119. Isolation and Assortment of Cell Nuclei from the Mammalian Cerebral Cortex

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The isolation of pure and metabolically active 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 particular to the cerebral tissue. Further, an apparently insuperable difficulty has been the separation of nuclei of various origins, i.e., neuronal, glial and endothelial nuclei. In this preliminary communication, a rapid aqueous method is described for the isolation of highly purified nuclear preparations with well-preserved metabolic activity. Separation of neuronal and glial nuclei has also been attempted with considerable success.

Isolation of nuclei. Adult guinea pigs of both sexes weighing 250–300 g were used in all experiments. Erythrocytes were removed by a short-time perfusion of the brain in situ. All the subsequent preparations were made at 0–4°C. Cerebral grey matter from 6 animals (approximately 10 g) was ground in 50 ml of 0.32 M sucrose containing 1.5 mM CaCl₂ using a Dounce type homogenizer provided with a plastic pestle (clearance 100–150 μ); grinding involved 8 strokes of the pestle, rotating at 1,500 rev/min, during 2 minutes. The suspension was filtered through 8 layers of gauze, and finally made up to 210 ml by adding appropriate volumes of 0.32 M sucrose–1.5 mM CaCl₂.

Step 1. Four 50.0 ml portions of the homogenate were centrifuged at 2,800 rev/min (1,000 gₜ) for 15 minutes. Supernatants were decanted, and the sediments were combined and resuspended in 12.5 ml of the sucrose-CaCl₂ medium. 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 rev/min (51,000 gₜ) for 10 minutes. This produced a tissue block compressed right above the boundary between the 0.32 M sucrose layer and the 2.3 M sucrose layer. The 0.32 M sucrose layer 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 block was taken out upside down, and its surface was thoroughly washed with 0.32 M sucrose in order to remove the adhering dense
sucrose. This tissue block was much alike in shape to a pudding and will be so designated below. One third of the upper part of the pudding appeared light brown, and the remaining lower part cream-colored. The upper portion turned pink with the incompletely perfused brain, and deeply red with the unperfused brain. The light brown portion was cut off using two razor blades operating manually from both sides, and three equivalent portions were resuspended, unless otherwise specified, in 6.5 ml of 0.32 M sucrose–1.5 mM CaCl₂ by a gentle hand homogenization in a Dounce type-plastic pestle homogenizer. This produced the P2 preparation. On microscopy of samples stained with aceto-orcein-fast green, light brown portion of the pudding consisted mainly of nuclei of various size, but also contained a certain amount of myelin sheath fragments and broken capillaries. The cream-colored portion consisted almost exclusively of myelin fragments, and of some contaminating mitochondria (Janus green stain); this part was discarded during the preparation.

**Step 3.** A 2.0 ml portion of the P2 suspension was layered on the top of the Ficoll density gradient consisting of 2 ml of 22 percent (w/v) Ficoll and 1 ml of 18 percent (w/v) Ficoll dissolved in 0.32 M sucrose, and three equivalent tubes were centrifuged at 25,000 rev/min for 10 minutes in the RPS 40 swing-out head. A white pellet at the bottom of the tube consisted mainly of nuclei, but some broken capillaries and a small amount of myelin sheath fragments were also present. Three pellets were combined and resuspended in 6.5 ml of 0.32 M sucrose (P3I preparation). As an alternative to the Ficoll gradient, a discontinuous density gradient consisting of 2 ml of 1.6 M sucrose and 1 ml of 1.2 M sucrose was used, and this produced the P3II preparation, suspended in, unless otherwise indicated, 6.5 ml of 0.32 M sucrose.

**Step 4.** 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 rev/min for 10 minutes. A thick white band at the interface between the 0.32 M sucrose layer and the 1.65 M sucrose layer consisted almost entirely of myelin components. In a thin white band in between the 1.65 M sucrose layer and the 2.0 M sucrose layer were found concentrated broken capillaries. 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, usually 3.0 ml. 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.

Another steps. In order to obtain the nuclear fraction rich in either the large (9-18 μ in diametre, in samples stained with aceto-orcein-fast green), or the small (6-8 μ in diametre) nuclei, the following preparations were made.

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 the discontinuous density gradient consisting of 2 ml of 2.4 M sucrose, 1 ml of 2.3 M sucrose, and 0.5 ml each of 2.0 M and 1.65 M sucrose in a tube fitting the BPS 40 head, and three equivalent tubes were centrifuged at 35,000 rev/min (100,000 gav) for 15 minutes. A white band at the interface between the 2.3 M sucrose layer and the 2.4 M sucrose layer 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 sample at 15,000 rev/min (18,400 gav) for 5 minutes, and resuspending the pellet in 1.0 ml of 0.32 M sucrose. This produced the P_L preparation.

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 CaCl₂, was layered on 1 ml of 2.3 M sucrose in a RPS 40 tube, and centrifuged at 25,000 rev/min for 10 minutes. This produced the second pudding which had a pink head portion and was slightly more fragile than the first pudding. The pink portion of the second pudding was cut off as described in the Step 2, and disintegrated in 0.4 ml of 0.32 M sucrose using a plastic rod, made up to 9 ml with 2.6 M sucrose, and mixed thoroughly with the rod. A 3 ml portion of this dense suspension was placed in a RPS 40 tube, and three equivalent tubes were balanced with appropriate volumes of 0.32 M sucrose. These were centrifuged at 35,000 rev/min for 15 minutes. After decantation, three pellets were thoroughly washed with 0.32 M sucrose, drained, and resuspended in an appropriate volume of 0.32 M sucrose (P_s preparation, generally 1.0 ml).

Morphology of isolated nuclei. On phase contrast microscopy, the large majority of isolated nuclei appeared either round or elliptical, and markedly differed in size. Nucleus of a large contour generally had one well shaped nucleolus which sharply contrasted with the light nucleoplasmic area. Nucleoli that were apparently segmented on one focus level turned to be roundly united on another focus level. In some nucleoli, however, the segmentation seemed to have actually occurred, and it will come into question whether this sort of segmentation of nucleolus represents an artefactual change arising from fractionation procedure. In smaller nuclei, nucleoplasm appeared darker, and the nucleolus-like granules were less clearly contrasted, and distributed...
over a wide range of nucleoplasmic area. The frequency of damaged nuclei was relatively small; less than 5 percent of the nuclei showed partially ruptured nuclear contour and herniation of the nucleoplasm. Electron microscopy of the P4I preparation and the P4II preparation showed that contamination of red blood cells, cell debris and mitochondria was virtually absent. Elements of the endoplasmic reticulum were, however, occasionally encountered. The majority of the sedimented nuclei showed the well-preserved nuclear membranes, in some instances with evident nuclear pores. They were provided with a finely textured nucleoplasm, and in typical cases, with well-defined nucleolus and ribosome-like granular structures. In some nuclei, however, portions of nucleoplasmic material tended to condense to varying extent.

An attempt to differentiate the origin of isolated nuclei was made with preparations stained by aceto-orcein-fast green. Generally, it will be a dangerous task to presume the origin of nuclei in isolated preparations. Particular difficulties were encountered in the differentiation of astroglial nucleus and neuronal nucleus of a smaller size, when the latter failed to show a well-defined nucleolus, though it appeared that the stain of nucleoplasm tended to be denser in astroglial nucleus than in the neuronal nucleus. The identification of microglial nuclei was next to impossible. In our tentative attempt, nucleus of a large contour, usually provided with one distinct nucleolus, though apparently segmented in some cases, and showing light area of nucleoplasm was taken to be neuronal origin; nucleus of a smaller contour, showing several nucleolus-like granules over the nucleoplasmic area, or in some cases apparently unprovided with these granules, and showing denser nucleoplasm was taken as oligodendroglial (cf. reference 4). Isolated nuclei were grouped into two classes, i.e. neuronal and non-neuronal. The neuronal consisted solely of neuronal nuclei differentiated as described above. The non-neuronal consisted 1) mainly of oligodendroglial and astroglial nuclei as differentiated on the above basis, 2) a certain amount of nuclei that were not properly identified, and possibly either of neuronal or of astroglial origin, and 3) of a negligible amount of endothelial nuclei (less than 0.5 percent of the total). The population of neuronal nuclei ranged from 55 to 65 percent and from 45 to 55 percent in the P4I preparation (Fig. 1) and in the P4II preparation respectively. In the preparation P_s (Fig. 2), the population of neuronal nuclei ranged between 70 and 80 percent. In contrast, 90–98 percent of the nuclei in the P_s preparation (Figs. 3–4) consisted of smaller nuclei, and the large majority of these was most likely to be of glial origin.

Gross chemistry of isolated nuclei. Nucleic acids were separated
Figs. 1-4. Photographs of nuclei isolated from the cerebral cortex of the adult guinea pig. Fig. 1 represents the P4I, Fig. 2 the P1, and Figs. 3 and 4 the P5 respectively. For the denotation of these nuclear preparations, see text. Samples were stained with aceto-orcein-fast green. Numbers indicate scales in micra. Numbers indicate scales in micra. DNA was determined using the diphenylamine
reaction,\(^1\) and RNA by the orcinol reaction\(^2\) and by the ultraviolet absorption. DNA was determined also by fluorometry.\(^9\) Protein was determined according to the method of Lowry et al.\(^11\) Purified calf thymus DNA, yeast RNA and bovine serum albumin respectively were used as standards. Approximately 30 percent of DNA originally present in the filtered homogenate was recovered in the P4I preparation; recoveries of DNA in the P4II, P\(_L\) and P\(_S\) preparations were about 45, 8, and 8 percent respectively.

Practically no activities of succinate dehydrogenase\(^10\) and glutamate dehydrogenase\(^5\) were detected in the present preparations, indicating that these were essentially free from contamination of mitochondria. Activities on a wet weight basis of acetylcholinesterase and cholinesterase\(^3\) in these nuclear preparations were found to be less than 0.1 percent of the value exhibited by the filtered homogenate. Also the activity of the sodium-plus-potassium-stimulated adenosinetriphosphatase system was virtually absent. These observations were taken to indicate that the present nuclear preparations were essentially free of contaminants deriving from membrane fragments of microsomal origin and of nerve-ending particles (vid. references 6, 10, 12, and 13). The present nuclear preparation retained the activities of ATP:NMN adenylyltransferase (reference 9, assayed with minor modifications) and of the DNA-dependent RNA polymerase system (reference 14, assayed with modifications), indicating that no serious activity loss of these nuclear enzymes has occurred during the preparation.

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References