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Fractionation of Nuclei from Brain by Zonal Centrifugation and a Study of the Ribonucleic Acid Polymerase Activity in the Various Classes of Nuclei

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1. The nuclei of the cells of the whole rat brain have been fractionated in a B-XIV zonal rotor with a discontinuous gradient of sucrose. Five fractions were obtained. Zone (I) contained neuronal nuclei (70%) and astrocytic nuclei (23%). Zone (II) contained astrocytic nuclei (81%) and neuronal nuclei (15%). Zone (III) contained astrocytic nuclei (84%) and oligodendrocytic nuclei (15%). Zone (IV) contained oligodendrocytic nuclei (92%) and zone (V) contained only oligodendrocytic nuclei. 2. The content of DNA, RNA and protein per nucleus was determined for each zone. Although the amount of DNA per nucleus is constant (7pg) the RNA varies from 4.5 to 2.5pg/nucleus and the protein from 38 to 17.6pg/nucleus. The neuronal nuclei have the greatest amounts of protein. The oligodendrocytic nuclei have the least content of RNA and protein. 3. The effects of pH, ionic strength, and Mg^{2+} and Mn^{2+} concentration on the activity of the nuclear system for synthesis *in vitro* of RNA have been investigated for unfractionated nuclei. From these studies a standard set of conditions for the assay of nuclear RNA polymerase has been established. 4. The activity of the RNA polymerase in each of the zonal fractions has been determined in the presence and in the absence of α-amanitin. Zone (II) is the most active, followed by zone (I). The nuclei of zones (IV) and (V) have comparable activity, which is 40% of that of zone (II). 5. The extent of incorporation of each of the four labelled nucleoside triphosphates by the nuclei from each zone has been measured. These values have been used to calculate the base composition of the RNA synthesized *in vitro* in each class of nucleus. 6. The effect of changes in the condition of assay of RNA polymerase in the different classes of nuclei has been investigated. Significant differences in the response to concentrations of metal ions and ammonium sulphate have been observed. 7. Homopolymer formation in each zone of brain nuclei has been determined. The extent of formation of the four homopolymers roughly parallels the RNA polymerase activity.

Brain tissue has a rapid rate of synthesis and degradation of both protein and RNA. At present the relation of the metabolism of protein and RNA to brain function is unknown (Glassman, 1969). Biochemical investigations of the problem are hampered by the complexity of the cellular structure of nervous tissue. As a consequence homogenates either of whole brain or of regions of the brain contain subcellular particles derived from a wide variety of cells. The attempts to overcome this difficulty by separating the many types of cells that occur in the brain have met with limited success. We have sought an alternative solution by attempting to fractionate the nuclei of brain cells on a scale adequate to provide sufficient material for comprehensive biochemical investigations.

The nucleus, as the major site of RNA synthesis, plays a dominant role in RNA and protein metabolism. However, studies of unfractionated nuclei are of limited value. The investigations of the nuclei from rodent liver have revealed wide differences in the biochemical activities of the various classes of liver cell nuclei (Johnston *et al.*, 1968*a*; Haines *et al.*, 1969, 1970; Johnston & Mathias, 1972). Hence a study of the activities of brain nuclei must be preceded by the development of a method for their fractionation. In this paper we report a procedure for separating rat brain nuclei into five classes and some of the chemical and biochemical properties of these nuclei.

Experimental

Isolation of nuclei

The preparation of brain nuclei was carried out according to the method of Løvtrup-Rein & McEwen (1966), with several modifications. The rats (male, albino Wistar strain) were killed by cervical dislocation and the brains were removed from the animals immediately. Brains were cleaned from meninges, rinsed in ice-cold NaCl (0.9%) and blotted with filter paper. All subsequent operations were conducted at

4°C. The brain tissue was carefully minced with scissors and homogenized by hand with a gentle twisting motion in a Potter homogenizer with a Teflon pestle (200 μm clearance) in 22 strokes. Homogenization medium A (0.32M-sucrose containing 3mM-$MgCl_2$ and 1mM-sodium cacodylate buffer, pH6.4, at 4°C was used (15ml/g of brain). The homogenate was filtered through nylon mesh boulting cloth (mesh 110) and centrifuged for 10min at 850*g*. The crude nuclear pellet from 35ml of filtered homogenate was then resuspended by using a Teflon pestle in 12ml of medium B (2.4M-sucrose containing 1mM-$MgCl_2$ and 1mM-sodium cacodylate buffer, pH6.4). The final concentration of sucrose of the resuspended nuclei was approx. 2.15M. The nuclei were purified by centrifugation for 60min at 5°C and 53000*g*. The purified pellets were resuspended in a total of 20ml of medium B. In all experiments 12–14 animals were used, the brain tissues from individual animals being pooled. In a typical preparation, 14 rats, each 90g body wt., yielded 19.5–20g of brain tissue. The yield of nuclei based on recovery of DNA was 65–75%. Losses of nuclei occur at several stages, including 8–10% loss in the myelin plug, which forms during the 53000*g* spin, on the walls of various vessels employed and by damage to nuclei, mainly during homogenization.

In certain experiments rabbit brains were used. The cerebrum and cerebellum were examined separately. In experiments with the cerebrum, two rabbits, each 2½kg body wt., were used, yielding 10.9g of tissue. In experiments with the cerebellum eight rabbits, 1½–2½kg body wt., were used, yielding 8.8g of tissue.

Zonal centrifugation of nuclei

All runs were carried out at 8–10°C. A gradient of sucrose was introduced into the MSE B-XIV titanium rotor (Measuring and Scientific Equipment Co. Ltd., Manor Royal, Crawley, Sussex, U.K.), by using an Isco Dialagrad (model 380) gradient pump while the rotor was running at 3000rev./min in an MSE 65 Super Speed centrifuge. The rotor was precooled to 2–3°C and a current of cold air (5°C) was circulated through the centrifuge bowl during loading of the gradient. The pumping rate was varied from 17ml/min to 12ml/min, depending on the viscosity of the solution being pumped into the rotor. The gradient comprised discontinuous zones of 1.3M- (overlay, 70ml), 2.0M- (50ml), 2.3M- (100ml), 2.4M- (80ml), 2.5M- (100ml), 2.6M- (100ml) and 2.85M-sucrose (to fill the rotor), all containing 1mM-$MgCl_2$, 1mM-sodium cacodylate buffer, pH6.4, and at 5°C. To prepare 2.85M-sucrose, the appropriate quantity of sucrose was added to water at 100°C, containing $MgCl_2$ and sodium cacodylate, with vigorous stirring. As soon as the sucrose had completely dissolved the vessel was transferred to a refrigerator at 8°C. The pH of the solution was not adjusted until immediately before use to prevent crystallization. The sample of purified nuclei (equivalent to approx. 19.5g wet wt. of tissue) in 20ml of 2.4M-sucrose (medium B) was introduced into the gradient between the 2.3M and 2.4M zones. The rotor was operated for 2h at 8–10°C and 45000rev./min. The contents of the rotor were then displaced, while the rotor was running at 3000rev./min, with a 45% (w/v) solution of sodium metrizoate (Isopaque, Nyegaard and Co. A/S, Oslo, Norway) in 1M-sucrose, pH7.0 (density $\simeq$ 1.45). (Metrizoic acid is 3-acetamido-5-*N*-methylacetamido-2,4,6-tri-iodobenzoic acid.) The effluent was passed through a flow cell of 1cm path-length in a Gilford 2000 extinction meter and monitored at 600nm. Displacement lasted for approx. 45min. The pumping rate was again varied according to the viscosity of the effluent. The effluent was collected manually in 10ml fractions in ice-cold 40ml tubes. In some experiments the concentration of sucrose in each of the fractions recovered from the zonal rotor was checked by using an Abbe refractometer. Each fraction was diluted by the addition of 30ml of medium C (0.32M-sucrose, 1mM-$MgCl_2$ and 1mM-sodium cacodylate buffer, pH6.4). The number of nuclei in each tube was determined from a sample counted with a Coulter Counter model F with 100 μm orifice (aperture setting 16, attenuation 0.500, threshold 6.5). The main tubes comprising each peak were pooled to give a total volume of 240–280ml per peak. From 15 to 20ml of the combined fractions was used for each assay of RNA polymerase, depending on the total number of nuclei present.

Cytoplasmic enzyme activities

Glucose 6-phosphatase. The assay was similar to that described by Stetten & Ghosh (1971) and was conducted in a medium, containing in a total volume of 0.5ml, 0.25ml of 0.1M-sodium cacodylate buffer, pH6.5, 0.15ml of 0.08M-glucose 6-phosphate and 0.1ml of nuclear or microsomal sample (1.5×10^7–3.0×10^7 nuclei, microsomal preparation equivalent to approx. 100 μg of protein). The reaction was initiated by addition of substrate. The incubation was carried out at 37°C for 10min, during which time the activity was linear. The reaction was stopped by addition of 1.5ml of silicotungstic acid (10%). The precipitate was collected by centrifugation at 1400*g* for 15min. The P_i in the supernatant was estimated by the method of Chen *et al.* (1956).

NADH- and NADPH-cytochrome c reductase. This was determined by the method of Sottocasa *et al.* (1967), in a final volume of 1.3ml. The reaction was initiated with NADH or NADPH. The activity was measured spectrophotometrically at 30°C by following the reduction of cytochrome *c* at 550nm in a Unicam SP. 8000 u.v. recording spectrophotometer.

For each assay 2.0×10^7–3.0×10^7 nuclei were used and microsomal fraction and mitochondria containing approx. 300μg of protein.

In both enzyme assays nuclei were recovered from the pooled diluted zonal fractions as for the RNA polymerase assay.

RNA polymerase activity

In most experiments the standard procedure for measuring the activity of DNA-dependent RNA polymerase was by determining the incorporation of [5-^{3}H]UTP into an acid-insoluble form. Each assay was conducted in a system containing, in a total volume of 0.5ml (final concn.): tris–HCl buffer, pH8.3 (0.1M); $MnCl_2$ (3mM) or $MgCl_2$ (5mM); $(NH_4)_2SO_4$, pH8.3 (0.3M or 0.4M); dithiothreitol (1mM); NaF (6mM); GTP, ATP, CTP and UTP (1mM); [5-^{3}H]UTP (1μCi, sp. radioactivity 2mCi/mmol; The Radiochemical Centre, Amersham, Bucks., U.K.); approx. 4×10^6–2×10^7 nuclei. α-Amanatin (1.3μg; Boehringer Ingelheim Ltd., Middx., U.K.) and actinomycin D (25μg; Merck Sharp and Dohme Ltd., Hoddesdon, Herts., U.K.) were added as shown in the tables. The nuclei were recovered from the pooled, diluted zonal fractions by centrifugation at 1400*g* for 60min. Either 15 or 20ml of diluted zonal fractions was used, depending on the total number of nuclei present in these fractions. The exact number of nuclei present in each tube was determined by DNA analysis of nuclei recovered from a sample treated in the same manner. The reaction mixture, excluding both tritiated and unlabelled nucleoside triphosphates, was then added to the nuclei in each assay tube, and the tubes were preincubated at 37°C for 10min. The reaction was initiated by addition of a mixture of the tritiated and unlabelled nucleoside triphosphates. After the reaction mixture had been incubated at 37°C for 20min, the reaction was terminated by addition of 0.5ml of ice-cold trichloroacetic acid (10%, w/v, in 0.02M-sodium pyrophosphate). The precipitate was washed four times with 5ml portions of 5% (w/v) trichloroacetic acid and twice with 5ml portions of ethanol–chloroform (3:1, v/v). The final precipitate was dried and digested for 15min with 0.2ml of formic acid.

In experiments to determine the nucleoside triphosphate requirement, the reaction medium contained 1μCi of [5-^{3}H]UTP (sp. radioactivity 2mCi/mmol) in the presence of combinations of three nucleotides (ATP, CTP, UTP; GTP, CTP, UTP; ATP, GTP, UTP).

In one series of experiments the extents of incorporation of [8-^{3}H]ATP, [8-^{3}H]GTP and [5-^{3}H]-CTP (1μCi; sp. radioactivity 2mCi/mmol) were determined and compared with that of [5-^{3}H]UTP in a series of incubations in which only one labelled nucleoside triphosphate was present in each tube in the presence of all four unlabelled nucleotides.

The extent of homopolymer formation in fractionated and unfractionated nuclei was determined in a reaction medium that included one of the four nucleoside triphosphates (1mM) and 1μCi of the corresponding tritiated nucleoside triphosphate (sp. radioactivity 2mCi/mmol).

Ribonuclease activity

The activity was measured under two conditions, one of which corresponded to that for the assay of RNA polymerase. The assay was conducted in each case in a total volume of 0.5ml.

Medium 1 was a modification of that used by Villabos *et al.* (1964) and contained: 50μl of 0.25% yeast RNA (highly purified, Calbiochem Ltd., London W.1, U.K.) in 0.05M-tris–HCl, pH8.2; 350μl of 0.05M-tris–HCl buffer, pH8.2; 100μl of nuclear sample (approx. 3×10^7 nuclei/assay). Medium 2 contained (final concn.): 50μl of 0.25% yeast RNA in 0.05M-tris–HCl buffer, pH8.2; 0.1M-tris–HCl buffer, pH8.2; $MnCl_2$ (3mM); $(NH_4)_2SO_4$, pH8.3 (0.3M); dithiothreitol (1mM); NaF (6mM); approx. 3×10^7 nuclei. The reaction was initiated by addition of RNA. The incubation was carried out at 37°C for 30min. The reaction was terminated by addition of 0.5ml of 0.25M-$HClO_4$ in aq. 96% ethanol. The blank in each case was treated identically with the reaction mixture, but the reaction was stopped immediately after addition of RNA. After standing on ice for 15min, the precipitate was spun at 1400*g* for 15min. The extinction of the supernatants was read at 260nm. Nuclei were recovered from the pooled, diluted zonal fractions as described for the RNA polymerase assay.

Measurement of radioactivity

Scintillation fluid (10ml), containing 200g of naphthalene, 15g of 2,5-bis-(5-t-butylbenzoxazol-2-yl)thiophen (CIBA Ltd., Horsham, Sussex, U.K.) in 1500ml of toluene and 1000ml of 2-ethoxyethanol, was added to labelled nuclei after digestion of the precipitate by formic acid. Radioactivity of the vials was counted in a Beckman liquid-scintillation counter. The efficiency for ^{3}H was 22.5%.

Microscopy of nuclear preparations

Each nuclear preparation was examined under oil immersion with a phase-contrast microscope (Wild M20; Heerbrugg, Switzerland; Zeiss Photomicroscope). The percentage of each class of nucleus in each of the pooled zonal fractions was always checked to ensure that this remained constant. The criteria employed to define the different types of nuclei are

those described by Glees (1955), Nurnberger (1958), Nurnberger & Gordon (1957), Glees & Meller (1968) and Rappoport *et al.* (1969). The neuronal nuclei included all nuclei with a centrally located single nucleolus and pale chromatin. Large pale oval or round nuclei with two, three or more paracentral nucleoli are those of astrocytic origin, and somewhat smaller, round dense nuclei with tightly packed chromatin and peripheral nucleoli are nuclei of oligodendrocytes. Small very dense rod-shaped nuclei without clear nucleoli are considered to be of microglial origin but are not generally distinguishable from oligodendroglial nuclei.

Phase-contrast photographs were taken with either a Polaroid camera attachment to the Wild M20 microscope or by using the Zeiss Ultraphot II. Electron micrographs were taken on a Siemans Elmiskop I electron microscope. The isolated nuclei were resuspended in chilled glutaraldehyde–formaldehyde fixative and left at 4°C overnight. The suspension was spun in plasma tubes to pellet the nuclei, which were then post-fixed in Dalton's osmic acid for 45–60min (Dalton, 1955). Dehydration was carried out in graded dilutions of ethanol (30, 50, 70, 90 and 95%; two 10min washes in each) and three washes in dry ethanol for 1h. After immersion (2min) in epoxypropane the dehydrated pellet was embedded in Araldite at room temperature for 18h. The Araldite was cured in an oven at 60°C for 48h and silver sections were cut on a Porter–Blum ultramicrotome and laid on copper grids, before staining with lead citrate for 30min.

Nuclear counts

Nuclei from the purified sample and those in fractions obtained directly from the zonal rotor, and diluted with medium C, were suitably diluted with 0.9% saline (Baxter Laboratories Ltd., Thetford, Norfolk, U.K.) and counted in a Coulter Counter model F with 100μm orifice (aperture setting 16, attenuation 0.500, threshold 6.5). The number of nuclei per unit volume of nuclear suspension was checked in a haemocytometer chamber. To eliminate inaccuracies due to a minor but variable degree of clumping, the nuclear counts of nuclei recovered from zonal fractions were determined indirectly by DNA analysis.

Nuclear volumes

A drop of nuclear suspension from fractions obtained directly from the zonal rotor, and diluted with medium C, was placed in a Thoma haemocytometer chamber. The nuclear diameters were measured under oil immersion with the phase-contrast microscope and a Leitz Wetzlar eyepiece micrometer. Both the major and minor axes of the prolate spheroidal nuclei were measured. The volumes were calculated from $\pi(ab^2)/6$, where a is the length of the major axis and b the length of the minor axis. For most fractions approx. 200 nuclei were measured. The calculations of volumes were done with an Olivetti Programma 101.

Chemical analyses

Nucleic acids. The content of DNA and RNA was measured by adding perchloric acid (final concn. 0.2M) to a nuclear suspension from the pooled zonal effluent, diluted with medium C and containing a known number of nuclei. DNA in the precipitate was then measured by the method of Burton (1956) as described by Widnell & Tata (1964) and with a modification introduced by Croft & Lubran (1965). RNA was determined by the orcinol reaction.

Protein. A known number of nuclei was recovered from the zonal fractions and protein determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Results and Discussion

Separation and characterization of nuclei

There have been several reports of the existence of polyploidy in the nuclei from brain tissue (Lapham, 1963, 1968; Lentz & Lapham, 1969; Herman & Lapham, 1969). However, it seems clear that the vast majority of the cells comprising this tissue are diploid. Attempts to fractionate the isolated nuclei of these cells by techniques based on rate sedimentation met with little success. This finding is similar to our previous experience of the difficulty in obtaining complete separation of the diploid stromal from the diploid parenchymal nuclei of rat liver (Johnston *et al.*, 1968*b*). Accordingly we turned our attention to the possibility of exploiting small differences in the buoyant density of the brain nuclei to achieve this fractionation.

Zonal fractionation of brain cell nuclei. The first problem to be solved in the attempt to fractionate the brain cell nuclei by isopycnic centrifugation was the choice of a suitable gradient. A number of gradients were examined, including glycerol, glycerol–sucrose and a range of sucrose gradients. The most suitable gradient was found to be a discontinuous sucrose gradient resembling that described by Løvtrup-Rein & McEwen (1966). It was necessary to have an outer zone of sufficiently high density to prevent any nuclei moving through to the rim of the rotor. For this purpose 2.85M-sucrose was used. Because of the extremely high viscosity of this solution it was impractical to pump out the contents of the rotor. Consequently for displacement of the rotor

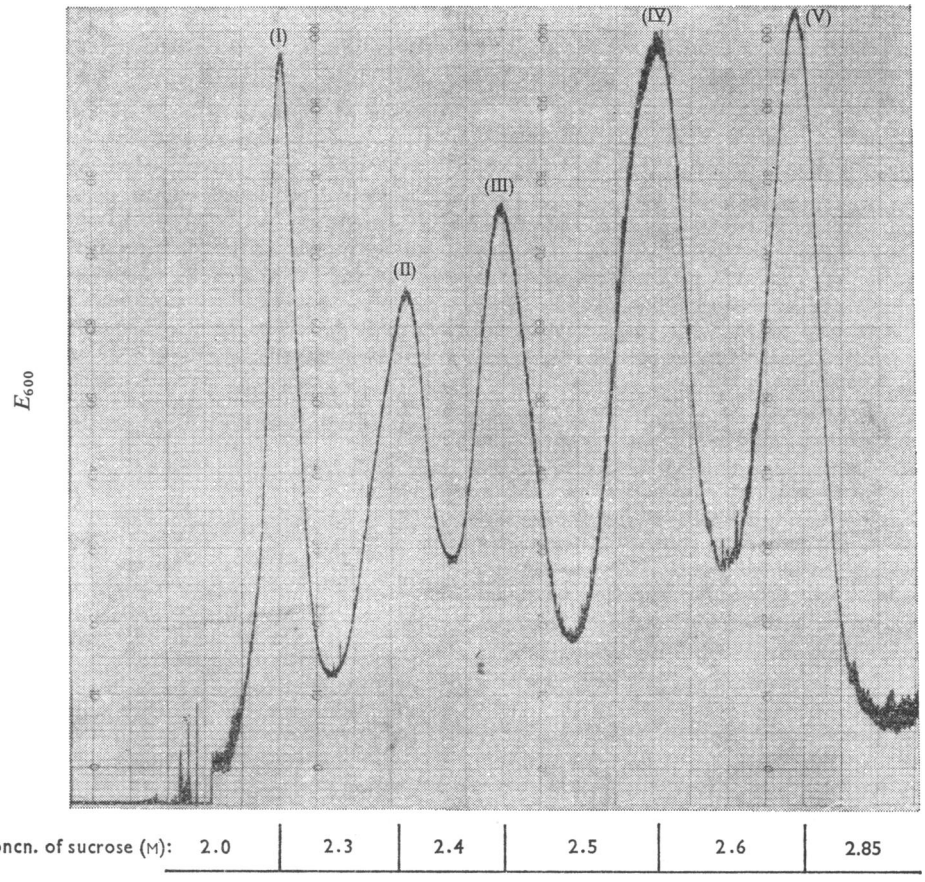


Fig. 1. *Chart of zonal profile obtained on emptying the rotor after running a sample of rat brain cell nuclei*

The brain nuclei were obtained from 14 male albino rats (90g body wt.), yielding 19.7g of brain tissue. The rotor was run at 45000rev./min for 2h at 10°C. The direction of sedimentation is from left to right. The peaks from left to right are zones (I), (II), (III), (IV) and (V) respectively. The ordinate gives extinction at 600ηm. The abscissa, indicating volume of effluent, with sucrose concentrations given below, is not linear with respect to volume of contents of rotor as the pump speed was varied during the emptying of the rotor.

contents a solution (45%, w/v) of Isopaque of high density and low viscosity in 1M-sucrose, pH7.0 (density $\simeq$ 1.45) was used. It was found that if the sample zone was introduced at the top of the gradient, a major proportion of the smaller glial nuclei were held back at the beginning of the gradient by the larger neuronal nuclei. This resulted in a high degree of contamination in the first zone. For this reason the sample was introduced between the 2.3M and 2.4M zones. This modification afforded a greater degree of purity in each of the resultant peaks. The chart obtained when nuclei from adult albino male rats weighing 90g were used is shown in Fig. 1. Five main zones of nuclei can be seen to form at the interfaces of the sucrose zones (I) 2.0M/2.3M, (II) 2.3M/2.4M, (III) 2.4M/2.5M, (IV) 2.5M/2.6M, (V) 2.6M/2.85M.

Characterization of the nuclei. The fractions corresponding to each zone were combined and identified microscopically according to the criteria summarized in the Experimental section (Plate 1). Zone (I) was found to comprise predominantly neuronal nuclei with a small proportion of astrocyte nuclei. Zones (II) and (III) contained mainly astrocyte nuclei, whereas zones (IV) and (V) are oligodendrocyte nuclei. The proportion of nuclei in each zone is given in Table 1, and the percentage of each class of nucleus in Table 2.

Table 1. *Total numbers of nuclei and percentage distribution of nuclei in the zones recovered after centrifugation*

Results are expressed as arithmetic mean values ± S.D. The number of experiments is shown in parentheses. In all experiments 14 male albino rats (90g), were used, yielding 19.7–20.4g of brain tissue.

Zone	10^{-6} × Total no. of nuclei (16)	Nuclei (% of total) (8)		
		Neurons	Astrocytes	Oligodendrocytes
(I)	169.6 ± 46.2	69.1 ± 8.4	22.9 ± 6.5	8.0 ± 4.6
(II)	152.2 ± 38.4	15.0 ± 4.7	80.8 ± 8.9	4.2 ± 4.2
(III)	228.6 ± 42.6	1.3 ± 0.7	84.0 ± 8.4	14.7 ± 2.0
(IV)	376.4 ± 75.3	0	8.5 ± 6.7	91.5 ± 6.7
(V)	349.1 ± 74.3	0	0	100

Table 2. *Total numbers of each class of nucleus and percentages of total nuclei in adult rat brain*

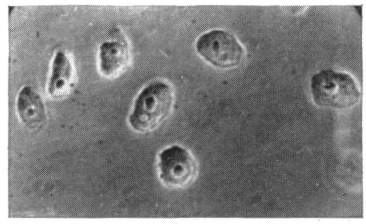
The values were calculated from the data in Table 1 on the assumptions that the neuronal nuclei in zones (I) and (II) were the same, and astrocytes in zone (I) were identical with those in zone (II) (astrocytes I), that the astrocytes in zones (III) and (IV) were the same (astrocytes II) and the oligodendrocyte nuclei in the first three zones were of both types.

Type of nucleus	10^{-6} × Total no. of nuclei	Nuclei (% of total)
Neuron	139.9	11.1 ± 2.3
Astrocyte I	162.3	12.7 ± 1.2
Astrocyte II	192.1	15.1 ± 2.1
Oligodendrocyte I	344.4	30.8 ± 3.0
Oligodendrocyte II	349.1	30.3 ± 3.9

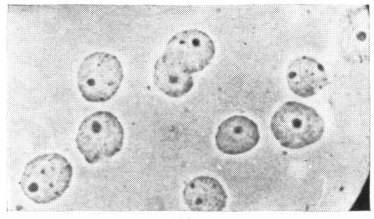
Reliable estimates of the proportion of the different types of cell in nervous tissue obtained from histological examination are not available. The nerve cells in the mammalian brain are embedded in perhaps ten times as many glial cells (Bremer, 1966) and oligodendrocytes are reported to be the most numerous cell in the brain (Galambos, 1964). Our results, although confirming these estimations, give a more accurate indication of the relative numbers of nuclei of each class found in the whole adult rat brain. Nuclear volumes in each zone were measured and plotted as histograms (Fig. 2). The amount of DNA, RNA and protein per nucleus in each of the main zones was determined. These results are given in Table 3.

The neuronal nuclei were mainly of irregular oval or round shape (see Plate 2) with one large clear central nucleolus and dispersed very pale chromatin, whereas the astrocyte nuclei, although of comparable size in many instances and also with pale, dispersed chromatin, were far more regular in their shape, being mainly round with smooth nuclear membranes and more than one prominent nucleolus. In certain instances there was difficulty in distinguishing between neuronal and astrocyte nuclei, particularly where only one of the nucleoli in the astrocyte nucleus was prominent. Size was not a suitable criterion for distinction because neuronal nuclei vary in volume from 350 to approx. 1000 μm^3 and astrocytes from 120 to 850 μm^3. However, the number of cases in which difficulty of identification arose was insignificant compared with the number of nuclei examined. Oligodendrocyte nuclei were small, round and dark with very condensed chromatin and several peripheral nucleoli. Those in zone (IV) of the zonal fractions had volumes from 120 to 200 μm^3; volumes of those in zone (V) were 50–126 μm^3. Microglial nuclei could not be identified specifically in any of the main fractions. The layer of granule cells in the cerebellum contains large numbers of small neurons (Eccles *et al.*, 1967), which have nuclei of diameter 5.5–7.0 μm (Lentz & Lapham, 1969). The nuclei contain condensed chromatin and small nucleoli (Shanthaveerappa & Bourne, 1965; Fox *et al.*, 1967). They are difficult to distinguish from the oligodendrocyte nuclei and in our fractionation are probably to be found in zones (III) and (IV). The isolated nuclei bear a strong resemblance to those of the intact tissue and appear to retain their characteristic morphology.

Attempts were made to fractionate nuclei from mouse brain and from rabbit cerebrum and cerebellum with a gradient similar to that applied to rat brain nuclei. Whereas the separation of mouse brain nuclei followed a pattern identical with that of the rat brain nuclei, giving similar results, the results obtained for the rabbit (Table 4) indicate that certain modifications to the gradient would be necessary to give a better separation, particularly in the least dense part of the gradient, because there is a relatively high number of neuronal nuclei in zone (II) and of astrocyte nuclei in zone (I). The very large neuronal nuclei (Purkinje cell nuclei in the cerebellum and Betz cell nuclei in the cerebrum) were always located in zone (I).



(*a*)



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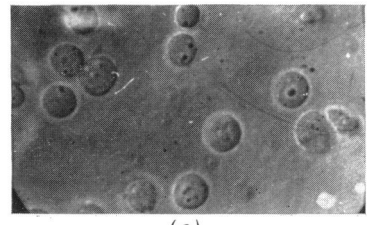

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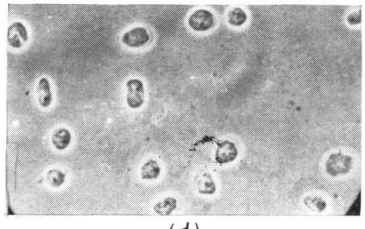

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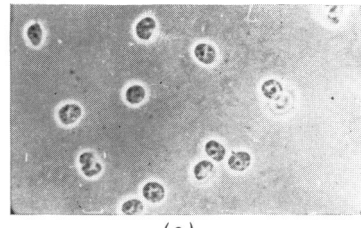
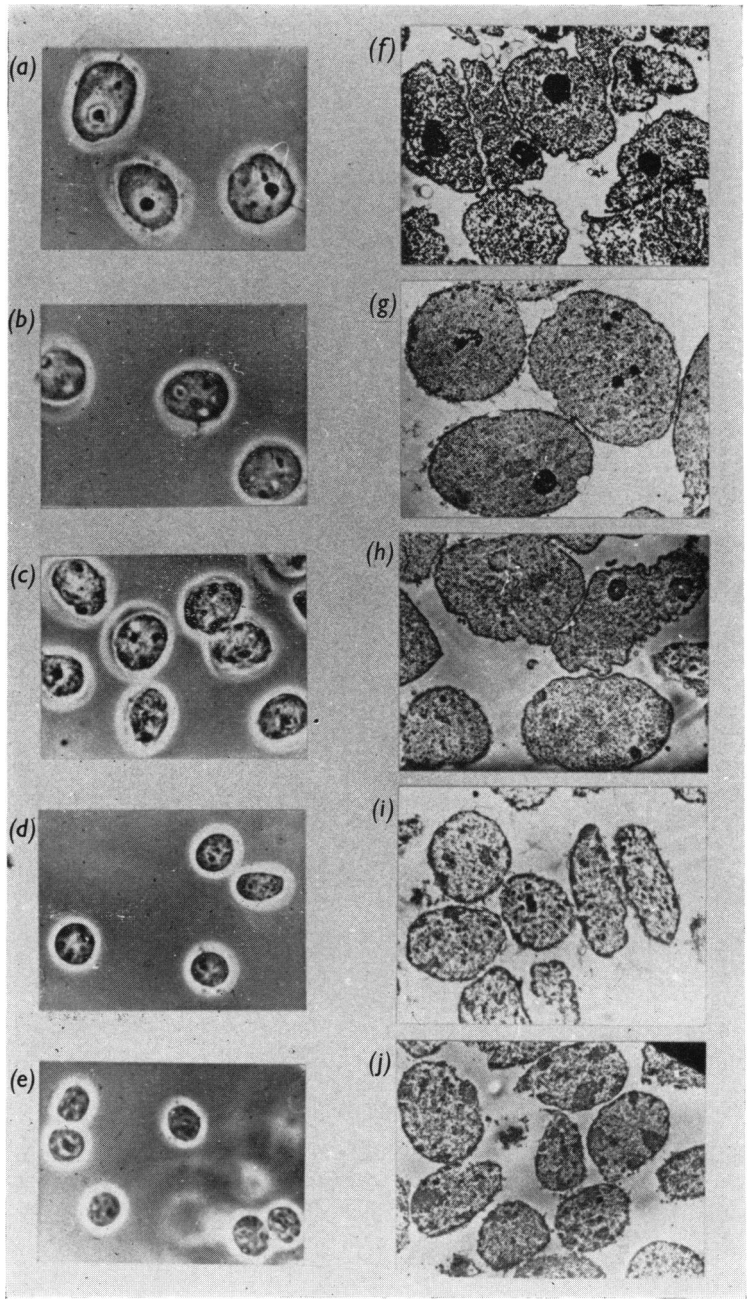


(*e*)

EXPLANATION OF PLATE 1

Unstained brain cell nuclei from rats (90*g body wt.*)

Photographs were taken under phase contrast with a Polaroid camera attachment to the Wild M20 microscope. The magnification is × 480. (*a*) Zone (I); (*b*) zone (II); (*c*) zone (III); (*d*) zone (IV); (*e*) zone (V).



EXPLANATION OF PLATE 2

Unstained brain cell nuclei from rats (90*g body wt.*)

Photographs were taken either under phase contrast with (*a–e*) a Zeiss Ultraphot II (magnification × 720) or by using (*f–j*) a Siemans Elmiskop I electron microscope (magnification × 3450). (*a*) and (*f*), Zone (I); (*b*) and (*g*), zone (II); (*c*) and (*h*), zone (III); (*d*) and (*i*), zone (IV); (*e*) and (*j*), zone (V).

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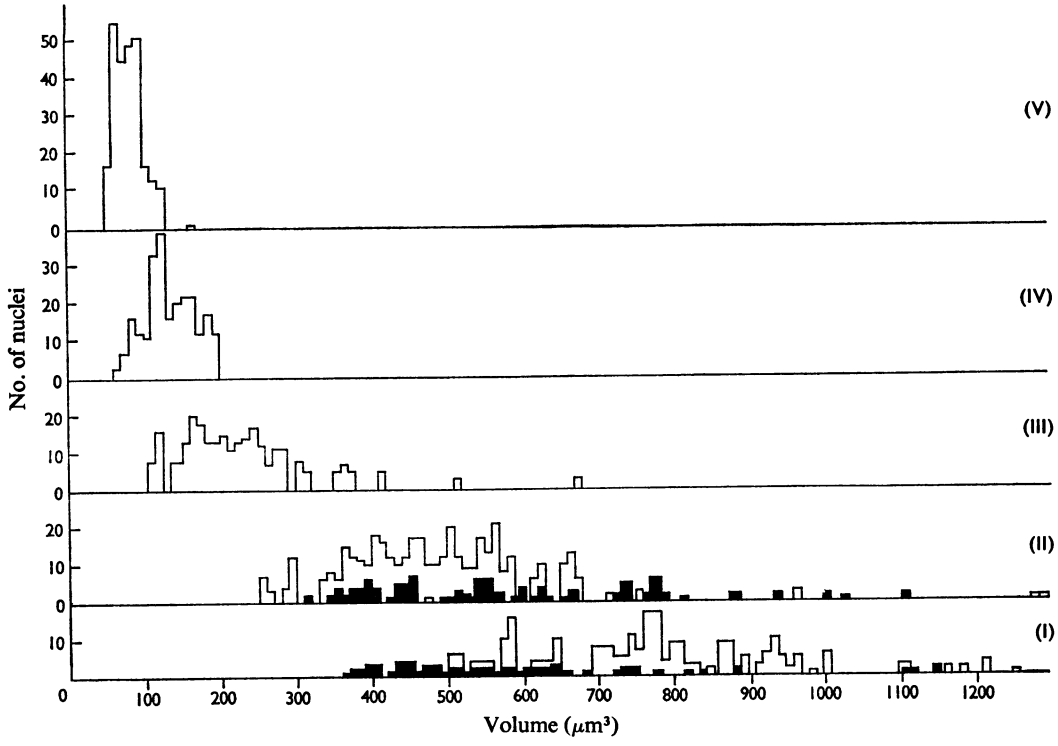

Fig. 2. *Histograms of the nuclear volumes of the nuclei from the five zones shown in Fig.* 1

The divisions on the ordinate each represent ten nuclei. Zones (I) to (V) are described in Fig. 1. For zone (I) the solid areas are for astrocyte nuclei and open areas are for neuronal nuclei. For zone (II) the solid areas are for neuronal nuclei and open areas are for astrocyte nuclei.

Table 3. *Content of DNA, RNA and protein of adult rat brain nuclei separated into zones by centrifugation*

The results are arithmetic mean values±S.D. from seven determinations.

	Content (pg/nucleus)			Ratios	
Zone	DNA	RNA	Protein	RNA/DNA	Protein/DNA
(I)	7.3±0.6	4.3±0.2	38.0±1.7	0.60±0.04	5.20±0.22
(II)	6.8±0.6	4.5±0.1	34.8±4.0	0.66±0.04	5.14±0.43
(III)	7.1±0.6	3.3±0.2	30.4±1.6	0.49±0.07	4.32±0.41
(IV)	7.2±0.7	2.6±0.3	18.3±0.8	0.36±0.01	2.47±0.35
(V)	7.4±0.3	2.5±0.1	17.6±1.8	0.32±0.01	2.40±0.35

All types of nuclei possess the same content of DNA, within the limits of experimental error. However, there are large differences in the amounts of protein and RNA present in the various classes. The neuronal nuclei have the highest amount of protein, considerably greater than either class of astrocytes and more than twice the content of protein in the oligodendrocyte nuclei. The range of the amounts of RNA present is not as great. The highest concentration is found in the astrocytes in zone (II). The oligodendrocytes in zones (IV) and (V) have virtually identical contents of RNA and protein, although they are distinguished by their mean volumes (Fig. 2). From the instant of homogenization, the nuclei which

Table 4. *Percentage distribution and total numbers of rabbit brain nuclei in the zones recovered after centrifugation*

The brains were obtained from two rabbits (each 2½kg), yielding 10.9g of cerebral tissue, and eight rabbits (1½–2½kg), yielding 8.8g of cerebellum. The results are approximate values. The nuclei in zone (III) from the cerebellum were difficult to identify. The nuclei from the granule cells may be located in this fraction. n.d., Not determined.

	Cerebrum				Cerebellum			
	Percentage distribution			10^{-6} × Total	Percentage distribution			10^{-6} × Total
Zone	Neurons	Astrocytes	Oligodendrocytes	no. of nuclei	Neurons	Astrocytes	Oligodendrocytes	no. of nuclei
(I)	65	25	10	118.1	50	40	10	263
(II)	42	42	16	90.5	40	50	10	80
(III)		75	20	68.2	n.d.	n.d.	n.d.	656
(IV)			>90	109.8			>90	486
(V)			98–100	117.9			98–100	355

heretofore had been in discrete and presumably different cytoplasmic environments are plunged into a common medium and are subjected to identical processes during purification and fractionation. Yet they retain marked differences in their chemical composition. This indicates that the protein and RNA components are firmly held within the nuclear membrane. Whilst within the intact cell many if not all of the cytoplasmic solutes of both high and low molecular weight may move freely in and out of the nucleus. These materials will be removed from the purified nuclei. Evidently the nuclei are characterized not only by morphological differences but also by major differences in the amounts of protein and RNA that they contain.

It is important to investigate the possibility that the variations in the ratios of both RNA and protein to DNA in the separated zones are due to different degrees of contamination. The contaminants most likely to contribute to high values of RNA and protein are the microsomal fraction and ribosomes or polyribosomes. Because of our method introducing the sample at an intermediate position in the stepped gradient, any free microsomal particles that are present, with buoyant densities of 1.14–1.2, will move upwards and accumulate in the overlay, whereas ribosomes would move towards the wall of the rotor. The difference in the amount of RNA per nucleus in zone (I) and zone (V) is 2pg. Since the ratio RNA/protein in rough microsomes is 1:5, the microsomal contamination would have a mass of 12pg/diploid quantity of DNA, if the difference in the RNA contents of zones (I) and (V) were attributable to contamination. This would mean that approx. 25% of the mass of zone (I) would have to be in the form of microsomal particles. Two lines of evidence eliminate this possibility. First, the examination of the material in zone (I) by electron microscopy shows the virtual absence of free microsomal particles or of any substantial numbers of cytoplasmic tabs attached to the nuclei, whose nuclear membranes appear to be mostly smooth. Secondly the results of the assay of two markers for microsomal particles in rat brain (Table 5) show that the contamination, if any, is uniform in the five separated zones. Although glucose 6-phosphatase is found in brain (Dodd *et al.*, 1971) more useful markers for endoplasmic reticulum appear to be NADH- or NADPH-cytochrome *c* reductase (Miller & Dawson, 1972).

The procedure devised by Løvtrup-Rein & McEwen (1966), which employs a discontinuous gradient with a total volume of 5ml in a swinging bucket rotor, gave rise to three nuclear fractions. The nuclei of astrocytes were found in a zone above that in which the neuronal nuclei were concentrated, and the oligodendrocyte nuclei formed a pellet. During the preparation of the nuclei they were exposed to Triton X-100. If the detergent treatment was omitted, the neuronal nuclei formed a band at lower density than the astrocytic nuclei (Burdman & Journey, 1969). A similar distribution of nuclei from rat cerebrum was found by Dravid & Duffy (1969). Separations in the zonal centrifuge give better resolutions and have the great advantage that quantities of material, adequate for biochemical investigations, can be handled.

RNA polymerase activity

Conditions for the assay. The nuclear DNA-dependent RNA polymerase has been solubilized and shown to exist in several forms (Roeder & Rutter, 1969, 1970; Kedinger *et al.*, 1970; Chesterton & Butterworth, 1971). These forms are characterized by differences in their response to Mn^{2+} and Mg^{2+} and to ionic strength. In addition they may be

Table 5. *Assay of cytoplasmic enzyme markers in zones of adult rat brains separated by zonal centrifugation*

Glucose 6-phosphatase activity is expressed as μmol of P_i liberated/10min per mg of protein. NADH- and NADPH-cytochrome *c* reductase activities are expressed as ΔE_{550}/10min per mg of protein.

	Activity		
Fraction	Glucose 6-phosphatase	NADH-cytochrome *c* reductase	NADPH-cytochrome *c* reductase
Liver microsomal fraction	1.41	18.44	4.06
Filtered homogenate	—	0.95	0.20
Post-850*g* supernatant	—	0.96	0.14
Mitochondria	—	0.55	0.07
Brain microsomal fraction	1.16	1.23	0.25
Unfractionated nuclei	0.10	0.15	0.03
Zone (I)	0.06	0.13	0.03
Zone (II)	0.07	0.10	0.02
Zone (III)	0.07	0.11	0.02
Zone (IV)	0.08	0.13	0.03
Zone (V)	0.06	0.11	0.03

differentiated by the extent to which they are inhibited by α-amanitin and by their localization in the nucleus. Enzyme form A, which prefers native DNA as a template, is localized in the nucleolus and is insensitive to α-amanitin. The nucleoplasmic enzyme, form B, is sensitive to α-amanitin and shows a preference for denatured DNA. It is presumed that the B form of the enzyme, which can be separated further into forms BI and BII (Kedinger *et al.*, 1971), is responsible for the synthesis of heterogeneous nuclear and messenger RNA and that the sensitivity to α-amanitin of the isolated enzyme is retained in the intact nucleus (Novello & Stirpe, 1969, 1970). Before measuring the relative activities of the different classes of brain nuclei it was necessary to establish optimum conditions for incorporation and the characteristics of the system for the synthesis of RNA in unfractionated nuclei. Some of these conditions were also examined on fractionated nuclei.

The activity of RNA polymerase in the unfractionated nuclei showed a fairly broad pH optimum, between pH8.2 and 8.8, by using a series of tris buffers. The rate of synthesis of RNA was virtually linear for the first 20min of reaction and then began to fall off and had nearly ceased after 40min. This pattern was observed no matter which of the nucleoside triphosphates was used in the labelled form. The responses of the brain nuclei to variations in the concentration of Mg^{2+}, Mn^{2+} and $(NH_4)_2SO_4$ resemble those of the nuclei of other tissues. The optimum conditions were found to be 3mM-Mn^{2+} in the presence of 0.4M-$(NH_4)_2SO_4$ and 5mM-Mg^{2+} with 0.3M-$(NH_4)_2SO_4$. Under these conditions the activity in the presence of Mn^{2+} was approximately three times that with Mg^{2+}. The rate of reaction was a linear function of the numbers of nuclei over a range from 2×10^6 to 4×10^7 nuclei per assay. In the presence of 3mM-$MnCl_2$ and 0.3M-$(NH_4)_2SO_4$ the incorporation was decreased to less than 10% of the maximal value by omitting any one of the four ribonucleoside triphosphates, or by the addition of α-amanitin (Table 6).

The ratio of the activities of enzyme form B to form A in the brains of 6-week-old rats was about 12:1 in comparison with approx. 2:1 at 1–2 months in the rat liver (Novello & Stirpe, 1970). This indicates that the RNA synthesized *in vitro* by the brain nuclei at high ionic strength is more DNA-like and that little ribosomal RNA synthesis in the nucleolus is taking place *in vitro* in brain in comparison with that in the liver.

The stability of the enzyme in unfractionated nuclei was examined over a period of 8h after isolation of the purified nuclei, under different conditions of sucrose concentration and temperature, in the presence and in the absence of α-amanitin (Table 6). The synthetic ability of the enzyme does not fall off significantly during this period and is relatively unaffected if the nuclei are suspended in concentrated sucrose (2.6M) at either 4°C or 11°C for this length of time. This indicates that the RNA polymerase activity in fractionated nuclei can be determined after zonal centrifugation without any major loss to the overall activity.

Activity in isolated and fractionated rat brain nuclei. The incorporation of ^{3}H-labelled UTP in the presence of all four unlabelled nucleotides was measured in each of the five zones resulting from zonal centrifugation of the isolated nuclei. The results are shown in Table 7. Major differences are apparent between the

Table 6. *Stability of RNA polymerase with time*

The reaction mixture included 3mM-$MnCl_2$ and 0.3M-$(NH_4)_2SO_4$. Incorporation of ^{3}H-labelled UTP was measured as an index of activity, which is expressed as $10^2 \times$ UTP incorporated (pmol/20min per μg of DNA). Approx. 2×10^7 nuclei were used per assay. The concentration of sucrose and the temperature at which the nuclei were kept before the RNA polymerase activity was determined are shown under conditions of incubation of nuclei. α-Amanitin (1.3μg in the final reaction medium) was used where indicated (+).

Conditions of incubation of nuclei			Activity			
Concn. of sucrose (M)	T (°C)	α-Amanitin	0h	3h	6h	8h
0.32	4	−	775	736	756	736
0.32	4	+	54	55	54	55
2.6	4	−	751	728	739	720
2.6	11	−	729	718	717	713

Table 7. *RNA polymerase activity in fractionated rat brain nuclei*

The incorporation of ^{3}H-labelled UTP (1μCi, specific radioactivity 2mCi/mmol) was measured in the presence of all four unlabelled nucleotides (final concn. 1mM each), 3mM-$MnCl_2$ and 0.3M-$(NH_4)_2SO_4$. Incorporation is expressed as $10^2 \times$ nucleotide incorporated (pmol/20min per μg of DNA). The results are expressed as arithmetic mean values ± S.D. for seven experiments.

Zone	Incorporation of nucleotide
(I)	777 ± 39
(II)	902 ± 8
(III)	521 ± 21
(IV)	394 ± 21
(V)	375 ± 34

Table 8. *Ribonuclease activity in fractionated brain nuclei*

Medium 1 contained yeast RNA and nuclei in tris-HCl buffer. Medium 2, which corresponds to the reaction mixture used for RNA polymerase studies excluding nucleotides, included yeast RNA, $MnCl_2$, $(NH_4)_2SO_4$, dithiothreitol, NaF and nuclei in tris-HCl buffer. For both media approx. 3×10^7 nuclei were used per assay.

Fraction	Activity (ΔE_{260}/30min per 10^9 nuclei) Medium 1	Medium 2
Unfractionated nuclei	8.6	3.6
Zone (I)	7.5	4.0
Zone (II)	11.8	3.1
Zone (III)	8.9	3.3
Zone (IV)	8.1	3.9
Zone (V)	6.5	3.2

three different types of nuclei, and also a large difference between the astrocytes in zone (II) and those in zone (III). That the much greater polymerase activity found in zones (I) and (II) is not due to elevated activity of ribonuclease in zones (IV) and (V) is shown in Table 8. The ribonuclease activity is lower in the medium used for assay of the polymerase than it is at the same pH but without $(NH_4)_2SO_4$, and is of a very low order. The activity per nucleus is virtually the same in all five zones.

The sensitivity of the nuclei to α-amanitin was investigated under conditions similar to those reported to be optimum for the purified rat liver polymerases (Novello & Stirpe, 1969), i.e. (for enzyme form A activity) 0.14M-KCl, 6mM-$MgCl_2$ and (for enzyme form B activity) 0.3M-$(NH_4)_2SO_4$, 3mM-$MnCl_2$. The activity in the absence of α-amanitin, under optimum conditions for form A, was determined. These results are shown in Table 9. The activity in all five zones when α-amanitin is present is less than 10% of maximum activity. The results under optimum conditions for form A (6mM-Mg^{2+}) are significantly lower than those for form B [3mM-Mn^{2+}, 0.3M-$(NH_4)_2SO_4$]. The incorporations when α-amanitin is present do not differ significantly under either set of conditions. These results indicate that most of the activity is due to form B of the enzyme and that little form A activity can be detected.

The estimations *in vitro* of the activity of RNA polymerase can be compared with the contents of RNA of the various types of nucleus shown in Table 3. The most active of the nuclei in the synthesis of RNA are the astrocytes of zone (II), which also have the highest content of RNA. The astrocytes in zone (III) have 60% of the RNA polymerase activity of

those in zone (II) and their content of RNA is 70% of that of the nuclei of zone (II). The oligodendrocytes peaks (zones IV and V) are of comparable activity and contain equivalent amounts of RNA. The ratio of the activity of the RNA polymerase in zone (II) to that of zone (V) is 2.4:1. The contents of RNA in the nuclei in these two zones are in the ratio 1.8:1. The content of RNA of any type of nucleus will be determined by the balance of a complex set of factors including the rates of synthesis and degradation within the nucleus and the rate of transport of RNA from the nucleus to the cytoplasm. In the cells of brain tissue a rough parallelism exists between the activity of RNA polymerase and the nuclear concentration of RNA.

The studies summarized in Table 7 of the incorporation of ^{3}H-labelled UTP revealed differences in the amount of UTP incorporated in a given time between the various classes of nuclei. It is of importance to determine whether or not these differences in rate are reflected in the types of RNA that are being synthesized. Because it was impractical to isolate the newly synthesized RNA and examine it either by physical methods or chemical analysis, we have resorted to an indirect method of determining the base composition of RNA synthesized *in vitro*. The incorporation of each of the four radioactively labelled nucleotides was measured in the presence of the three other unlabelled nucleotides in each of the five zonal fractions in the presence of Mn^{2+} and $(NH_4)_2SO_4$, either all in the same experiment or in parallel experiments with the incorporation of ^{3}H-labelled UTP measured as a standard of activity. The results of these experiments are shown in Table 9. Whereas the nuclei from zone (II) are the most active in the incorporation of UTP, CTP and ATP, those from zone (I) have the greatest activity with GTP. From experiments involving the uptake of labelled adenine and cytidine Løvtrup-Rein (1970) concluded that *in vivo* astrocytic nuclei and neuronal nuclei incorporated radioactive precursors into RNA to similar extents whereas the incorporation into glial nuclei occurred at only a quarter of the rate. The interpretation of this observation *in vivo* is complicated by uncertainties regarding turnover and pool sizes. A similar series of experiments was conducted in the presence of α-amanitin and results are also shown in Table 9. From the results in this table the base ratios (A+U)/(G+C), A/G and U/G were determined and are shown in Table 10. From the percentages of nuclei found in each zone (Table 1) and the results from Table 9, the values for RNA synthesis for each type of nucleus were calculated and are shown in Fig. 3. These calculations are based on the assumptions that the neuronal nuclei in zone (II) are the same as those in zone (I), the astrocyte nuclei in zones (I) and (II) are identical (astrocytes I) and those in zones (III) and (IV) are identical

Table 9. *RNA polymerase activity in fractionated rat brain nuclei, in the absence and in the presence of α-amanitin, with different labelled nucleotides*

In parallel experiments uptake of ^{3}H-labelled UTP was always measured so that results from different experiments could be compared directly. In the columns headed Mg^{2+}, 6mM-$MgCl_2$ and 0.15M-KCl were used. In the columns headed Mn^{2+}-$(NH_4)_2SO_4$, 3mM-$MnCl_2$ and 0.3M-$(NH_4)_2SO_4$ were used. The presence (+) or absence (−) of 1.3 μg of α-amanitin is indicated. ^{3}H-labelled UTP, ATP, CTP and GTP were each 1 μCi (sp. radioactivity 2mCi/mmol).

^{3}H-labelled precursor ...		$10^2 \times$ Incorporation of nucleotide (pmol/20min per μg of DNA)											
		UTP				ATP		CTP			GTP		
		Mg^{2+}		Mn^{2+}-$(NH_4)_2SO_4$		Mg^{2+}	Mn^{2+}-$(NH_4)_2SO_4$	Mg^{2+}	Mn^{2+}-$(NH_4)_2SO_4$		Mg^{2+}	Mn^{2+}-$(NH_4)_2SO_4$	
Zone	α-Amanitin ...	+	−	+	−	+	−	+	+	−	+	+	−
(I)		59	352	55	777	33	367	56	54	679	33	40	377
(II)		67	364	65	902	26	407	45	44	947	50	59	277
(III)		36	333	37	521	23	327	38	48	650	39	39	227
(IV)		15	139	24	394	22	211	33	33	389	27	27	166
(V)		25	87	34	375	25	199	31	31	412	17	19	171

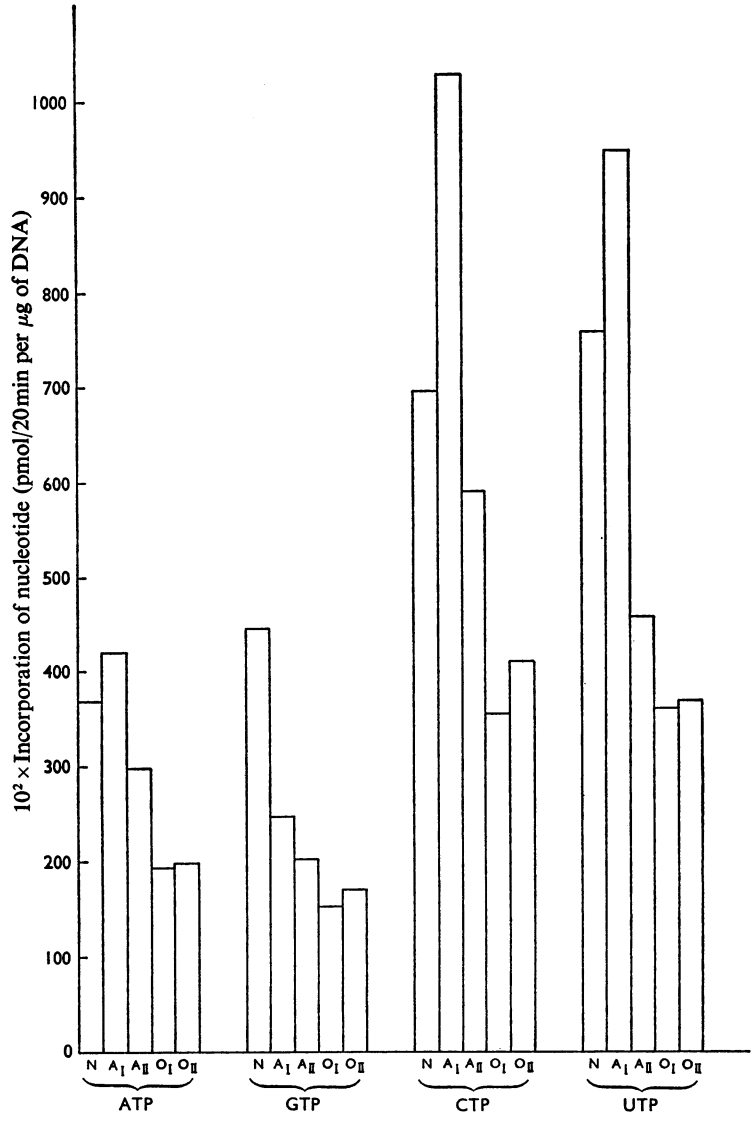

Fig. 3. *RNA polymerase activity in each type of nucleus in the rat brain*

The results shown here are calculated from those in Tables 1 and 9. The activity is expressed as 10^2 ×nucleotide incorporated (pmol/20min per μg of DNA). N, Neuronal nuclei; A_I and A_{II}, astrocyte nuclei; O_I and O_{II}, oligodendrocyte nuclei (see Table 1).

(astrocytes II). The contamination of the first three zones with oligodendrocytes was assumed to contain equal numbers of each type of oligodendrocyte. From these results the percentage of the total incorporation of each labelled precursor into each class of nucleus and the base ratios (A+U)/(G+C), A/G and U/G were calculated. The results are shown in Table 11.

The data in Table 11 give a guide to the relative rates of incorporation of the four nucleotides. All types of brain nuclei are characterized by high incorporation of C and U in comparison with A and G. Nuclei of astrocytes II and oligodendrocytes II have the highest G+C incorporation. There are distinct differences between the two classes of astrocytes. Astrocytes I have a high U and C incorporation

compared with that of astrocytes II. The two classes of oligodendrocyte nuclei are similar in overall chemical composition (Table 3) and incorporate the four ribonucleotide triphosphates in approximately the same proportions, although minor differences between them may exist. They differ in size.

Effect of variation of the condition of assay on the RNA polymerase activity of the fractionated nuclei. In the experiments discussed above, concerned with the activities of the RNA polymerase of the various classes of brain nuclei, standard conditions determined from investigations of unfractionated nuclei have been employed. This enables a direct comparison to be made of one type of nucleus with another, which has revealed significant differences in the enzyme activities and in the base composition of the RNA that has been synthesized. However, it is important to establish the extent to which these differences in the rates of RNA synthesis are dependent on the exact conditions of assay. Accordingly we have determined the effect of varying the $(NH_4)_2SO_4$ and $MnCl_2$ concentrations in each of the five zonal fractions and the results are shown in Figs. 4 and 5. In the presence of 3mM-$MnCl_2$ the nuclei from zone (I) and zone (III) have maximal activity with 0.4M-$(NH_4)_2SO_4$, which declines by 15% if the concentration of $(NH_4)_2SO_4$ is decreased to 0.3M. The other types of nuclei have their optima at 0.3M-$(NH_4)_2SO_4$. It is noteworthy that at 0.5M-$(NH_4)_2SO_4$ the neuronal nuclei display greatest activity, whereas activity of the astrocyte I nuclei has fallen to almost 50% of that of the neuronal nuclei and is less than that of astrocyte II nuclei. The rapid decline in the enzyme activity of neuronal and astrocytic nuclei at concentrations of $(NH_4)_2SO_4$ above 0.4M could be due to several effects of the very high ionic strength. It is possible that the enzyme is either being removed from the template or its structure is in some way being modified. Alternatively the high ionic strength could have an effect on other proteins in association with the template. The different patterns of the activation by $(NH_4)_2SO_4$ in each class of nucleus would suggest that differences exist in the physical relationship between the enzyme and deoxyribonucleoprotein. It is possible that different forms of the enzyme might exist in each type of nucleus. Alternatively there may be an alteration in the proportions of different forms of the enzyme in the various sorts of nuclei. The responses of the oligodendrocyte nuclei in zones (IV) and (V) to alterations in $(NH_4)_2SO_4$ concentration above 0.3M are in marked contrast to those of the other types of nuclei. In the presence of 0.3M-$(NH_4)_2SO_4$ all types of nuclei have maximal activity at 3mM-$MnCl_2$. It is noteworthy that the nuclei from zones (I) and (II) show significantly greater activation at 2mM-$MnCl_2$ than do nuclei of the other types. It is evident that although there are differences in the optimum conditions for the determination *in vitro* of the RNA polymerase of each of the various classes of brain nuclei, these are not sufficient to obliterate the marked

Table 10. *Base ratios for each zone of fractionated rat brain nuclei*

The results are calculated from those in Table 9 obtained in the presence of Mn^{2+}–$(NH_4)_2SO_4$, and in the absence of α-amanitin.

	Base ratios		
Zone	$\frac{A+U}{G+C}$	$\frac{A}{G}$	$\frac{U}{G}$
(I)	1.09	0.97	2.07
(II)	1.07	1.47	3.27
(III)	0.96	1.44	2.29
(IV)	1.09	1.27	2.38
(V)	0.98	1.17	2.17

Table 11. *Incorporation of the different ^3H-labelled precursors by each type of nucleus expressed as a percentage of the total and as calculated base ratios*

The results are calculated from those in Table 9 obtained in the presence of Mn^{2+}–$(NH_4)_2SO_4$ and in the absence of α-amanitin, and from Table 1 (percentage of each type of nucleus in each zone), so that the values given are for each class of nucleus rather than for each zone.

	Percentage incorporation				Base ratios		
Type of nucleus	ATP	GTP	CTP	UTP	$\frac{A+U}{G+C}$	$\frac{A}{G}$	$\frac{U}{G}$
Neurone	16.3	19.6	30.7	33.4	0.99	0.83	1.70
Astrocyte I	15.9	9.4	38.9	35.8	1.07	1.69	3.81
Astrocyte II	19.3	13.0	38.1	29.6	0.95	1.48	2.27
Oligodendrocyte I	18.2	14.3	33.5	34.0	1.09	1.27	2.38
Oligodendrocyte II	17.3	14.9	35.8	32.0	0.97	1.16	2.12

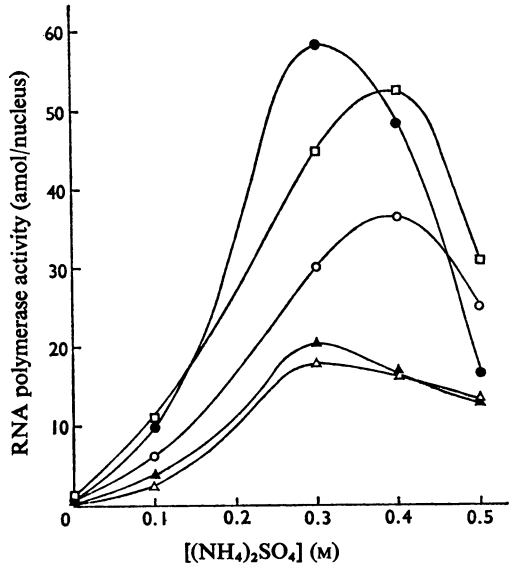

Fig. 4. *Effect of varying the concentration of ammonium sulphate on RNA polymerase activity in each of the five zonal fractions*

The reaction medium included 3mM-$MnCl_2$. Incorporation of ^{3}H-labelled UTP (sp. radioactivity 2mCi/mmol) was measured as an index of activity. Approx. 2×10^7 nuclei were used per assay. □, Zone (I); ●, zone (II); ○, zone (III); ▲, zone (IV); △, zone (V).

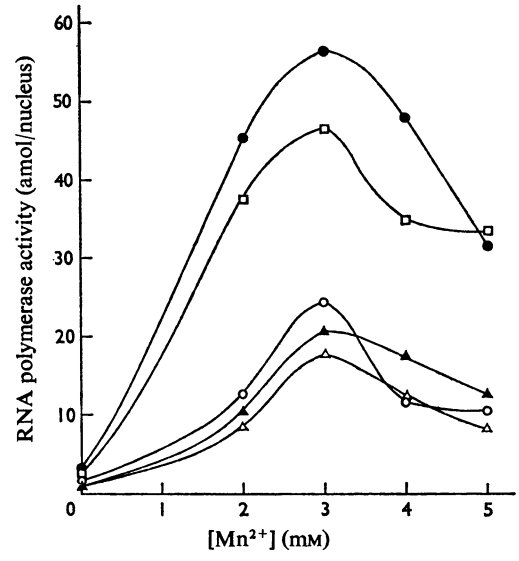

Fig. 5. *Effect of varying the concentration of manganese chloride on RNA polymerase activity in each of the five zonal fractions*

The reaction medium included 0.3M-$(NH_4)_2SO_4$. Incorporation of ^{3}H-labelled UTP (sp. radioactivity 2mCi/mmol) was measured as an index of activity. Approx. 2×10^7 nuclei were used per assay. □, Zone (I); ●, zone (II); ○, zone (III); ▲, zone (IV); △, zone (V).

disparities in their relative activities under our standard conditions. Nevertheless it is worth emphasizing that comparisons of the activities of different classes of nuclei conducted under a standard set of conditions may yield misleading results. This is because of the variable responses of nuclei from different types of cell to alterations in the concentration of metal ions, ionic strength and other factors that influence the rate of RNA synthesis. The physiological significance of any given set of conditions for the assay of RNA polymerase is difficult to assess. However, it will be seen from Figs. 4 and 5 that, except at very high concentrations of $(NH_4)_2SO_4$, the nuclei from the various zones retain marked differences in the activity of the enzyme over a wide range of conditions.

Formation of homopolymers in fractionated brain nuclei

The incorporations that we have observed, which require the simultaneous presence of the four ribonucleoside triphosphates, have all the characteristics of reactions catalysed by DNA-dependent RNA polymerase (nucleoside triphosphate–RNA nucleotidyltransferase, EC 2.7.7.6). However, it is possible that concomitantly with incorporations catalysed by this enzyme, there may be variable amounts of homopolymer formation (Edmonds, 1965).

The incorporation of single nucleoside triphosphates into unfractionated nuclei was determined and may be compared with the activity in the presence of all four nucleotides (Table 12). In certain instances α-amanitin was added to measure only enzyme form A activity. The conditions used here were those necessary for optimum activity in the presence of all four nucleotides.

For both the nuclei of whole brain (Table 12) and those that have been subjected to zonal fractionation (Table 13) the homopolymer formation represents 10% or less of the extent of incorporation of the corresponding ribonucleoside triphosphate in the presence of all four triphosphates. The homopolymer formation is insensitive to α-amanitin and is affected only to a minor extent by actinomycin D. It roughly parallels the RNA polymerase activity of the various classes of nuclei.

Table 12. *Rate of single and four nucleoside triphosphate reactions in unfractionated nuclei in the presence or absence of α-amanitin and actinomycin D*

The activity is expressed as $10^2 \times$ nucleotide incorporated (pmol/20min per μg of DNA). The reaction medium contained 3mM-$MnCl_2$ and 0.3M-$(NH_4)_2SO_4$. Approx. 2×10^7 nuclei were used per assay. α-Amanitin (1.3μg) and actinomycin D (25μg) were used where indicated. ^{3}H-labelled ATP, CTP, GTP and UTP were each 1μCi (sp. radioactivity 2mCi/mmol).

^{3}H-labelled precursor	Nucleotides	Activity		
		No addition	+α-Amanitin	+Actinomycin D
ATP	ATP, CTP, GTP, UTP	371	46	37
	ATP	45	42	30
CTP	ATP, CTP, GTP, UTP	762	53	54
	CTP	62	43	37
GTP	ATP, CTP, GTP, UTP	228	38	34
	GTP	16	10	10
UTP	ATP, CTP, GTP, UTP	741	55	33
	UTP	17	19	13

Table 13. *Rate of single nucleoside triphosphate reactions in fractionated nuclei in the presence or in the absence of α-amanitin*

The activity is expressed as $10^2 \times$ nucleotide incorporated (pmol/20min per μg of DNA). The reaction medium contained 3mM-$MnCl_2$, 0.3M-$(NH_4)_2SO_4$ and 1.3μg of α-amanitin (where included). ^{3}H-labelled ATP, GTP, CTP and UTP were each 1μCi (sp. radioactivity 2mCi/mmol).

Zone	^{3}H-labelled precursor ...	Activity				
		ATP		GTP	CTP	UTP
			+α-Amanitin			
(I)		95	92	15	93	30
(II)		88	100	17	99	40
(III)		82	83	14	54	18
(IV)		46	48	1	36	12
(V)		34	38	1	25	11

General discussion

We have been able to separate the nuclei isolated from the whole brain of young adult rats into five main classes. The quantities of nuclei obtained by our method, which involves isopycnic centrifugation in a zonal rotor, are adequate for chemical, biochemical and microscopic studies. Microscopic examination indicates that only one of these zones is pure. However, the maximum degree of contamination, which occurs in zone (I), does not exceed 30% and one zone (zone V) appears to contain only oligodendrocyte nuclei.

The difference in the ability of the different classes of nuclei to synthesize RNA is obviously of importance with regard to the functions of each type of nucleus. The separation of the nuclei into classes facilitates the study of their functional differences. It will be of particular value to extend these studies to fractionated nuclei from specific areas of the brain.

It is worthwhile to relate the differences in RNA polymerase activity to the morphological differences existing between the different classes of nuclei. It has been shown that in calf thymus lymphocytes the extended euchromatin fraction is capable of more active RNA formation than the dense heterochromatin fraction (Frenster *et al.*, 1963). This observation would seem to apply to brain cell nuclei, because neuronal and astrocyte I nuclei, which both have lightly staining, disperse chromatin, have higher RNA polymerase activity then small oligodendrocytes with dense tightly packed chromatin. Astrocyte II nuclei, although having more dense chromatin

than neuronal nuclei or nuclei of astrocytes I, and more euchromatin than oligodendrocytes, show intermediate RNA polymerase activity.

Base ratios for the various RNA species in rat brain have been reported (Mahler *et al.*, 1966; Jacob *et al.*, 1966). The base ratio (A+U)/(G+C) for ribosomal RNA from rat brain cortex is 0.576, that of ribosomal RNA derived from cerebral cortex, cerebellum and brain stem is 0.661; tRNA has the ratio 0.653; that of rapidly labelled RNA (presumably mRNA) is 1.13. Our results for all types of brain nuclei most closely approximate to the last-named, i.e. the rapidly labelled or so called 'DNA-like' RNA. Oligodendrocytes II and astrocytes II show slightly more resemblance to base ratios for rRNA, but are generally more like rapidly labelled RNA. This conclusion concerning the type of RNA that is synthesized *in vitro* can be compared with the results from studies of the RNA synthesis in the absence and presence of α-amanitin. Polymerase form B (nucleoplasmic enzyme) is responsible for synthesis of mRNA or heterogeneous nuclear RNA, whereas polymerase form A (nucleolar enzyme) synthesizes rRNA. In the presence of α-amanitin, which inhibits polymerase B, our results have shown that less than 10% of the activity that we are measuring under optimum conditions is due to enzyme form A; i.e. we are measuring very little synthesis of rRNA. Most of the RNA being synthesized is heterogeneous RNA or mRNA, a conclusion in agreement with the base-ratio studies. The argument that a higher proportion of the total RNA synthesis of brain is devoted to messenger RNA synthesis rather than ribosomal RNA in comparison with liver is supported by observations on the activity of brain RNA in stimulating protein synthesis (Bondy & Roberts, 1967) and hybridization experiments (Bondy & Roberts, 1968).

It is important, however, to consider the high concentrations of $(NH_4)_2SO_4$ necessary to activate the enzyme. Pogo *et al.* (1967) demonstrated a changing pattern of RNA synthesis in liver nuclei treated with Mn^{2+} and $(NH_4)_2SO_4$. Under conditions of high ionic strength they showed that the RNA being synthesized is more 'DNA-like'. These results suggest that at high ionic strength a different set of loci on the DNA template are exposed for transcription and one would expect a much larger proportion of 'DNA-like' RNA to be synthesized. This is the finding with brain cell nuclei.

Bondy & Waelsch (1965) reported higher activity of RNA polymerase in brain than in liver. The investigations of Kato & Kurokawa (1970) indicated that the reaction requiring all four ribonucleoside triphosphates is more active in neuronal nuclei than in glial nuclei and that in liver nuclei the rate of reaction is much lower than in various types of cerebral nuclei. The extent of activation with $(NH_4)_2SO_4$ is less in liver than in brain. Brain nuclei undergo discernible changes in $(NH_4)_2SO_4$ at concentrations greater than 0.1 M: the nuclei clump into a gel. This does not happen with liver nuclei. The observations by these two groups, although less detailed than our studies, are in good agreement with them.

It is possible that concomitant with RNA synthesis homopolymer formation may occur. Dravid *et al.* (1971) have demonstrated the presence of an ATP-polymerizing enzyme in rat brain nuclei independent of DNA and primed by poly(A) itself. The presence of $(NH_4)_2SO_4$, or GTP, UTP or CTP, was inhibitory. Darnell *et al.* (1971) have shown an A-rich sequence in mRNA of HeLa cells and Edmonds *et al.* (1971) have demonstrated poly(A) sequences in the heterogeneous nuclear RNA and rapidly labelled polyribosomal RNA of HeLa cells. This poly(A) accounts for about 0.5% of such RNA. Kato & Kurokawa (1970) in their studies of poly(A) synthesis in partially fractionated brain cell nuclei found that GTP, CTP and UTP inhibited the poly(A) synthesis and promoted RNA synthesis. They attribute a high rate of incorporation of CTP to neurones. Formation of poly(C) has been claimed in an aggregate enzyme from cerebral nuclei (Mandel *et al.*, 1967). Daneholt & Brattgård (1966) showed higher rates of incorporation of [^{3}H]cytidine into nerve cells and [^{3}H]adenine into glial cells *in vivo*.

Our results for unfractionated and fractionated nuclei show relatively high poly(C) and poly(A) synthesis in both neuronal and glial nuclei, compared with poly(U) and poly(G) synthesis. It is noteworthy that poly(A) synthesis is the same in the presence and absence of α-amanitin. Edmonds *et al.* (1971) showed no poly(A) synthesis in the nucleolus and no poly(A) sequences in rRNA. We can therefore conclude that poly(A) is synthesized independently of RNA polymerase. It is therefore difficult to assess the extent to which homopolymer formation contributes to RNA synthesis under normal conditions. It is not possible to relate, for example, high incorporation of C into RNA to high poly(C) synthesis, because the likelihood is that, in the presence of the other nucleoside triphosphates, poly(C) synthesis is inhibited. The investigation of the natural occurrence of these homopolymers in the different types of nuclei would elucidate the extent to which any homopolymer formation contributes to the overall RNA synthesis both *in vivo* and *in vitro*.

The studies reported in this paper have revealed major differences between the various classes of brain nuclei that we have separated, in their overall chemical composition and in the activity of an important nuclear enzyme system. These differences, coupled with the retention by the isolated nuclei of their characteristic appearances, support the view that isolation of the nuclei does not lead to loss of

differentiation. The delineation of the factors that control the activities of the brain nuclei must await extensive studies of RNA synthesis *in vivo*, of the activities of other nuclear enzymes and of the types and relative amounts of nuclear proteins.

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