The Activity of Deoxyribonucleic Acid Polymerase and Deoxyribonucleic Acid Synthesis in Nuclei from Brain Fractionated by Zonal Centrifugation

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(Received 21 May 1973)

1. The DNA polymerase (EC 2.7.7.7) activity in purified intact brain nuclei from infant rats was investigated. The effects of pH, Mg^{2+} , glycerol, sonication and storage of the nuclei under different conditions were examined and a suitable assay system was established. 2. The nuclei from infant brain cells were fractionated by zonal centrifugation in a discontinuous sucrose gradient into five zones: zone (I) contained neuronal nuclei (59%) and astrocytic nuclei (41%); zone (II) contained astrocytic nuclei (81%) and neuronal nuclei (19%); zone (III) contained astrocytic nuclei (82%) and oligodendrocytic nuclei (18%); zone (IV) contained oligodendrocytic nuclei (92%) and zone (V) contained oligodendrocytic nuclei (100%). 3. The content of DNA, RNA and protein for each fraction was measured. 4. The distribution of DNA polymerase activity in the fractionated infant and adult rat brain nuclei was determined. The highest activity was found in the neuronal nuclei from zone (I) and the following zones exhibited a progressive decline. In contrast with the nuclei from infant rats those from adults had a much higher activity and expressed a preference for native DNA as template. 5. The deoxyribonuclease activity in all classes of nuclei was measured with [3H]DNA as substrate. A general correspondence in the pattern of the relative activities in the nuclear fractions with the distribution of DNA polymerase was found. 6. The incorporation of [3H]thymidine into nuclear DNA in infant and adult rat brain was investigated. The specific radioactivity of the DNA in the 10-day-old rats was highest in zone (V) whereas in the nuclei of adult rats, which exhibited a comparatively low incorporation, the highest specific radioactivity was associated with zones (I) and (V).

The study of the cellular development of brain tissue is of great importance in uncovering the functions of the many types of cells that this tissue contains. According to some reports (Fujita, 1967; Altmann, 1969) the proliferation of the neuronal cells ceases shortly after birth whereas the glial cells retain the capacity for division.

It has been shown that the total DNA content of the rat brain increases up to 20 days of age and remains constant thereafter (Mandel *et al.*, 1964; Adams, 1966; Sung, 1969; Mori *et al.*, 1970). These early changes in the rate of DNA synthesis mainly reflect the cellular proliferation of the brain (Mandel *et al.*, 1964; Lentz & Lapham, 1969; Giuditta, 1971) and are accompanied by similar variations in the amount or activity of the enzyme responsible for DNA replication (Brasel *et al.*, 1970; Chiu & Sung, 1970; Giuffrida *et al.*, 1972). The bulk of cellular DNA is present in the nuclei, where also its biosynthesis occurs. Therefore the biological activity of the nuclear DNA polymerases and the means of its regulation are of particular interest.

Rat brain DNA polymerases in total brain tissue

and nuclear extracts have been investigated in some detail (Chiu & Sung, 1971, 1972a,b; Murthy & Bharucha, 1971; Giuffrida *et al.*, 1970; Bharucha & Murthy, 1971). The use of unfractionated nuclear or whole-tissue extracts could be a major complication for interpretation of the experimental results owing to the heterogeneity of type of cells in the brain.

A proper understanding of the biological role and control of nuclear DNA polymerase activity would be facilitated by investigations of the enzyme within the intact fractionated nuclei at different stages of their development. In view of this, in the present studies some of the general properties of the DNA polymerase in intact brain nuclei have been investigated together with the distribution of DNA polymerase activity in the various nuclear types at different stages of cell development.

Experimental

Reagents

Unlabelled deoxyribonucleoside triphosphates were obtained from Boehringer (Mannheim) Ltd.,

Ealing, Middx., U.K. [^3H]Thymidine 5'-triphosphate (sp. radioactivity 23.9 Ci/mmol) and [^3H]thymidine (sp. radioactivity 15 Ci/mmol) were supplied by The Radiochemical Centre, Amersham, Bucks., U.K. Calf thymus DNA (type I) and crystalline pancreatic ribonuclease were purchased from Sigma (London) Chemical Co., London, S.W.6, U.K. Highly purified yeast RNA and Pronase were obtained from Calbiochem Ltd., London W.1, U.K. Sodium metrizoate (Isopaque) was supplied by Nyegaard and Co., A/S, Oslo, Norway.

^3H -labelled HeLa-cell DNA (sp. radioactivity $2\mu\text{Ci}/\text{mg}$) was prepared by exposing HeLa cells to [^3H]thymidine for 72 h and subsequent isolation of the DNA by the method of Gilmour and Paul (1968). The cultures of HeLa cells were grown by Professor K. R. Rees.

Isolation and fractionation of nuclei

Unless otherwise indicated, all operations were carried out at $0-4^\circ\text{C}$. Albino rats of the Wistar strain of ages and weights specified in the text were used.

The animals were killed by decapitation or cervical dislocation. The brains were removed immediately and pooled to obtain samples of approx. 10 g for unfractionated preparations and 25 g for zonal fractionation. The nuclei were isolated by the method of Løvtrup-Rein & McEwen (1966) as modified by Austoker *et al.* (1972). The preparations were purified by centrifugation through 2.0 M-sucrose, 1 mM with respect to MgCl_2 , 1 mM-sodium cacodylate buffer, pH 6.4, for 60 min at 50000g. The yield of nuclei was approximately 100×10^6 nuclei/g of brain tissue. Phase-contrast microscopy showed the nuclei to be intact and free of cytoplasmic contamination.

The nuclei were separated into classes by zonal centrifugation in a discontinuous gradient ranging from 2.0 to 2.8 M-sucrose as described by Austoker *et al.* (1972).

The contents of the rotor were displaced with a 45% (w/v) solution of sodium metrizoate in 1 M-sucrose and passed through a flow cell of 1 cm path length in a Gilford 2000 extinction meter and monitored at 600 nm. The effluent was collected manually in large fractions corresponding to the single peaks. Nuclei were recovered by first diluting the fractions 1:4 (v/v) with 0.32 M-sucrose, 1 mM with respect to MgCl_2 , pH 6.4. Samples containing suitable numbers of nuclei were sedimented by centrifugation, resuspended in a small volume of 0.32 M-sucrose and used for enzymic or chemical assays on the same day of their preparation, unless otherwise stated.

The integrity and purity of the nuclear fractions were checked with a phase-contrast microscope (type M20; Wild, Heerbrugg, Switzerland). The criteria for identification of the different types of nuclei were those used by Austoker *et al.* (1972).

Counting of the nuclei

The nuclei were counted immediately after suitable dilution with 0.9% (w/v) NaCl (Baxter Laboratories Ltd., Thetford, Norfolk, U.K.) in a Coulter Counter model F with a 100 μm orifice tube (aperture setting 16; attenuation 0.500, threshold 6.5).

The nuclear counts were also determined indirectly by DNA analysis.

Extraction of 'soluble' and 'particulate' forms of DNA polymerase

These were obtained from the nuclear preparations by the method of Chiu and Sung (1972a).

DNA polymerase activity

DNA polymerase activity was assayed by the initial rate of incorporation of [^3H]thymidine 5'-triphosphate into an acid-insoluble product.

The standard incubation mixture contained in a volume of 0.25 ml: 50 nmol each of dATP, dGTP, dCTP and [^3H]dTTP (sp. radioactivity $40\mu\text{Ci}/\mu\text{mol}$), 150 μg of calf thymus DNA, 15 μmol of Tris-HCl buffer, pH 8.5, 1.25 μmol of MgCl_2 , 0.5 μmol of ATP, 0.25 μmol of 2-mercaptoethanol and a suitable amount of nuclei.

The incubation was carried out at 37°C for 30 min with continuous shaking. The reaction was terminated by the addition of 0.5 ml of ice-cold 0.1 M-sodium pyrophosphate followed by 1 ml of 17% (w/v) trichloroacetic acid. The resulting precipitate was washed three times with 5% (w/v) trichloroacetic acid, twice with ethanol-chloroform (3:1, v/v), dried and dissolved in 0.2 ml of 98% formic acid.

Each reaction was performed in triplicate. Assays with both native and denatured DNA as template were run simultaneously.

The time-course of incorporation of [^3H]thymidine 5'-triphosphate was tested with four different amounts of nuclei for periods of 10, 15, 20, 30, 60 and 120 min of incubation, with both native and denatured DNA. The incubation time of 30 min and protein concentrations of 600 μg (approx. 12×10^6 nuclei) or less were used in most experiments so as to be well within the limits of linearity.

One unit of DNA polymerase activity was defined as the incorporation of 1 pmol of [^3H]TTP into acid-insoluble product in 1 min at 37°C . Specific activity was expressed as units per mg of DNA.

Deoxyribonuclease activity

The activity was measured by following the release of radioactive acid-soluble fragments from native or denatured [^3H]DNA.

The incubation was carried out under the standard

conditions for the assay of DNA polymerase in the presence of the four deoxyribonucleoside triphosphates and ^3H -labelled DNA as substrate. The reaction was linear with respect to release of ^3H -labelled acid-soluble material for at least 60 min. It was terminated by the addition of 0.2 ml of non-radioactive carrier DNA (2 mg/ml) and 0.5 ml of ice-cold 0.5 M HClO_4 . After 30 min at 0°C the precipitate was removed by centrifugation and the radioactivity of the released acid-soluble products was measured.

One unit of deoxyribonuclease activity was defined as $1\text{ }\mu\text{g}$ of ^3H DNA rendered acid-soluble/min at 37°C . Specific activity was expressed as units per mg of DNA.

dATPase (deoxyadenosine triphosphate)

dATPase activity was assayed by the liberation of P_i from dATP used as substrate. The incubation mixture was as described by Chiu & Sung (1970), with minor modifications. The supernatant was analysed for P_i by the method of Weil-Malherbe & Green (1951).

One unit of dATPase activity was defined as the production of $1\text{ }\mu\text{mol}$ of P_i /min at 37°C . Specific activity was expressed as units per mg of nuclear DNA.

Incorporation of ^3H thymidine into nuclear DNA in vivo

The animals (either 25 10-day-old or 14 adult rats) were injected intraperitoneally with $2\text{ }\mu\text{Ci}$ of ^3H -thymidine (sp. radioactivity 15 Ci/mmol)/g body wt. Adult male rats were also injected intracisternally under ether anaesthesia with $0.3\text{ }\mu\text{Ci}$ of ^3H -thymidine/g body wt., and 12 h after injection the rats were killed by decapitation or cervical dislocation. The brains were pooled and the nuclei purified and fractionated.

Samples of the nuclei recovered after the centrifugation were washed three times with 5% (w/v) trichloroacetic acid, twice with ethanol-chloroform (3:1, v/v), dried and digested with 0.2 ml of 98% formic acid.

Measurement of radioactivity

The samples dissolved in formic acid were mixed with 10 ml of scintillation solution containing 200 g of naphthalene, 15 g of BBOT [2,5-bis-(5-*t*-butylbenzoxazol-2-yl)thiophen] (CIBA Ltd., Horsham, Sussex, U.K.) in 1500 ml of toluene and 1000 ml of 2-ethoxyethanol.

Radioactivity was determined by using a Beckman or Intertechnique liquid-scintillation spectrometer with 23% counting efficiency for ^3H . Corrections

for differences in quenching among the samples were performed by external standardization.

Chemical analysis

Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. RNA was determined by the orcinol reaction (Mejbaum, 1939) with purified yeast RNA as standard. DNA was measured by the two-wavelength method of Spirin (1958) or by the diphenylamine reaction as modified by Croft & Lubran (1965). All optical measurements were made with a Gilford 2000 extinction meter.

Results

Unfractionated brain nuclei from 10-day-old rats

DNA polymerase activity. (i) Reaction requirements. The intact nuclei incorporated deoxyribonucleotides into an acid-insoluble product (Table 1). The activity was strongly dependent on the presence of exogenous

Table 1. *Requirements for DNA polymerase activity in unfractionated nuclei of 10-day-old rats*

The nuclei (12×10^6 , $70\text{ }\mu\text{g}$ of DNA) were assayed for DNA polymerase activity by the initial rate of incorporation of $[\text{Me-}^3\text{H}]$ thymidine 5'-triphosphate ($1\text{ }\mu\text{Ci}$, sp. radioactivity $40\text{ }\mu\text{Ci}/\mu\text{mol}$) into an acid-insoluble product as described in the Experimental section. The results are expressed as percentages of the activity of the complete assay system, which incorporated 2.0 pmol of ^3H dTTP/min with native DNA and 4.2 pmol of ^3H dTTP/min with heat-denatured DNA as template.

Assay system	DNA polymerase activity (% of incorporation)	
	Native DNA	Denatured DNA
Complete	100	100
-DNA	16.5	8.1
- MgCl_2	45.8	29.2
-SH groups	93.3	77.7
-dATP	62.6	47.0
-dGTP	71.5	53.5
-dCTP	66.2	44.9
-dATP, dGTP and dCTP	54.4	33.5
-ATP	86.9	49.5
+Phosphoenolpyruvate ($1\text{ }\mu\text{mol}$) and pyruvate kinase ($5\text{ }\mu\text{g}$)	96.9	85.1
+Glycerol ($300\text{ }\mu\text{mol}$)	121.0	117.0
Blank	16.0	9.0

DNA as a template. When it was omitted the incorporation was decreased to 10% of that in the complete system. The enzyme utilized either native or heat-denatured DNA, with preference for the denatured DNA. For maximum incorporation all four deoxyribonucleoside triphosphates were required. However, in common with other nuclear preparations, the DNA polymerase exhibited a high degree of activity in the absence of one or three of the deoxynucleoside triphosphates. The extent of the decrease varied depending on the triphosphate omitted and was more obvious with denatured DNA as template. Thiol compounds, such as 2-mercaptoethanol or dithiothreitol, slightly increased the enzyme activity. ATP stimulated the activity with heat-denatured template by 50% but had little effect on the activity with native DNA. However, the incorporation was very slightly affected by the presence or absence of an ATP-generating system. Addition of glycerol (1.6 mM final concentration) to the incubation mixture enhanced the DNA polymerase activity.

As there was a possibility that the restricted permeability of the membrane of the intact nuclei could prevent the free penetration and contact of the substrates (especially the DNA template) with the enzyme, the effect of rupture and sonication of the nuclei on the incorporation was checked (Table 2). These treatments did not stimulate incorporation of [^3H]thymidine triphosphate. The sonication caused a

loss of enzyme activity, especially with heat-denatured DNA.

(ii) Effect of Mg^{2+} ions. Omission of Mg^{2+} ions caused a decrease in incorporation of 55% with native and 70% with denatured DNA as template. A broad optimum between 5 and 10 mM- Mg^{2+} was observed with denatured DNA as template and between 5 and 8 mM- Mg^{2+} with native DNA.

(iii) pH optimum. Maximal DNA polymerase activity with either native or denatured DNA was reached at approximately pH 8.5. A fairly sharp fall in the activity was observed at higher pH values. Between pH values of 7 and 10 the relative capacity of the nuclei to utilize denatured or native DNA as template was unchanged.

(iv) Stability. Nuclei suspended in 0.32 M-sucrose with and without glycerol and 2-mercaptoethanol were assayed after 1, 2, 3 and 30 days of storage at 0° and -20°C. At -20°C, the enzymic activity was fully preserved in the presence of glycerol and 2-mercaptoethanol. At 0°C the glycerol and thiol groups compensated the effect of the higher temperature, but when kept without them the enzyme underwent a rapid loss of activity, with a steep decline to 55% of the initial value during the first 3 days. In all cases the activity with denatured DNA as template was much more affected than that with native DNA.

Deoxyribonuclease activity. The low deoxyribonuclease activity in the nuclear preparation was

Table 2. *Effect of sonication or disruption of unfractionated brain nuclei of 10-day-old rats on their DNA polymerase activity*

Nuclei were sonicated in batches (5 ml) with a 100 W ultrasonic disintegrator fitted with a titanium microprobe (Measuring and Scientific Equipment Ltd., Crawley, Sussex, U.K.). The nuclei (13×10^6 /assay) were subjected to sonication for three 30 s periods, followed each time by a 15 s pause at 0°C, or homogenized and extracted with 0.01 M-Tris-HCl buffer, pH 7.4, for 15 min at 0°C. The rate of DNA polymerase activity was measured by the incorporation of [^3H]thymidine 5'-triphosphate (sp. radioactivity 40 $\mu\text{Ci}/\mu\text{mol}$) into acid-insoluble product. The results are expressed as enzyme units per mg of nuclear DNA.

Template	...	DNA polymerase activity (units/mg of DNA)	
		Native DNA	Denatured DNA
Non-sonicated nuclei		28.6	59.4
Sonicated nuclei		25.6	39.6
Disrupted nuclei		28.8	59.6

Table 3. *Content of total soluble and particulate forms of DNA polymerase in 10-day-old rat brain nuclei*

Unfractionated nuclear preparations from 20 g of brain tissue from 25 rats were separated into two equal parts, one of intact nuclei and the other of nuclei disrupted with a glass homogenizer and sequentially extracted with 0.01 M-Tris-HCl buffer (pH 7.4)-2 mM-2-mercaptoethanol (soluble-form DNA polymerase) and twice with 0.2 M-sodium phosphate buffer (pH 7.4)-2 mM-2-mercaptoethanol (particulate-form DNA polymerase). The results are expressed as enzyme units (pmol of [^3H]dTTP incorporated/min) per g of brain.

Template	...	DNA polymerase activity (units/g of brain)	
		Native DNA	Denatured DNA
Intact nuclei		32.7	61.9
Disrupted nuclei		32.0	62.5
(a) Tris extract		9.7	42.4
(b) Phosphate extract (i)		19.5	17.1
(c) Phosphate extract (ii)		2.6	3.5

demonstrated with ^3H -labelled HeLa-cell DNA as substrate. After incubation of 12×10^6 nuclei for 30 min about 0.18% of the denatured and 0.06% of the native substrate was rendered acid-soluble.

Solubilization of DNA polymerase. Recently the existence of a particulate form of DNA polymerase in rat brain has been reported (Chiu & Sung, 1972a). They reported it to be present in a larger amount in adult animals and to be inactive and undetectable unless solubilized by extraction with phosphate buffer. We thus decided to examine the solubilization of the nuclear DNA polymerase that we have detected in 10-day-old rats.

The nuclear preparation was separated into two equal parts, one of intact nuclei and the second of disrupted nuclei. They were first extracted with Tris-HCl and then with phosphate buffer, and the results are recorded in Table 3.

With native DNA as template two-thirds of the activity is recovered as the so-called particulate form in the extracts with phosphate buffer, whereas with denatured DNA most of the activity is in the form of the soluble polymerases. It is noteworthy that the DNA polymerase activity of the disrupted nuclei is identical with that of intact nuclei and is equal to the combined total of the activity in the three extracts. The data in Table 3 demonstrate that there is no masked activity in the intact nuclei, which can therefore be used directly for investigations of total DNA polymerase.

Fractionated nuclei of young and adult rats

Rat brain nuclei from 8- and 10-day-old animals were fractionated by isopycnic centrifugation into five distinct zones (Figs. 1a and 1b). The nuclei were morphologically similar to the fractions of adult rat brain nuclei (Austoker *et al.*, 1972) and were identified microscopically by the same criteria.

Zone (I) consisted mainly of neuronal nuclei, with about 35–40% contamination by astrocyte nuclei. Zones (II) and (III) were composed of astrocyte nuclei with about 15% contamination by neuronal and oligodendrocyte nuclei. Zone (IV) was composed of oligodendrocytes contaminated by microglial nuclei and in zone (V) were found almost pure oligodendrocyte nuclei (Table 4). The fractions, especially zones (II)–(V), probably also contained proliferating neuroblast and 'microneuronal' nuclei.

Comparison of these results with those of Austoker *et al.* (1972) emphasizes the fact that the relative proportion of different types of nuclei in each of the fractionated nuclear zones and the size of the zones varied significantly with the age of the animals (Fig. 1, and Austoker *et al.*, 1972). The 8-day-old rats had a large third peak and apparently smaller second, fourth and fifth peaks. With 10-day-old rats, an increased first peak was obtained, which formed the

Fig. 1. Fractionation of brain nuclei by zonal centrifugation from (a) 8-day-old and (b) 10-day old rats

In all experiments 25 infant rats (20g body wt.) were used, yielding approx. 25g of brain tissue. The nuclei were fractionated by 2h centrifugation at 45000 rev./min at 10°C in the B XIV titanium zonal rotor. The rotor was slowed to 3000 rev./min for unloading. The light-scattering profile of the effluent at 600nm was recorded by using a 1 cm flow cell. Full-scale deflection represents $0.8E_{600}$ unit. The direction of sedimentation is from left to right. The interfaces between the sucrose solutions (molar concn.) at which the fractions were obtained are indicated above each peak. The solid line indicates the fractions pooled in the separate zones. The abscissa indicating the volume of effluent is not linear with respect to volume of contents of rotor as the pump speed was varied during the emptying of the rotor. The zones (I)–(V) are marked. For further details see the text.

major band, whereas the nuclear profile of the adult rats contained small second and third peaks but noticeably increased fourth and fifth peaks which predominated over the others.

A comparison of the total number of nuclei in the zones recovered after centrifugation (Table 5) indicated that the variations in the light-scattering profiles of the effluent from the zonal rotor (Fig. 1) reflect the changes in the number of nuclei in the five main zonal fractions.

(i) *Chemical analyses.* The amount of DNA, RNA and protein per nucleus was measured. Table 6 shows that the DNA content in the nuclei of animals of a particular age was relatively constant. For this reason, it was used as a reference unit throughout all experiments with the fractionated nuclei.

Greater variation was observed in the RNA and

protein contents. They reach their highest value in zone (I) and gradually decrease in the following groups of nuclei.

(ii) *DNA polymerase activity.* In 10-day-old rats, pronounced differences in the specific DNA polymerase activity were found between the nuclei in the five main zones. These differences were observed both with native and denatured templates. With denatured DNA as template the highest amount of enzyme activity was associated with nuclei from zone (I) and decreased successively in the following zones to reach a comparatively small value with the nuclei of the last peak. The variations in the specific activity of the

Table 4. *Percentage distribution of different classes of nuclei after zonal centrifugation of brain cell nuclei from 10-day-old rats*

The results are expressed as averages of five experiments $\pm$ S.E.M.

Zone	Percentage distribution of different classes of nuclei		
	Neurons	Astrocytes	Oligodendrocytes
(I)	58.6 $\pm$ 1.4	35.7 $\pm$ 2.3	6.0 $\pm$ 1.2
(II)	16.6 $\pm$ 4.1	80.4 $\pm$ 4.5	3.0 $\pm$ 0.1
(III)	1.7 $\pm$ 0.2	81.6 $\pm$ 3.7	16.7 $\pm$ 3.7
(IV)	0	8.0 $\pm$ 1.1	92.0 $\pm$ 1.2
(V)	0	0	100

Table 5. *Numbers of nuclei in the separate zones recovered after zonal centrifugation*

In all experiments 25 rats were used, yielding 18.2–21.5 g of brain tissue. The results are expressed as averages of five experiments with rats of each age.

Zone	10 ⁻³ $\times$ Numbers of nuclei per g of brain tissue		
	8-day-old rats	10-day-old rats	Adult rats*
(I)	26460	31480	8480
(II)	7440	8320	7610
(III)	31160	20610	11430
(IV)	22070	17300	18820
(V)	8060	9120	17450

* From Austoker *et al.* (1972).

Table 6. *DNA, RNA and protein contents in 10-day-old rat brain nuclei fractionated by zonal centrifugation*

Nuclei were isolated and fractionated from brain cells and the content of DNA, RNA and protein was determined as described in the Experimental section. The values are the averages of six determinations $\pm$ S.E.M.

Zone	Content (pg/nucleus)		
	DNA	RNA	Protein
(I)	5.3 $\pm$ 0.4	4.3 $\pm$ 0.7	49.8 $\pm$ 0.5
(II)	5.7 $\pm$ 0.4	4.0 $\pm$ 0.8	35.0 $\pm$ 0.8
(III)	5.6 $\pm$ 0.6	4.3 $\pm$ 0.7	34.3 $\pm$ 1.7
(IV)	5.5 $\pm$ 0.8	2.8 $\pm$ 0.4	30.6 $\pm$ 1.3
(V)	5.9 $\pm$ 0.4	2.5 $\pm$ 0.3	26.2 $\pm$ 0.3

separate fractions with native DNA closely resembled those with denatured DNA, except for the second peak of nuclei, where the DNA polymerase activity reached the highest value (Fig. 2). However, all fractions, as did the unfractionated nuclei, showed a preference for heat-denatured DNA over native DNA as template.

In all experiments, fractionated nuclei from 8-day-old rats followed the same pattern of changes in the specific enzyme activity despite the fact that they showed a somewhat different profile of separation.

In view of reports that proliferation of the neurons ceases very shortly after birth, whereas the glial cells retain the capacity for division, we have investigated the DNA polymerase activity and in addition the incorporation of [3 H]thymidine *in vivo* into nuclear DNA of adult rats. The highest DNA polymerase activity was associated again with the nuclei from zone (I) and the remaining fractions exhibited a progressive decline. In contrast with the results with nuclei from 10-day-old rats, in all fractions there was a preference for the native DNA as a template rather than denatured DNA, and their enzymic activity was considerably higher than that of nuclei of 7–10-day-old rats.

(iii) *Deoxyribonuclease activity.* The deoxyribonuclease activities under conditions that were used to measure synthesis were determined because the extent of incorporation of precursors into DNA will represent the balance between the rate of synthesis and the rate of degradation. The true assessment of the relative activities of the different zones of nuclei therefore requires a knowledge of the breakdown of DNA under conditions that were used to measure its synthesis.

With nuclei from 10-day-old rats there were significant differences in the specific activity with either native or denatured [3 H]DNA as substrate (Fig. 3). The activity was markedly higher in the first zone, decreased gradually in the next three zones and was slightly increased in the last fraction of nuclei. However, the data indicated a comparatively low nuclease activity in all classes of nuclei. When the amount of substrate hydrolysed during 30 min was expressed as a percentage of DNA used as template in the DNA polymerase assay it varied from 0.05 to 0.35%. It should be noted that the differences in the specific deoxyribonuclease activity in general corresponded to those in the DNA polymerase activity between the separate zones.

Fig. 2. DNA polymerase activity in the different classes of brain nuclei of 10-day-old (a) and adult (b) rats

Fractionation of the nuclei and conditions were as described in the Experimental section. The results are expressed as arithmetic means. Vertical bars represent the range of values derived from five experiments. Template: □, native DNA; ▨, denatured DNA. For details of zones (I)–(V) see the text.

Fig. 3. Deoxyribonuclease activity in the different types of brain nuclei from 10-day-old (a) and adult (b) rats

Nuclei were fractionated and deoxyribonuclease activity was assayed as described in the Experimental section. The results are the average of five experiments. Vertical bars indicate the range of values. Substrate: □, native [^3H]DNA; ▨, denatured [^3H]DNA.

The different classes of nuclei from adult rats exhibited a low nuclease activity which followed in general the pattern of activity of the nuclei from 10-day-old rats. The deoxyribonuclease activity with denatured DNA as a substrate in the adult rats was somewhat higher. However, expressed as percentage of the DNA used as template it varied from 0.5 to 0.05% and closely resembled the changes in their DNA polymerase activity in that the highest activity was found in zone (I), and it gradually decreased in the following fractions.

(iv) *dATPase activity.* The rates of dATPase activity in the five nuclear zones are recorded in Fig. 4. A powerful hydrolytic activity towards dATP occurs in all zones. The differences in specific activity between the zones follow roughly the same pattern as those for deoxyribonuclease and DNA polymerase activity.

(v) *Incorporation of [^3H]thymidine into nuclear DNA.* Although the highest specific activities of DNA polymerase, deoxyribonuclease and dATPase occur in zone (I), these data provide no information about which fraction of nuclei is mainly involved in biosynthesis of DNA, because the assays of the DNA

polymerase activity *in vitro* do not distinguish between replication and repair of the genome. It was of importance to investigate the incorporation of radioactive thymidine into the nuclear rat brain DNA *in vivo*.

From the results obtained by intraperitoneal administration of the labelled precursor in 10-day-old rats (Fig. 5) it was evident that there are considerable differences in the activities of the separate classes of nuclei. The specific radioactivity of the DNA reached its highest value in the oligodendrocyte fractions and the DNA was more highly labelled in the nuclei of zone (V) compared with those of zone (IV), whereas the incorporation of the DNA precursor into the nuclei of the first three zones was almost identical and about 30% that of the last two zones.

Under the same experimental conditions the incorporation of [^3H]thymidine into DNA in adult rats was about one-thirtieth of the activity of the nuclei of infant rats (Fig. 5). The low specific radioactivity of the DNA made impossible the accurate estimation of differences in the activity of the separate classes of nuclei.

After intracisternal administration of the label it

Fig. 4. dATPase activity in fractionated brain nuclei of 10-day-old rats

The results are expressed as arithmetic means for three experiments. Vertical bars represent range of values.

was found that the incorporation of [³H]thymidine occurred in considerable amounts in all classes of brain nuclei. The specific radioactivity of the DNA gradually increased from zone (II) to zone (V) but was highest in the first zone, consisting mostly of neuronal nuclei (Fig. 5).

Discussion

The properties of the DNA-dependent DNA polymerase in intact brain nuclei from 10-day-old rats exhibited many similarities to those of other mammalian DNA polymerases. The activity was strongly dependent on the presence of exogenous DNA and Mg²⁺ ions (Margolis, 1969; Haines *et al.*, 1970; Lindsay *et al.*, 1970; Bharucha & Murthy, 1971). The pH optimum with both native and denatured DNA as template was 8.5 and a broad Mg²⁺ optimum between 5 and 10mM was found (Friedman & Mueller, 1968; Lindsay *et al.*, 1970). The DNA polymerase activity was not influenced by the presence of thiol compounds and there was considerable incorporation of [³H]dTTP in the absence of one or more deoxyribonucleoside triphosphates (Patel *et al.*,

Fig. 5. Incorporation of [³H]thymidine into nuclear DNA of brain from 10-day-old (a) and adult (b) rats *in vivo*

The animals were injected intraperitoneally with 2 μ Ci of [³H]thymidine (sp. radioactivity 15 Ci/mmol)/g body wt. Adult rats were also injected intracisternally with 0.3 μ Ci of [³H]thymidine/g body wt., and 12h after injection the rats were killed. Nuclei were isolated and fractionated as described in the Experimental section. The specific radioactivity of the nuclear DNA was measured. Vertical bars represent the range of values for each fraction derived from three experiments. □, Intracisternal injection; ▨, intraperitoneal injection.

1967; Greene & Korn, 1970; Lindsay *et al.*, 1970; Haines *et al.*, 1970). The enzyme activity was stimulated and preserved by the addition of glycerol to the purification medium or the incubation mixture (Coffey *et al.*, 1968; Margolis, 1969).

However, as in most mammalian systems, the enzyme utilized both native and denatured DNA, with a preference for the denatured DNA template. These two forms of enzymic activity differed in their response to sonication, Mg²⁺ concentration and storage. These observations are in agreement with some recent studies on rat brain DNA polymerases in which two distinct enzymes with some different properties and preference for DNA template have been separated (Chiu & Sung, 1972a,b).

The brain nuclei from adult rats showed high DNA polymerase activity with a preference for native DNA as template. Similar findings with total brain extracts have been reported (Chiu & Sung, 1970) and may

reflect changes in the prevailing type of enzyme activity with age (Chiu & Sung, 1972b).

In our experiments it has been established that the use of intact nuclei, in which the DNA polymerase is exposed to minimal external influences, does not result in the restriction of the penetration of the enzyme substrates and enables the determination of the total extractable activity of the enzyme (Table 3).

The second part of the investigation was concerned with the chemical analyses and distribution of the enzyme activity in the fractionated brain nuclei. The differences revealed in the protein, DNA and RNA contents of nuclei from 10-day-old rats resembled in general those in the fractionated brain nuclei from adult rats (Austoker *et al.*, 1972). Whether these differences can be related to the functional activity of the different classes of brain cell nuclei cannot be assessed without further detailed studies of the types and quantities of nuclear RNA and proteins.

The [^3H]thymidine incorporation into nuclear DNA of infant rats *in vivo* demonstrated that the highest rate of DNA synthesis was associated with the nuclei in zones (IV) and (V). The increased incorporation in the nuclei from these zones may reflect the postnatal multiplication of the oligodendrocyte cells and can also be due to the presence of actively proliferating neuroblast, spongioblast and microneural nuclei (Altmann, 1969) in these fractions. After intraperitoneal injection of [^3H]thymidine very low radioactivity was found in all classes of adult brain nuclei (Fig. 5). The low rate of [^3H]thymidine incorporation may be a consequence of the completed development of the brain cells of the older animals, after which they retain very little or no capacity to divide (Fujita, 1967; Altmann, 1969). Similar observations for the incorporation of [^3H]thymidine *in vivo* have been reported by Burdman (1972) for neuronal and glial nuclei. Alternatively the freedom of access of [^3H]thymidine, administered by the intraperitoneal route, to the brain may be diminished in the older animals.

The activity of the different nuclear fractions after intracisternal administration of [^3H]thymidine shows more distinctly the relationship between the incorporation by the different classes of nuclei. However, the interpretation of these results is more complicated, as it is possible that concomitant with the neuronal [^3H]thymidine incorporation additional proliferation of the cells may also occur in response to neural damage (Vaughn & Skoff, 1972), which can take place during this route of administration of the DNA precursors. However, uncertainty about the pool sizes and specific radioactivities of precursors of DNA in the many types of cell found in whole brain prevent an unequivocal interpretation of the experiments *in vivo*.

In measurements of the DNA polymerase activity *in vitro*, we found that it reached its highest value in the

nuclei from zone (I), whereas the remaining fractions exhibited a progressive decline. A relatively high activity was associated with the nuclei from adult rats (Fig. 2). The measurements of the deoxyribonuclease activity and the pattern of the relative deoxyribonuclease and dATPase activities (Figs. 3 and 4) made it impossible to suggest that the variations in the DNA polymerase activity in the nuclear fractions are due to differences in their nuclease or dATPase activity.

The observations on the distribution of the DNA polymerase activity measured *in vitro* are obviously in contrast with the results obtained for [^3H]thymidine incorporation *in vivo* and the expected rate of DNA replication in fully differentiated cells.

The measurements of DNA polymerase *in vitro* may represent the total replication and repair activity of the nuclei and the assay may not give a true indication of the system for replication. Alternatively the replication complex may be selectively inhibited as a consequence of isolation of the nuclei. Further, the high DNA polymerase activity of the brain nuclei from adult rats could be associated with a process of renewal of the DNA, the existence of which has been suggested by Pelc (1963, 1964). According to his proposal fully developed non-dividing cells which are metabolically active may periodically renew their DNA. In such cases this process is more likely to involve the DNA polymerase repair system and could be associated mainly with the neuronal cells. However, it should be emphasized that exogenous DNA, either native or denatured, is present in the assays of the DNA polymerase, and this may lead to the detection of activity that is latent in its absence.

The differences in the activity of the separate classes of nuclei could be due to differences in the total amount of the enzyme or to differences in the rate of its DNA polymerase activity. The resolution of this problem will require further isolation and quantitative measurement of the different forms of DNA polymerase activity in the separate types of brain nuclei.

M. A. S. gratefully acknowledges a Fellowship from the Food and Agriculture Organization. This work has been supported by the Science Research Council.

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