Studies on Ribonucleic Acid and Homopolyribonucleotide Formation in Neuronal, Glial and Liver Nuclei

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1. Various types of nuclear preparations, with different ratios of neuronal to glial nuclei, were isolated from guinea-pig cerebral grey matter and ox cerebral grey matter and white matter. Conditions appropriate for the separate assay of RNA and poly A formation were described. Comparative rates of RNA and poly A formation were studied in cerebral and liver nuclei. 2. RNA polymerase activity per nucleus is higher in neuronal nuclei than in glial nuclei. In liver nuclei, the activity is much lower than in cerebral nuclei. The physical relationship between RNA polymerase and deoxyribonucleoprotein seems to differ in neuronal, glial and liver nuclei. 3. Poly A polymerase activity in liver nuclei is selectively activated by Mn^{2+} and inhibited by GTP, CTP and UTP. On a DNA basis, the activity in an aggregate enzyme is the same as in intact nuclei. Poly A polymerase activity per nucleus is much higher in liver nuclei than in neuronal nuclei. Glial nuclei show an intermediate activity. 4. It is suggested that, in neuronal nuclei, the synthesis of RNA is more prominent than that of poly A under conditions where both polymers are formed simultaneously. This contrasts with liver nuclei, where more poly A is made than RNA. 5. In neuronal nuclei, the rate of CTP incorporation is much higher than in glial and liver nuclei. This incorporation is most probably due to poly C synthesis.

DNA-primed RNA polymerase (nucleoside triphosphate-RNA nucleotidyltransferase, EC 2.7.7.6) (Barondes, 1964; Pete, Wintzerith, Mandel & Mandel, 1964; Bondy & Waelsch, 1964, 1965; Dutton & Mahler, 1968) and also homopolyribonucleotide formation (Jacob & Mandel, 1966; Mandel, Dravid & Pete, 1967; Dravid, Duffy & Haglid, 1969) in the brain have been the topic of several recent investigations. None of these investigations, however, gave particular attention to the differentiation of neurones and glial cells, an important prerequisite to the study of cerebral tissues. After the development of a differential isolation of neuronal and glial nuclei (Kato & Kurokawa, 1966, 1967) it was shown that the activity per nucleus of ATP-NMN adenylyltransferase (EC 2.7.7.1), another nuclear enzyme, was unequal in neuronal and glial nuclei (Kurokawa, Kato & Inamura, 1966, 1967). Significantly higher protein-synthetic activity per nucleus has been demonstrated in neuronal nuclei compared with glial nuclei (Inamura, Kato & Kurokawa, 1968). Also, little attention had been paid to the balance between RNA and homopolyribonucleotide formation in the whole cell nucleus. The main concern in

the present work was to study RNA and homopolyribonucleotide formation, and their comparative rates and balance among neuronal, glial and liver nuclei. A preliminary account of this work has been published (Kato & Kurokawa, 1968).

MATERIALS AND METHODS

Isolation of nuclei. Nuclear samples were isolated from guinea-pig cerebral cortex, ox cerebral cortex and sub-cortical white matter by procedures described by Kato & Kurokawa (1967), but on an enlarged scale. The Hitachi RPS40 rotor was replaced by the RPS25 rotor, and volumes of dense sucrose were increased from 1 ml to 5 ml for 1.2M- and 1.65M-sucrose, and from 2 ml to 10 ml for 1.6M- and 2.1M-sucrose. This enabled handling of as much as 50–60 g of fresh tissue at a time. Ox cerebral nuclei were successfully isolated from the brain, which had been kept in the cold for 2–3 h before the initial homogenization. The nuclear sample in which the population of neuronal nuclei averaged 51% is, for brevity, expressed as P_{51} . This is equivalent to the sample described as P4II in our previous papers and P_3 is equivalent to that described as P_5 (Kato & Kurokawa, 1967; Kurokawa *et al.* 1966). Ranges of population of neuronal nuclei are 47–53%, 26–41%, 1–6%, <1% and <2% in the P_{51} (guinea-pig grey matter), P_{36} (ox grey matter),

P₃ (guinea-pig grey matter), P₀ (ox grey matter) and P₁ (ox white matter) samples respectively. P₃₆ (ox grey matter), P₁ (ox white matter) and highly purified liver nuclei were isolated by a procedure identical with that used for obtaining the P₅₁ (guinea-pig grey matter). P₀ (ox grey matter) nuclei were isolated by the procedure for P₃ nuclei (guinea-pig grey matter). On a DNA basis, recoveries of nuclei were 64% for P₅₁ (guinea-pig grey matter) and 54% for guinea-pig liver nuclei. Isolated nuclei were homogeneously dispersed in 0.32M-sucrose, usually at the concentration of 10⁸ nuclei/ml.

Microscopy. Each nuclear preparation was stained with aceto-orcein-fast green, and nuclei of neuronal, glial and endothelial origins were differentiated as described by Kato & Kurokawa (1967). The number of nuclei per unit volume of nuclear suspension was counted with unstained samples using a Bürker-Türk haemocytometer.

Four-nucleoside-triphosphate reaction. This was assayed in medium A (0.5ml), which contained (final concentrations): tris-HCl buffer, pH 8.5 (0.1M); MgCl₂ (5mM); β -mercaptoethanol (5mM); (NH₄)₂SO₄ (0.3M) adjusted to pH 8.5 with aq. NH₃; GTP, CTP and UTP (0.5mM each); ATP (0.1mM); 0.15 μ Ci of [8-¹⁴C]ATP and approx. 2 $\times$ 10⁷ fresh nuclei (equivalent to about 140 μ g of DNA). When one of the other nucleoside triphosphates was labelled (0.2 μ Ci), the concentrations of non-radioactive nucleoside triphosphates were 0.5mM each. The system was not saturated with respect to incorporation of ATP when each nucleoside triphosphate was added at 1.0mM and below. In some experiments 5mM-MgCl₂ was replaced by 2mM-MnCl₂. The bivalent cation used is specified in parentheses as four-nucleoside-triphosphate reaction (Mg²⁺) or four-nucleoside-triphosphate reaction (Mn²⁺). The four-nucleoside-triphosphate reaction (Mg²⁺) is regarded as being equivalent to DNA-dependent RNA polymerase.

Single-nucleoside-triphosphate reaction. This was assayed in medium B (0.5ml), which contained (final concentrations): tris-HCl buffer, pH 8.0 (0.1M); MnCl₂ (2mM); β -mercaptoethanol (5mM); (NH₄)₂SO₄ (30mM) adjusted to pH 8.0 with aq. NH₃; one of the four nucleoside triphosphates (0.2mM); 0.5 μ Ci of the corresponding ¹⁴C-labelled nucleoside triphosphate and approx. 2 $\times$ 10⁷ fresh nuclei. Where necessary, the nucleoside triphosphate included in medium B is specified in parentheses as e.g. single-nucleoside-triphosphate reaction (ATP). Single-nucleoside-triphosphate reaction (ATP) and single-nucleoside-triphosphate reaction (CTP) are taken to be equivalent to poly A and poly C synthetase respectively.

Treatment of the reaction product. After incubation at 37°C for 15min, the reaction was terminated by the addition of 0.5ml of cold 10% (w/v) trichloroacetic acid in 0.02M-sodium pyrophosphate. The precipitate was treated in the cold in one of the following two ways. In method I, acid-soluble components were removed by washing the precipitate four times with 10ml portions of 5% (w/v) trichloroacetic acid, and lipid materials were removed by successive washings with 10ml of 95% (v/v) ethanol, twice with 10ml of ethanol-chloroform (3:1, v/v), with 10ml of ethanol-ether (3:1, v/v) and with 10ml of ether. The final precipitate was digested with 0.5ml of 0.3M-KOH at 37°C for 1h (Hutchinson & Munro, 1961). After being adjusted to pH 9 with 60% (v/v) HClO₄, the digest was kept overnight at 4°C, 0.05ml of 5% (v/v)

Koniducks (wetting agent for photographic films; Konishiroku Photo Ind. Co., Tokyo, Japan) was added, and the mixture was brought to 1.5ml final volume with water and centrifuged. A measured portion of the supernatant was applied to a glass-fibre disc (GA-100; Toyo Roshi Co., Tokyo, Japan) (Malt & Miller, 1967) and dried. In method II the mixture was poured on to a glass-fibre disc immediately after the reaction had been stopped with trichloroacetic acid. The precipitate, collected on the surface of the disc, was then washed with 25ml of 5% (w/v) trichloroacetic acid, 25ml of ethanol-chloroform (3:1, v/v) and 15ml of ether, and dried. The radioactivity retained in the disc was determined with a Beckman DPM-100 liquid-scintillation spectrometer, with toluene scintillator containing 0.4% (w/v) of 2,5-diphenyloxazole. In method I, counting efficiency was calculated on the basis of an increment of radioactivity resulting from the addition of a known amount of [8-¹⁴C]ATP to the disc. This was generally found to be about 75% and corrections were made accordingly. With method II, relative rates of incorporation only are shown.

In some additional experiments the final precipitate with method I was extracted with 10% (w/v) NaCl at 100°C for 10min (Weiss, 1960). In this way almost the same amount of radioactivity was extracted as with alkaline digestion. The radioactivity from the final precipitate with method II was approx. 105% of that from the alkaline digest with method I.

DNA. This was assayed by fluorimetry with calf thymus DNA as standard (Kissane & Robins, 1958).

Reagents. ATP, GTP, CTP, UTP, RNA (from yeast, type XI) and DNA (from calf thymus, type I) were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. [8-¹⁴C]ATP, [8-¹⁴C]GTP, [2-¹⁴C]CTP and [2-¹⁴C]UTP were from Schwarz BioResearch Inc., Mount Vernon, N.Y., U.S.A. Bovine pancreatic deoxyribonuclease and snake-venom phosphodiesterase were from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Sephadex G-25 was from Pharmacia, Uppsala, Sweden. Actinomycin D was a gift from Merck, Sharpe and Dohme, Rahway, N.J., U.S.A.

RESULTS

Four-nucleoside-triphosphate reaction

Conditions for the reaction. The four-nucleoside-triphosphate reaction in cerebral P₅₁ nuclei is linear only for a relatively short time (Fig. 1). In the absence of ammonium sulphate, the reaction levels off more rapidly than in the presence of 0.3M-ammonium sulphate. The time-course of the four-nucleoside-triphosphate reaction (Mn²⁺) does not differ essentially from that of the four-nucleoside-triphosphate reaction (Mg²⁺).

In the four-nucleoside-triphosphate reaction (Mn²⁺), a broad pH optimum at about pH 8.5 is observed in cerebral nuclei, but not in liver nuclei (results not presented). In the four-nucleoside-triphosphate reaction (Mg²⁺), the incorporation of ATP is maximal at about pH 8 in liver nuclei, though the optimum is by no means clear-cut. In cerebral nuclei, the rate of incorporation gradually

Fig. 1. Time-course of the four-nucleoside-triphosphate reaction in cerebral P_{51} nuclei. Reaction mixture contained 0.1 M-tris-HCl buffer, pH 8.5, 5 mM- β -mercapto-ethanol, 0.5 mM each of GTP, CTP and UTP, 0.1 mM-ATP, 0.15 μ Ci of [8-¹⁴C]ATP and approx. 2×10^7 nuclei. In addition, 5 mM-MgCl₂ (○) or 2 mM-MnCl₂ (●) was included. —, Reaction in the presence of 0.3 M-(NH₄)₂SO₄; ----, reaction in the absence of (NH₄)₂SO₄.

increases within the pH range 7.5–9.5 (results not presented).

Either Mn^{2+} or Mg^{2+} is essential for the four-nucleoside-triphosphate reaction (Fig. 2). Mn^{2+} stimulates the reaction most effectively at a concentration of 2 mM. The stimulatory effect of Mg^{2+} is less than that of Mn^{2+} , and it gradually increases as the concentration rises at least up to 10 mM. When GTP, CTP and UTP are omitted from medium A, the incorporation of ATP is much decreased but the activation by bivalent cations occurs in a way similar to that in the presence of the four nucleoside triphosphates (Fig. 2).

Aging the nuclei at -20°C for 3 days lowers the rate of the four-nucleoside-triphosphate reaction (Mg^{2+}) to 75% of the control value, but little further decrease occurs during storage at -20°C for at least 20 days longer. In the present experiments, fresh nuclei only were used for assay. When nuclear structure is completely destroyed by ultrasonic treatment (10 kHz for 20 min at $0-4^\circ\text{C}$), the rate of the four-nucleoside-triphosphate reaction (Mg^{2+}) is lowered to 20% of the control value.

Effects of ammonium sulphate. In the four-nucleoside triphosphate reaction (Mg^{2+}), the ATP incorporation into cerebral P_{51} nuclei is maximally

Fig. 2. Effect of $MgCl_2$ or $MnCl_2$ on the four-nucleoside-triphosphate reaction in cerebral P_{51} nuclei. —, Reaction in medium A (see the Materials and Methods section), with either $MgCl_2$ (○) or $MnCl_2$ (●); ----, reaction in medium A but without GTP, CTP and UTP.

Fig. 3. Effect of (NH₄)₂SO₄ on the four-nucleoside-triphosphate reaction in cerebral P_{51} nuclei. The reaction was carried out in medium A, but at various ionic strengths, and with either 5 mM-MgCl₂ (○) or 2 mM-MgCl₂ (●).

stimulated by 0.45 M-ammonium sulphate, whereas the four-nucleoside-triphosphate reaction (Mn^{2+}) is maximally stimulated by 0.3 M-ammonium

sulphate (Fig. 3). In liver nuclei, ammonium sulphate activates the incorporation, but the extent of activation is much less in comparison with cerebral P_{51} nuclei (Fig. 4). Also, in sharp contrast with cerebral nuclei, no optimum concentration for activation is observed with liver nuclei. Cerebral P_{51} nuclei undergo visually discernible changes in reaction mixtures containing ammonium sulphate. In the absence of this salt, the mixture remains homogeneously turbid during the incubation. When the concentration of ammonium sulphate is raised above 0.1 M, the P_{51} nuclei clump into a gel. Liver nuclei do not undergo any such physical alterations, even in the presence of 0.3 M-ammonium sulphate.

Fig. 4. Effect of $(\text{NH}_4)_2\text{SO}_4$ on the four-nucleoside-triphosphate reaction in liver nuclei. The reaction conditions were the same as in Fig. 3, with either 5 mM-MgCl₂ (○) or 2 mM-MnCl₂ (●).

Characterization of the four-nucleoside-triphosphate reaction. Nucleoside triphosphates other than ATP are also actively incorporated into both cerebral and liver nuclei (Table 1). The rate of incorporation differs for the four nucleoside triphosphates, for various types of nuclei, and also with the activating bivalent cation. In liver nuclei, with 2 mM-Mn²⁺ in medium A, the incorporation of ATP is relatively higher than that of other nucleoside triphosphates. Higher incorporation of ATP in the presence of

Fig. 5. Rate of the four-nucleoside-triphosphate reaction as a function of number of nuclei. ○, Cerebral P_{51} ; △, cerebral P_3 ; □, liver nuclei, assayed in medium A with 5 mM-MgCl₂. ●, P_{51} ; ▲, P_3 ; ■, liver nuclei, assayed in medium A containing 2 mM-MnCl₂.

Table 1. *Four-nucleoside-triphosphate reaction in cerebral and liver nuclei with different labelled nucleoside triphosphates: sensitivity to actinomycin D*

Medium A, modified so that the final concn. of each nucleoside triphosphate was 0.5 mM, each labelled with ¹⁴C (0.2 μCi) in the presence of either 2 mM-MnCl₂ or 5 mM-MgCl₂. Approx. 2×10^7 nuclei were used for each assay. The activity unit is $10^7 \times \text{mol}$ of ATP incorporated/g of DNA in 15 min at 37°C.

Nuclei	Cation	Actinomycin D (50 μg/ml)	Activity			
			ATP	GTP	CTP	UTP
Cerebral P_{51}	Mn^{2+}	—	25.4	37.7	27.4	24.7
		+	4.0	4.8	4.9	2.7
	Mg^{2+}	—	22.9	32.1	24.4	21.1
		+	3.4	4.4	3.9	2.6
Cerebral P_3	Mn^{2+}	—	22.1	18.9	15.2	15.4
		+	4.1	3.3	3.1	2.4
	Mg^{2+}	—	16.4	19.1	15.6	12.3
		+	3.4	2.6	4.3	2.0
Liver nuclei	Mn^{2+}	—	1.92	1.59	1.52	1.11
		+	1.70	1.08	0.84	0.64
	Mg^{2+}	—	0.86	1.44	0.89	0.60
		+	0.74	0.95	0.68	0.37

Mn^{2+} is also observed in cerebral P_3 , but not P_{51} , nuclei. In the presence of actinomycin D (50 $\mu g/ml$) the four-nucleoside-triphosphate reaction in cerebral nuclei is inhibited by 80–90% whichever nucleoside triphosphate is labelled. In liver nuclei actinomycin D affects the incorporation to a smaller extent. The four-nucleoside-triphosphate reaction with labelled ATP in liver nuclei is most resistant to actinomycin D.

Comparative rates of the four-nucleoside-triphosphate reaction (Mg^{2+}) in neuronal, glial and liver nuclei. The relation between the rate of the reaction and the number of nuclei in medium A is shown in Fig. 5. Comparative rates of the reaction in various types of nuclei, assayed with a fixed number of nuclei (2×10^7) in medium A, are shown in Table 2. In P_{36} nuclei, the rate per nucleus is definitely higher than in P_0 and P_1 , and in P_{51} nuclei it is higher than in P_3 , as shown also in Fig. 5. This implies that in neuronal nuclei the four-nucleoside-triphosphate reaction (Mg^{2+}) is much more active than in glial nuclei. In liver nuclei the rate of the reaction is much lower in comparison with various types of cerebral nuclei.

Single-nucleoside-triphosphate reaction

Incorporation of single nucleoside triphosphates into various types of nuclei. Nuclei were incubated in medium B with each of the four nucleoside triphosphates and the comparative rates of incorporation were examined (Table 3). In liver nuclei, ATP is incorporated 8–40 times as actively as other nucleoside triphosphates. In glial nuclei (P_3) ATP is incorporated less actively than in liver nuclei, but still more actively than other nucleoside triphosphates. In contrast, the incorporation of CTP is more active in the P_{51} nuclei.

Conditions for the single-nucleoside-triphosphate reaction (ATP). The incorporation of ATP into

liver nuclei levels off within 20 min of incubation. The optimum pH for the reaction is 8.0 (results not presented). Mn^{2+} activates the incorporation most effectively at 2 mM, but Mg^{2+} has only a slight activating effect (Fig. 6).

In liver nuclei, incorporation of ATP is maximally activated by 30 mM-ammonium sulphate (Fig. 7). In cerebral P_{51} nuclei, however, an activating effect of ammonium sulphate is not apparent.

When liver nuclei are ultrasonically treated (10 kHz for 20 min at 0–4°C), the rate of the single-nucleoside-triphosphate reaction (ATP) is increased from 10^{-6} mol of ATP incorporated/g of DNA in 15 min in intact nuclei to 1.22×10^{-6} mol of ATP incorporated/g of DNA in 15 min in ultrasonically treated nuclei. This contrasts with the reduction in rate of the four-nucleoside-triphosphate reaction (Mg^{2+}) in cerebral P_{51} nuclei under the same conditions. In an aggregate enzyme prepared from liver

Table 3. *Rate of single-nucleoside-triphosphate reaction in cerebral and liver nuclei*

Medium B, but with 0.2 mM-nucleoside triphosphate for P_{51} and P_3 nuclei and 0.1 mM-nucleoside triphosphate for liver nuclei, was used. Approx. 2×10^7 nuclei were used for each assay. The activity unit is $10^7 \times$ mol of ATP incorporated/g of DNA in 15 min at 37°C.

Nuclei	Activity			
	ATP	GTP	CTP	UTP
Cerebral P_{51}	3.19	1.44	5.06	1.02
Cerebral P_3	3.86	1.73	1.64	1.12
Liver nuclei	10.47	0.49	1.25	0.24

Table 2. *Rate of four-nucleoside-triphosphate reaction (Mg^{2+}) in cerebral and liver nuclei*

Medium A containing 5 mM- $MgCl_2$ was used. The number of nuclei used for each assay was 2×10^7 . The activity unit is $10^{18} \times$ mol of ATP incorporated/nucleus in 15 min at 37°C. GM, grey matter; WM, white matter. Values are means $\pm$ s.d. of numbers of assays given in parentheses.

Species	Nuclei	Activity
Guinea pig	Cerebral P_{51} (GM)	10.50 ± 1.40 (6)
	Cerebral P_3 (GM)	9.59 ± 0.78 (6)
	Liver nuclei	1.41 ± 0.23 (6)
Ox	Cerebral P_{36} (GM)	5.36 ± 0.77 (6)
	Cerebral P_0 (GM)	2.88 ± 0.35 (6)
	Cerebral P_1 (WM)	1.24 ± 0.58 (6)

Fig. 6. Effect of $MgCl_2$ or $MnCl_2$ on the single-nucleoside-triphosphate reaction (ATP) in liver nuclei. The reaction was carried out in medium B (see the Materials and Methods section), but with either $MgCl_2$ ($\square$) or $MnCl_2$ ($\blacksquare$) as indicated.

Fig. 7. Different activation of the single-nucleoside-triphosphate reaction (ATP) and the four-nucleoside-triphosphate reaction (Mn^{2+}) by $(NH_4)_2SO_4$ in cerebral P_{51} (○ and ●) and liver (□ and ■) nuclei. —, Single-nucleoside-triphosphate reaction (ATP) in medium B; ----, four-nucleoside-triphosphate reaction (Mn^{2+}) (reproduced from Figs. 3 and 4).

Fig. 8. Inhibition by GTP (○), CTP (□) and UTP (Δ) of the single-nucleoside-triphosphate reaction (ATP) in liver nuclei. ●, Value in the simultaneous presence of the three nucleoside triphosphates; each one of GTP, CTP and UTP was in the concentration shown on abscissa, the total concentration being three times the value shown.

nuclei by the method of Weiss (1960), the rate of the single-nucleoside-triphosphate reaction (ATP) is 1.12×10^{-6} mol of ATP incorporated/g of DNA in 15 min.

Effects of nucleoside triphosphates and actinomycin D on the single-nucleoside-triphosphate reaction (ATP). GTP, CTP and UTP inhibit the single-

nucleoside-triphosphate reaction (ATP) in liver nuclei, and with the three nucleoside triphosphates present the inhibition is cumulative (Fig. 8). The inhibition is observed not only with intact nuclei but also with ultrasonically treated nuclei and aggregate enzyme (results not presented).

The single-nucleoside-triphosphate reaction (ATP) is not inhibited, but rather slightly enhanced, by actinomycin D ($50 \mu g/ml$). In the presence of the other three nucleoside triphosphates, however, actinomycin D was inhibitory (Table 4). These effects of nucleoside triphosphates and actinomycin D differ in degree among several types of nuclei (Table 4). In medium B the four-nucleoside-triphosphate reaction (Mn^{2+}) proceeds simultaneously with the single-nucleoside-triphosphate reaction (ATP) when other nucleoside triphosphates are present, and the balance between these two reactions is expected to differ according to the type of nuclei (see the Discussion section).

Characterization of the single-nucleoside-triphosphate reaction (ATP). 10mM-Pyrophosphate completely inhibits the ATP incorporation. NMN when added to medium B at concentrations between 0.01mM and 1.0mM markedly elevates the ATP incorporation (3.5-fold at 0.5mM). This is most probably due to the formation of an ADP-ribose polymer (Chambon, Weill, Doly, Strosser & Mandel, 1966; Sugimura, Fujimura, Hasegawa & Kawamura, 1967; Nishizuka, Ueda, Nakazawa & Hayaishi, 1967), since the effect of 0.5mM-NMN is completely counteracted by 0.5–1.0mM-NAD or 0.5–1.0mM-nicotinamide, and ATP incorporation in the absence of NMN is unaffected by either NAD or nicotinamide.

Liver nuclei were incubated for 30 min at $37^\circ C$ in medium (0.5ml) containing 50mM-phosphate buffer, pH 7.0, 5mM- $MgCl_2$ and deoxyribonuclease ($50 \mu g/ml$). The reaction was terminated by adding 10ml of ice-cold 50mM-phosphate buffer, pH 7.0. Nuclei were washed off deoxyribonuclease by three centrifugations and collected by centrifugation in the cold. These were suspended in 0.5ml of medium B (see the Materials and Methods section) and the rate of $[8-^{14}C]$ ATP incorporation was measured. In the case indicated, the above treatment was repeated once or twice more for 60 or 90 min incubation. In liver nuclei thus treated with deoxyribonuclease, 90, 99 and 99.3% of the DNA is removed within 30, 60 and 90 min of incubation respectively. Incorporation of ATP is enhanced in deoxyribonuclease-treated nuclei. Thus when the amount of DNA is decreased to 1% of that in fresh nuclei, the incorporation is increased to 150% of the original value. However, further treatment of nuclei with deoxyribonuclease causes a fall in the rate of incorporation. DNA, when added to the reaction mixture containing deoxyribonuclease-treated nuclei in the

Table 4. *Effects of nucleoside triphosphates and actinomycin D on the single-nucleoside-triphosphate reaction (ATP) in cerebral and liver nuclei*

Medium B containing 0.5 μ Ci of [8- ^{14} C]ATP (0.2 mM) was used. GTP, CTP and UTP, when present, were at 0.5 mM each, and actinomycin D was 50 μ g/ml. The reaction product was treated by method II (see the Materials and Methods section), and percentage relative activities only are shown. The 100% values (controls) for each nuclear preparation are similar to those listed in Table 5.

Conditions	Relative activity (%)					
	Guinea pig			Ox		
	P ₅₁	P ₃	Liver	P ₃₆	P ₀	P ₁
ATP (control)	100	100	100	100	100	100
ATP + actinomycin D	119.5	99.6	108.0	103.2	108.0	120.5
ATP + GTP, CTP, UTP	85.0	63.0	25.4	118.2	57.3	25.8
ATP + GTP, CTP, UTP and actinomycin D	40.8	22.7	9.2	30.3	29.4	24.3

Table 5. *Rate of single-nucleoside-triphosphate reaction (ATP) in cerebral and liver nuclei*

Medium B was used. The number of nuclei used for each assay was 2×10^7 . The activity unit is $10^{18} \times$ mol of ATP incorporated/nucleus in 15 min at 37°C. GM, grey matter; WM, white matter. Values are means $\pm$ S.D. of the number of assays given in parentheses.

Species	Nuclei	Activity
Guinea pig	Cerebral P ₅₁ (GM)	2.55 ± 0.11 (5)
	Cerebral P ₃ (GM)	3.44 ± 0.47 (5)
	Liver nuclei	9.25 ± 1.63 (10)
Ox	Cerebral P ₃₆ (GM)	1.04 ± 0.27 (4)
	Cerebral P ₀ (GM)	1.61 ± 0.32 (6)
	Cerebral P ₁ (WM)	1.78 ± 0.41 (7)

amount equivalent to that in fresh nuclei (150 μ g), results in a suppression of ATP incorporation, activities being 95, 64 and 67% of the control value at 30, 60 and 90 min of incubation respectively.

As far as the bivalent cation is concerned, 2 mM-Mn²⁺ plus 20 mM-Mg²⁺ in medium B activates maximally the single nucleoside triphosphate reaction (ATP). Under these conditions, the presence of GTP, CTP and UTP inhibits the reaction by 69%. When 2 mM-Mg²⁺ plus 20 mM-Mg²⁺ is included in medium B, and the concentration of ammonium sulphate is raised to 0.3 M, the ATP incorporation is suppressed to 57% of the value with 30 mM-ammonium sulphate. Under these conditions, addition of GTP, CTP and UTP inhibits the incorporation by 50%.

Comparative rates of the single-nucleoside-triphosphate reaction (ATP) in neuronal, glial and liver nuclei. The rate of ATP incorporation among various types of nuclei was assayed with a fixed number of nuclei (2×10^7) in medium B (Table 5). Activity per nucleus was highest in liver nuclei. In P₃ nuclei the activity was higher than in P₅₁, and

Table 6. *Effect of nucleoside triphosphates and actinomycin D on the single-nucleoside-triphosphate reaction (CTP) in guinea-pig cerebral nuclei*

Medium B containing 0.2 μ Ci of [2- ^{14} C]CTP (0.2 mM) was used. The reaction product was treated by method II (see the Materials and Methods section), and percentage relative activities only are shown. Final concentrations are 0.5 mM for ATP, GTP and UTP, and 50 μ g/ml for actinomycin D.

Conditions	Relative activity (%)	
	P ₅₁	P ₃
CTP (control)	100	100
CTP + actinomycin D	80.4	113
CTP + ATP, GTP, UTP	230	77.1
CTP + ATP, GTP, UTP and actinomycin D	88.3	50.1

in P₀ and P₁ nuclei it was higher than in P₃₆. This implies that in glial nuclei, the single-nucleoside-triphosphate reaction (ATP) is more active than in neuronal nuclei.

Single-nucleoside-triphosphate reaction (CTP). As stated above, the incorporation of CTP was relatively higher in cerebral P₅₁ than in P₃ nuclei (Table 3). In other experiments we observed that CTP was incorporated 1.5 times as actively in P₃₆ nuclei (ox grey matter) as in P₀ (ox grey matter) (results not presented). In the P₅₁ nuclei, the four-nucleoside-triphosphate reaction (Mn²⁺) was promoted by the simultaneous presence of the other three nucleoside triphosphates (Table 6). Actinomycin D completely counteracts the four-nucleoside-triphosphate reaction (Mn²⁺), although it inhibits the single-nucleoside-triphosphate reaction (CTP) only to a limited extent. The effects of nucleoside triphosphates and actinomycin D on CTP incorporation in P₃ nuclei differ from those in P₅₁ nuclei and are comparable with those observed in the

single-nucleoside-triphosphate reaction (ATP) in liver nuclei (see Table 4).

DISCUSSION

Nature of the four-nucleoside-triphosphate reaction (Mg^{2+}). The reaction is sensitive to actinomycin D, although the degree of inhibition differs between cerebral and liver nuclei (Table 1). The presence of Mg^{2+} and the simultaneous presence of all four nucleoside triphosphates are absolutely required for the reaction (Fig. 2). The reaction is activated by ammonium sulphate, markedly in cerebral P_{51} nuclei and to a smaller extent in liver nuclei (Figs. 3 and 4). All of these observations indicate that the four-nucleoside-triphosphate reaction (Mg^{2+}) is equivalent to the DNA-primed RNA polymerase reaction first described in an aggregate enzyme from rat-liver nuclei (Weiss, 1960). The single-nucleoside-triphosphate reaction (ATP) will not proceed in the reaction mixture for the four-nucleoside-triphosphate reaction (Mg^{2+}) because the absence of Mn^{2+} , high concentration of ammonium sulphate and the simultaneous presence of GTP, CTP and UTP all work to inhibit the single-nucleoside-triphosphate reaction (ATP) (Figs. 6, 7 and 8).

Nature of the four-nucleoside-triphosphate reaction (Mn^{2+}). In the presence of 0.3M-ammonium sulphate, the four-nucleoside-triphosphate reaction (Mn^{2+}) is far more active than the four-nucleoside-triphosphate reaction (Mg^{2+}) in both cerebral (Figs. 1, 2 and 3) and liver nuclei (Fig. 4). In the absence of ammonium sulphate, however, the four-nucleoside-triphosphate reaction (Mn^{2+}) is slightly less active than the four-nucleoside-triphosphate reaction (Mg^{2+}) in both cerebral (Figs. 1 and 3) and liver nuclei (Fig. 4). This agrees with the properties of the DNA-primed RNA polymerase reaction in rat-liver nuclei (Widnell & Tata, 1966; Novello & Stirpe, 1969).

In the four-nucleoside-triphosphate reaction (Mn^{2+}) of liver nuclei, ATP is incorporated more actively than the other nucleoside triphosphates (Table 1), but this incorporation proves to be most resistant to actinomycin D. This is most probably due to the concomitant occurrence of the single-nucleoside-triphosphate reaction (ATP), since even after being inhibited by 0.3M-ammonium sulphate, the single-nucleoside-triphosphate reaction (ATP) in liver nuclei is still more active than the four-nucleoside-triphosphate reaction (Mn^{2+}) (Fig. 7). In contrast with liver nuclei, RNA synthesis will predominate in the four-nucleoside-triphosphate reaction (Mn^{2+}) in cerebral P_{51} nuclei where, with 0.3M-ammonium sulphate, the single nucleoside triphosphate reaction (ATP) is far less active compared with the four-nucleoside-triphosphate reaction (Mn^{2+}) (Fig. 7).

Nature of the single-nucleoside-triphosphate reaction (ATP). In liver nuclei, this reaction is activated by low concentrations of ammonium sulphate, but higher concentrations of the salt are inhibitory (Fig. 7). This mode of activation is in sharp contrast with the four-nucleoside-triphosphate reaction (Mg^{2+}), where more RNA is formed at the higher concentration (Fig. 3). Mn^{2+} stimulates the single-nucleoside-triphosphate reaction (ATP), most effectively at 2mM, but Mg^{2+} shows only a slight activating effect (Fig. 6). The reaction is inhibited by other nucleoside triphosphates (Fig. 8), and slightly activated by actinomycin D (Table 4). These observations indicate the involvement of a reaction other than RNA polymerase.

The AMP moiety of ATP is thought to be incorporated into the product since the reaction is completely inhibited by pyrophosphate. Formation of an ADP-ribose polymer can be ruled out because of the lack of inhibition by NAD or nicotinamide (see the Results section).

Liver nuclei were incubated in medium B and then treated by method I. The precipitate, before the digestion with potassium hydroxide, was treated with pancreatic deoxyribonuclease, which did not result in any release of labelled adenine. In further experiments the reaction product in medium B containing liver nuclei was extracted by the method of Kirby (1956). The remaining $[8-^{14}C]ATP$ was largely removed by five precipitations of the product with ethanol. The final precipitate was then dissolved in 0.1% (w/v) potassium acetate and filtered through a Sephadex G-25 column (2cm $\times$ 48cm), equilibrated with 0.1% potassium acetate; this eliminated the last trace of labelled ATP. Digestion of the final product with snake-venom phosphodiesterase, with the addition of an amount of highly polymerized RNA, resulted in a progressive liberation of labelled adenine lasting for more than 2h with a simultaneous increase in E_{260} in the supernatant obtained after precipitation with perchloric acid. It may be concluded that the incorporation of ATP in the single-nucleoside-triphosphate reaction (ATP) is not due to the terminal attachment of AMP to DNA or RNA molecules, but to the formation of poly A.

Comparative rates of RNA and poly A formation in neuronal, glial and liver nuclei. Under the present assay conditions RNA polymerase activity per nucleus in liver nuclei is much lower than in cerebral nuclei (Tables 1 and 2, and Fig. 5), whereas the rate per nucleus of poly A formation is much higher in liver nuclei than in cerebral nuclei (Table 5). Considering the population of neuronal and glial nuclei in P_{51} and P_{36} fractions, the difference between neuronal and liver nuclei in the rates of RNA and poly A synthesis becomes much greater. Glial nuclei show intermediate values. It is noteworthy

that in glial nuclei from white matter (P_1) RNA polymerase activity is lower (Table 2), and poly A polymerase activity is higher (Table 5), than in glial nuclei from grey matter (P_0). In our observations it was seen that a mixture of equal numbers of cerebral P_{51} and liver nuclei gave an intermediate RNA polymerase activity, suggesting that the differences are not due to freely diffusible activators or inhibitors.

Bondy & Waelsch (1964) reported that RNA polymerase activity is higher in cerebral than in liver nuclei. It should be noted that these workers used [$8\text{-}^{14}\text{C}$]ATP and Mn^{2+} in their reaction medium and corrected their values of RNA polymerase for a blank obtained after incubation of nuclei in the absence of the other three nucleoside triphosphates. In this blank medium poly A formation is expected to proceed at a much higher rate in liver than in cerebral nuclei (see Fig. 7 and Table 5). This will make the value of RNA polymerase unduly low in liver nuclei and unduly high in cerebral nuclei. However, higher activity of RNA polymerase in brain compared with liver nuclei is claimed in experiments with labelled UTP, with which it is stated that the radioactivity in the non-nucleotide-dependent blank is decreased to less than 5% of the total (Bondy & Waelsch, 1965).

Barondes (1964), working with an aggregate enzyme, found that the RNA polymerase of brain was on the average only 37% as active as the liver enzyme, when activity was expressed per unit of DNA in the enzyme preparation. His assay medium contained 0.3M-potassium chloride, which had been demonstrated to be effective for activating RNA polymerase, though somewhat less so than ammonium sulphate (Goldberg, 1961). During the preparation of aggregate enzyme, a portion of the RNA polymerase can be released from the nuclei without concomitant release of DNA (cf. Liao, Sagher & Fang, 1968). It is also probable, although not proved, that the extent of release of RNA polymerase differs among various types of nuclei, and if this is the case, RNA polymerase activity on a DNA basis assayed with aggregate enzyme is not necessarily a relative measure of the polymerase activity in the whole nucleus.

Different mode of existence of RNA polymerase in cerebral and liver nuclei. RNA polymerase in isolated nuclei is activated by ammonium sulphate differently in cerebral P_{51} and liver nuclei (Figs. 3 and 4). A possible explanation for the ammonium sulphate activation of RNA polymerase in the aggregate enzyme is that the high ionic strength of the salt dissociates DNA-histone complexes and uncovers more of the DNA primer (Chambon, Ramuz & Doly, 1965). Removal of histones from rat-liver nuclei stimulated RNA polymerase activity when this was assayed in the absence of

ammonium sulphate, but not when RNA synthesis had already been stimulated by the presence of high salt concentration (Pegg & Korner, 1967). It has also been shown that soluble RNA polymerase from *Escherichia coli*, free of endogenous DNA, is markedly inhibited by ammonium sulphate, but the inhibition is greatly diminished when the enzyme is first allowed to complex with exogenous DNA (Chambon *et al.* 1965). The fact that the four-nucleoside-triphosphate reaction is differently activated by ammonium sulphate in cerebral P_{51} and liver nuclei strongly suggests the difference in the physical relationship between RNA polymerase and deoxyribonucleoprotein in these types of nuclei. It is noteworthy that visually discernible changes in reaction mixtures containing ammonium sulphate are observed in cerebral P_{51} nuclei, but not in liver nuclei, which may reflect the difference in deoxyribonucleoprotein between these types 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, Allfrey & Mirsky, 1963). Higher activity of RNA polymerase in neuronal nuclei in comparison with glial and liver nuclei may be relevant to this observation, since microscopy shows that neuronal nuclei have more lightly stained chromatin than glial and liver nuclei (cf. Kato & Kurokawa, 1967).

It has been shown that the Mg^{2+} -activated RNA polymerase reaction forms rRNA and is located primarily in the nucleolus, whereas the Mn^{2+} and ammonium sulphate-activated RNA polymerase reaction forms a more DNA-like RNA and occurs primarily in the extranucleolar chromatin (Widnell & Tata, 1966; Maul & Hamilton, 1967). Requirement for Mn^{2+} is specific, as the upward shift of the U/G incorporation ratio by the addition of ammonium sulphate is observed only when Mn^{2+} is present (Pogo, Littau, Allfrey & Mirsky, 1967). A similar upward shift of the U/G incorporation ratio was also observed in our experiments, when Mg^{2+} was replaced by Mn^{2+} in the reaction medium containing 0.3M-ammonium sulphate (Table 1). Thus the U/G incorporation ratio increases from 0.42 to 0.70 and from 0.64 to 0.81 in liver and glial nuclei respectively. In cerebral P_{51} nuclei, however, the U/G ratio remains virtually unchanged, the value being 0.66 irrespective of whether Mg^{2+} or Mn^{2+} is present. It is thus suggested that RNA polymerase activity in the extranucleolar chromatin within neuronal nuclei *in vivo* is more active compared with that in liver and glial nuclei.

Poly A formation in liver nuclei as compared with similar activities in micro-organisms and in other mammalian tissues. The purified preparations of microbial RNA polymerase are reported to form

poly A with DNA as a template (Chamberlin & Berg, 1962, 1964; Fox & Weiss, 1964; Stevens, 1964). The reaction specifically requires Mn^{2+} , and is inhibited in the presence of other nucleoside triphosphates.

Specific requirement of Mn^{2+} (Fig. 6) and inhibition by nucleoside triphosphates (Fig. 8) in the poly A formation are also observed in liver nuclei. The possibility that this inhibition is due to a removal of Mn^{2+} by added nucleoside triphosphates was ruled out by experiments where ATP incorporation was assayed in medium B containing 2mM- Mn^{2+} plus 20mM- Mg^{2+} (see the Results section). Added GTP, CTP and UTP bind some of the bivalent cations. The concentration of Mn^{2+} is, however, expected to remain almost unchanged, since Mg^{2+} is preferentially bound by nucleoside triphosphates because of its higher concentration. When the concentration of ammonium sulphate is 30mM, which is optimum for poly A formation, addition of GTP, CTP and UTP inhibits the reaction by 69%. When the concentration of ammonium sulphate is raised to 0.3M, the reaction is inhibited to a smaller extent (50%). With 0.3M-ammonium sulphate, RNA formation will predominate on the addition of GTP, CTP and UTP, and this will mask the inhibitory effect of these nucleotides on poly A formation.

Native DNA in the nucleus does not seem to play an essential role in priming the poly A formation in liver nuclei, as shown in experiments with deoxyribonuclease (see the Results section). No adequate explanation for this is possible at present. In microbial RNA polymerase, it has been established that native DNA is more effective than heat-denatured DNA for RNA synthesis, and single-stranded DNA is more effective than helical DNA for homopolyribonucleotide synthesis (Chamberlin & Berg, 1964; Fox & Weiss, 1964). ATP polymerase purified from calf thymus was activated by Mg^{2+} and required poly A as a template (Edmonds & Abrams, 1960, 1962). Requirement for a bivalent cation was not specified in poly A polymerase activity prepared from chorioallantoic membrane of chick embryo, although the inhibition of this activity by other nucleoside triphosphates was reported (Venkataraman, 1962).

Balance between RNA and poly A formation among various types of nuclei. When GTP, CTP and UTP are added to medium B containing labelled ATP, poly A formation is inhibited whereas RNA formation is stimulated. On the subsequent addition of actinomycin D, the stimulated RNA formation is inhibited, leading to a further decrease in ATP incorporation (Table 4). The results presented in Table 4 indicate that, under these conditions, RNA formation in cerebral P_{51} and P_{36} nuclei is more easily promoted than in liver nuclei. This promotion of RNA synthesis in P_{51} and P_{36} nuclei

can be attributed to the high RNA polymerase activity in neuronal nuclei. In liver nuclei inhibition of poly A synthesis appears to be more prominent than the promotion of RNA synthesis, in agreement with the high poly A polymerase activity in liver nuclei (Table 5). Glial nuclei, P_3 , P_0 and P_1 behave like liver nuclei, but unlike cerebral P_{51} and P_{36} nuclei (Table 4).

Single-nucleoside-triphosphate reaction (CTP) in neuronal nuclei. In cerebral P_{51} nuclei, the rate of [^{14}C]CTP incorporation is much higher than those of the other three [^{14}C]nucleoside triphosphates (Table 3). Taking into account the lower rate of CTP incorporation in cerebral P_3 nuclei where glial nuclei preponderate, active incorporation of CTP can be attributed to a property of neuronal nuclei. Inhibition of the single-nucleoside-triphosphate reaction (CTP) by the other three nucleoside triphosphates cannot be demonstrated in P_{51} nuclei (Table 6), presumably because highly active RNA formation promoted under these conditions masks the inhibition. On the basis of actinomycin D sensitivity (Table 6), the single-nucleoside-triphosphate reaction (CTP) in the present experiment can be taken to represent poly C synthesis. Formation of poly C has already been claimed in an aggregate enzyme from cerebral nuclei (Mandel *et al.* 1967), but without particular reference to neuronal nuclei.

The demonstration of the difference in the rates of poly A and poly C synthesis in neuronal and glial nuclei will require some reservation until an experiment is performed where the nature of several molecular species of RNA in micro-dissected neurones and glial cells is discussed on the basis of their base ratios (cf. Hyden, 1967). Higher rates of incorporation of [^3H]cytidine into nerve cells and of [^3H]adenine into glial cells *in vivo* (Danesholt & Brattgard, 1966), for example, may partly be a reflexion of higher poly C and poly A synthesis in neuronal and glial nuclei respectively. However, the natural occurrence of these homopolymers in brain cells remains to be explored.

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