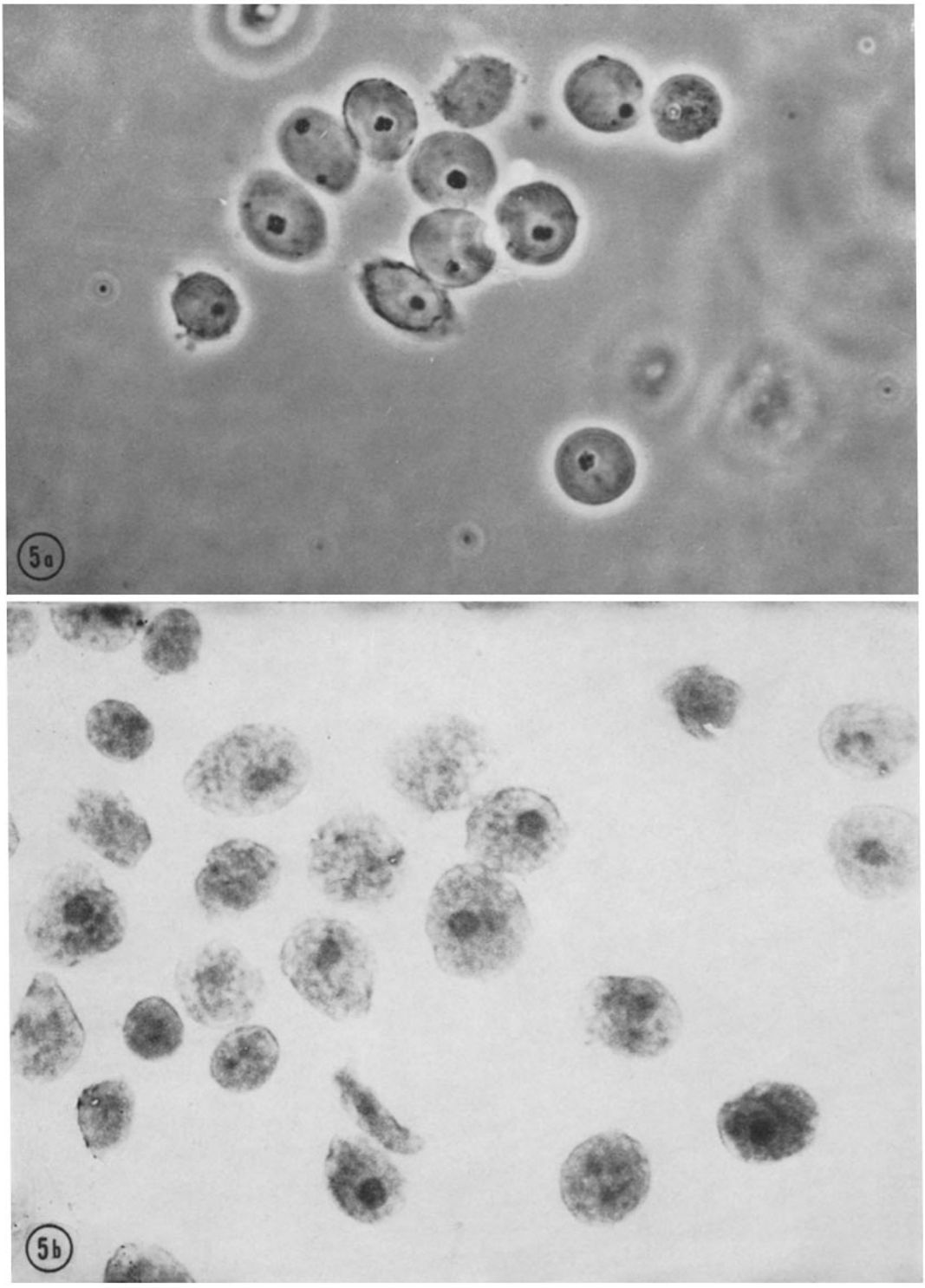


FIGURE 5 Neuronal nuclei from rat brain collected in band 4 of the sucrose density gradient fractionation. One prominent nucleolus is to be noted. Fig. 5 *a* phase contrast. × 600. Fig. 5 *b* crystal violet staining. × 1000.

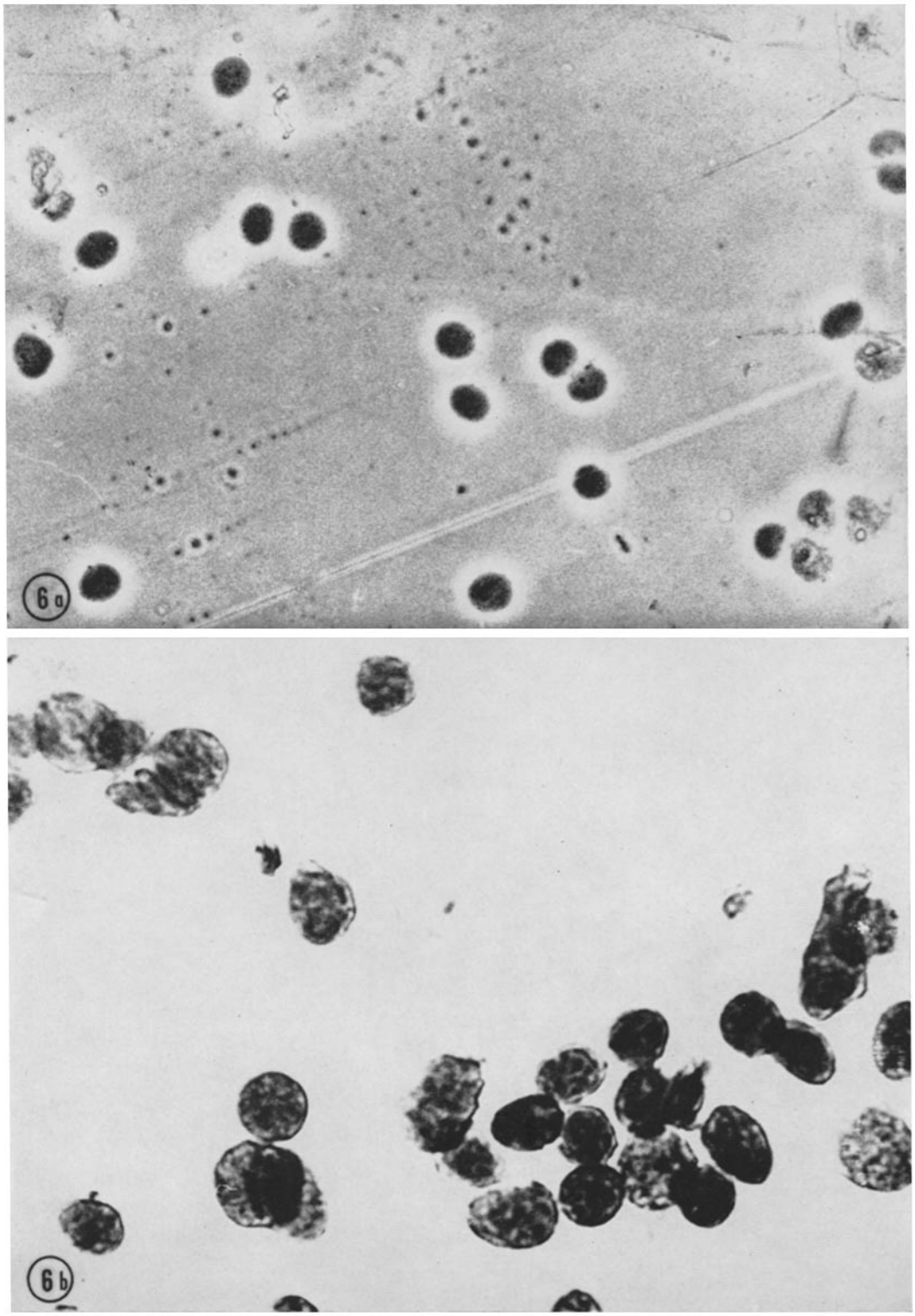


FIGURE 6 Mixture of oligodendroglial and microglial nuclei from the pellet after sucrose density gradient centrifugation. Fig. 6 *a* phase contrast. × 400. Fig. 6 *b* normal illumination after crystal violet staining. × 1000.

nuclei from the other fractions does not exceed 5 to 10%. DNA measurements after density gradient separation showed that over 50% of the DNA put on the gradient is recovered in the fractions. In other words, 20 to 25% of the nuclei of the homogenate can, by this technique, be recovered as homogeneous nuclear fractions. One g of fresh brain tissue is sufficient to carry out the whole procedure and to obtain a separation as shown in Fig. 3.

This method for isolating and fractionating brain nuclei seems to satisfy the three conditions put forward by Chauveau et al. (2) for this type of technique: preservation of the morphology, homogeneity of the fractions, and sufficiently high yield.

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