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The Metabolism of High-Molecular-Weight Ribonucleic Acid, including Polyadenylated Species, in the Developing Rat Brain

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High-molecular-weight RNA was isolated from rat brain at various times after the intracranial administration of [^{32}P]P_i. The synthesis of 28S and 18S rRNA could be detected within 1h of the injection of the radioactive precursor and appeared to be more pronounced, relative to other high-molecular-weight RNA, in the brains of older rats compared with those of newborn rats. Polyadenylated RNA, representing most mRNA and their precursors, was isolated by chromatography on oligo(dT)-cellulose. The contribution of this polyadenylated RNA to total RNA synthesis was investigated in the cerebral cortex and the phylogenetically older brain stem at different stages in the development of the rats by using a 5h period of labelling as an arbitrary index of transcription. In the brain stem the proportion of labelled polyadenylated RNA comprised 27–30% of the total RNA. The corresponding values for the cortex decreased from 34% in newborn rats to 23% in 40–150-day-old rats. These data indicated that proportionately more polyadenylated RNA is synthesized in the cortex of the newborn than in the adult rat and that there is a progressive decrease in the synthesis of polyadenylated RNA relative to rRNA during development.

In the adult mammalian brain there is almost no cellular division and the predominantly non-replicative state is reached early in the development and differentiation of the brain. In the rat brain, which develops postnatally (Davison & Dobbing, 1968), cellular division proceeds up to the second week after birth, although there are different rates of proliferation in different regions, since in the cortex almost 50% of the adult cellular complement is present at birth, whereas in the cerebellum the corresponding value is about 10% (Balázs, 1974). During this period the developing rat brain continues to accumulate rRNA. This accumulation of RNA, based on DNA content, ends abruptly after the cessation of cellular division and then is reversed slightly until a constant rRNA content is reached (Adams, 1966; Balázs *et al.*, 1968). It has also been reported that the turnover of rapidly labelled cerebral RNA hybridizing to DNA, i.e. presumptive mRNA, was faster in newborn than in adult rats (Bondy & Roberts, 1968). Thus there are distinctive changes in the metabolism of RNA during the period of postnatal development, which has been shown to be extremely sensitive to various factors [e.g. undernutrition of the animal as well as hormonal and amino acid imbalances (see *Biochem. Soc. Spec. Publ.* **1** (1973)]. The ability of polyadenylated RNA, i.e. mRNA and precursors (Lim & Canellakis, 1970; Brawerman, 1974), to bind to oligo(dT)-cellulose (Aviv & Leder, 1972) has made it possible for us to investigate the metabolism of polyadenylated RNA in relation to that of rRNA during rat brain development.

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Materials and Methods

Chemicals

A.R.-grade chemicals were used in all cases. All glassware was treated with 0.05M-NaOH to destroy ribonuclease activity before rinsing with water. [^{32}P]P_i was obtained from The Radiochemical Centre, Amersham, Bucks., U.K., as a sterile acidic solution (10mCi/ml).

Animals

Wistar rats of both sexes, bred in this laboratory, were used in all experiments. Iso-osmotic neutralized [^{32}P]P_i in 0.9% NaCl was injected in volumes that varied with the age and size of the animal. Usually 0.1ml/g wet wt. of brain was injected intracranially and deposited over the right hemisphere of the cerebellum of animals under ether anaesthesia. In young rats, the hypodermic needle was forced through the skull, whereas in adults the needle was guided into the intracranial space via the foramen magnum. The animals recovered within a few minutes of the treatment and young rats were returned to mothers when

necessary. At appropriate times after the injection rats were decapitated and the skull was cut open in a hemicircle, starting from the foramen magnum to expose the brain. The cerebellum was removed *in situ* with a small spatula. When the brain was divided into regions the cerebral hemispheres were loosened and peeled from the brain stem at the region of the corpus callosum. The hemispheres were separated from the brain stem in a caudal–rostral direction and cuts were made at the external capsule. The olfactory lobes were removed from the remaining part of the brain, which was then considered as the brain stem in subsequent analysis to differentiate it from the cortex. The brain stem included the thalamus and striatum in the rostral portion and the medulla oblongata caudally. The meninges and other blood vessels were then teased away. In experiments involving the use of forebrains, the brain was transected at the level of the pons–medulla and the olfactory lobes were dissected out.

Nucleic acid extraction

The brain preparations were immediately homogenized either in 0.25M-sucrose containing 50mM-Tris/HCl, pH7.6, 25mM-KCl and 1mM-$MgCl_2$ or in medium containing 1% (v/v) tri-isopropylnaphthalenesulphonic acid, 6% (w/v) *p*-aminosalicylic acid, 8mM-Tris/HCl, pH8.0 (TNS medium). A loose-fitting Potter–Elvehjem Teflon homogenizer in a glass vessel (Thomas and Co., Philadelphia, Pa., U.S.A.) was used to disperse the tissue in sucrose solution with 15 strokes by hand, or if in TNS medium with a motor-driven pestle at very low speeds (300rev./min). In sucrose the tissue concentration was 20% (w/v), adjusted to 10% (w/v) after homogenization by addition of concentrated TNS medium. The final concentration of both was thus 10% in the TNS medium. Nucleic acid extraction was carried out at room temperature by a modification of the method of Kirby (1968) by five successive extractions of the aqueous phase with phenol (500g), *m*-cresol (70ml) and 8-hydroxyquinoline (0.5g) previously equilibrated with 10mM-Tris/HCl, pH7.6. This phenol medium was used as a mixture (1:1, v/v) with chloroform containing 1% (v/v) 3-methylbutan-1-ol (isoamyl alcohol) to facilitate quantitative extraction of polyadenylated RNA (Perry *et al.*, 1972). After the first extraction, aqueous phase and the remaining interphase were adjusted to contain 0.5M-NaCl and re-extracted with fresh phenol medium. All phase separations were performed by centrifuging the mixtures in a MSE 4L centrifuge at 3000*g* for 10min. The final aqueous phase was mixed with 2vol. of ethanol and left overnight at −20°C to precipitate the nucleic acids. The ethanol precipitate was washed by resuspension in 70% (v/v) ethanol containing 0.1M-sodium acetate and then dissolved in 10mM-Tris/HCl, pH7.6, containing 0.2% sodium dodecyl sulphate in the same volume as the original 10% (w/v) homogenate. The nucleic acids were allowed to dissolve completely, over a period of several hours. The homogeneous solution was then adjusted to contain 2.0M-LiCl, and complete precipitation of high-molecular-weight RNA occurred over a period of at least 15h at 4°C. This precipitate was washed twice by resuspension in small volumes of 2.0M-LiCl and subsequently dissolved in the Tris/sodium dodecyl sulphate solution. This solution was adjusted to contain 0.15M-sodium acetate, pH6.0, and after the addition of 2vol. of ethanol, the RNA precipitate was allowed to form overnight at −20°C. This purified RNA was finally dissolved in a small volume of the Tris/sodium dodecyl sulphate solution immediately before electrophoresis on polyacrylamide gels or chromatography on oligo(dT)-cellulose.

Polyacrylamide-gel electrophoresis

This was performed essentially as described by Loening (1967) on 2.0–2.4% (w/v) polyacrylamide gels. Absorbance of the gels was measured at 260 and 280nm on a Gilford 240 spectrometer with a linear gel-scanning attachment either directly after electrophoresis or after fixation in 7% (v/v) acetic acid. Gels were frozen and sliced into 1mm fragments with a Mickle gel slicer. The radioactivity of dried slices was assayed in 5ml of a toluene-based scintillation fluid containing 0.75% (w/v) butyl-PBD [5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole; Ciba Ltd. Horsham, Sussex, U.K.] in a Beckman LS 230 spectrometer. Recently radioactivity in gel slices has been measured after placing them on 16mm cine-film connected to an automated assembly incorporating a Geiger–Müller counter attached to a rate meter and recorder. This equipment was purchased from J. and P. Engineering, Reading, Berks., U.K., and was based on a design by Dr. U. E. Loening.

Oligo(dT)-cellulose chromatography

^{32}P-labelled polyadenylated RNA was isolated on columns containing 200mg of oligo(dT)-cellulose (Searle Ltd., High Wycombe, Bucks., U.K.) by a modification of the method described by Aviv & Leder (1972). The matrix was treated with 0.1M-NaOH and then equilibrated with 0.4M-NaCl, 10mM-Tris/HCl, pH7.4, and 0.2% sodium dodecyl sulphate (high-salt solution). The ^{32}P-labelled RNA sample was applied in 2ml of the high-salt solution. The elution of non-polyadenylated RNA with the high-salt solution was monitored in successive 2ml fractions by Cerenkov radiation (Lim *et al.*, 1970)

until no trace of radioactivity could be detected in the effluent. The polyadenylated RNA bound to the oligo(dT)cellulose was then eluted with 10mM-Tris/HCl, pH7.4, and 0.2% sodium dodecyl sulphate (low-salt solution); again elution was monitored by Cerenkov radiation, which is a more sensitive method of assaying for the presence of RNA than u.v.-absorbance measurements. The RNA in the low-salt solution was precipitated by addition of 3vol. of ethanol after first adjusting the solution to 0.15M-sodium acetate, pH6.0, and adding yeast RNA as carrier.

Assay of radioactivity in purified RNA

The purity of ^{32}P-labelled RNA prepared by phenol extraction–LiCl precipitation was assessed by a modification of the method of Bollum (1968). Samples (20–100μl) of ^{32}P-labelled RNA in Tris/sodium dodecyl sulphate solution were pipetted on to Whatman GF/C glass-fibre filters (radius 2.5cm). Dried filters were assayed for radioactivity in 5ml of the toluene-based scintillation fluid. This represented total radioactivity of the sample. After radioactivity counting, the filters were soaked and then washed in ice-cold 10% (w/v) trichloroacetic acid/1% (w/v) $Na_4P_2O_7$ for 30min. The filters were then placed on Millipore filter supports and successively washed with fresh trichloroacetic acid solution, ethanol and finally ether. The dried filters were next assayed for radioactivity in 5ml of the scintillation fluid. This represented trichloroacetic acid-precipitable radioactivity. The filters were recovered from the fluid, which was found to contain no radioactivity after removal of filters. Dried filters were soaked in 5% (w/v) trichloroacetic acid and then heated in the same solution at 90°C for 15min. The filters were subjected to the ethanol–ether washing and drying procedure before measurements of radioactivity, which was considered to be residual radioactivity after the removal of RNA.

Results

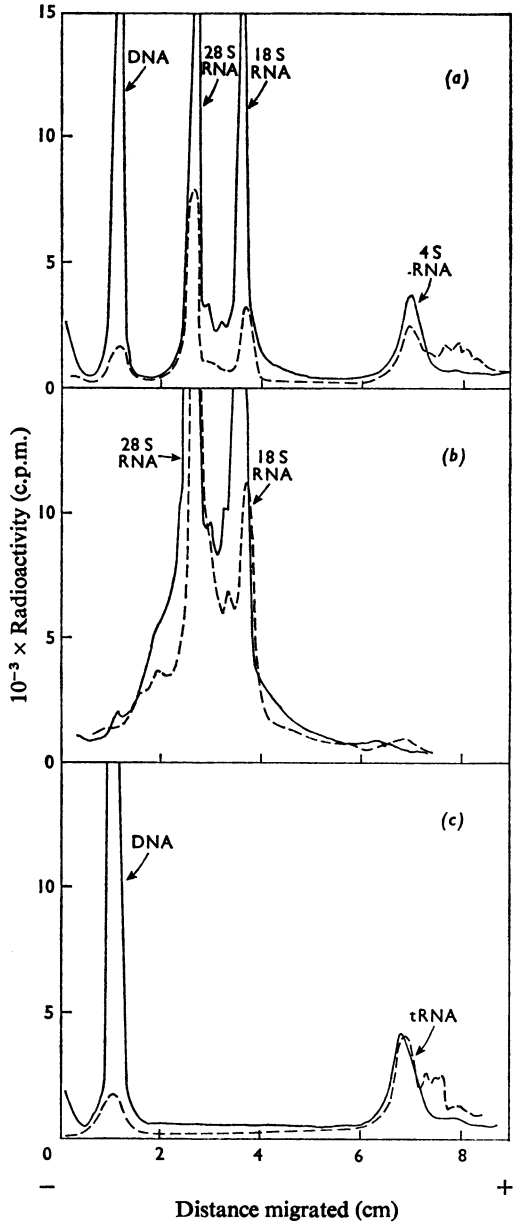
Incorporation of [^{32}P]P_i into high-molecular-weight RNA

[^{32}P]P_i was chosen as a precursor for brain RNA in order to label all four nucleotide constituents, which would result in substantial incorporation of radioactivity in the RNA, relative to that observed when either radioactive uridine or orotic acid was used. The ^{32}P-labelled nucleic acids could be conveniently assayed in solution by Cerenkov radiation; this facilitated the study of RNA species (e.g. mRNA, which constitute only a small proportion of the total cellular RNA), as direct measurements of radioactivity could be made on effluents from chromatography columns, before precipitation and analysis on polyacrylamide gels. For investigations on the metabolism of high-molecular-weight RNA including rRNA and mRNA, as well as their precursor forms, it was necessary to eliminate from the phenol extract of nucleic acids (*a*) DNA, which is labelled extensively in young rats as it is synthesized, and (*b*) tRNA, the terminal turnover of which would interfere with accurate assessment of the incorporation of precursors into RNA. Precipitation procedures involving the use of 2M-LiCl provided the most suitable way of obtaining purified preparations of the high-molecular-weight RNA, since both tRNA and DNA are soluble in 2M-LiCl. As shown in Fig. 1(*a*), nucleic acids, prepared by ethanol precipitation of phenol extracts of the forebrains of 10-day-old rats 10h after the intracranial administration of [^{32}P]P_i, contained considerable radioactivity in rRNA and tRNA as well as in DNA. Radioactivity could be detected in the region of the polyacrylamide gel immediately after that of tRNA. This radioactivity was not derived from nucleic acids, as will be shown below. RNA precipitated by 2M-LiCl and analysed similarly on polyacrylamide gels consisted entirely of high-molecular-weight forms, as shown by the adsorbance and radioactivity in the region containing rRNA (Fig. 1*b*). The nucleic acids soluble in LiCl consisted of DNA and tRNA, with no evidence of rRNA (Fig. 1*c*). The radioactivity in material of high mobility also remained soluble. Thus the LiCl precipitation procedures resulted in selective and quantitative separation of high-molecular-weight RNA from other nucleic acids. The high-molecular-weight RNA labelled after 10h consisted largely of rRNA and it was difficult to detect labelling of either mRNA or precursors to this and rRNA.

The presence of these precursor RNA species could be detected after a short period of labelling of the nucleic acids. Fig. 2 represents an analysis of rapidly labelled high-molecular-weight RNA as well as illustrating yet another advantage of the LiCl precipitation procedures. The incorporation of precursor into nucleic acids is rather low at 1h and restrictions on the amount of nucleic acids analysed on polyacrylamide gels made it difficult to detect labelling of the nucleic acids, other than in DNA, at the upper portion of the gel, or tRNA (Fig. 2*a*). With the elimination of DNA, the high viscosity of which contributes largely to the restriction on the amount of nucleic acid layered on gels, much more high-molecular-weight RNA could be analysed electrophoretically. The analysis then revealed previously undetectable radioactivity in a heterogeneous population of RNA of high molecular weight (Fig. 2*b*). These labelled high-molecular-weight RNA species probably included precursors of both rRNA and mRNA.

Polyribonucleotide nature of the 2M-*LiCl precipitate of phenol extracts of brain*

Phenol extracts of ^{32}P-labelled brain preparations included not only nucleic acids, which form the main constituent, but also highly labelled contaminants. After very short periods of labelling, the contribution of these contaminants to the total radioactivity of the ethanol precipitate was high (Table 1), as shown by their solubility in trichloroacetic acid, normally used for precipitating the nucleic acids. Their relative contribution to the radioactivity of ethanol precipitates fell from 95% in 3h of labelling to about 50% by 48h because of the continued and sustained synthesis of RNA. These contaminants are highly acidic and on gel electrophoresis migrate near the solvent front, ahead of tRNA. When the nucleic acid extracts were subjected to the LiCl precipitation procedures, it was found that the radioactivity in the high-molecular-weight RNA fraction was exclusively precipitated with 5% (w/v) trichloroacetic acid at all times after the administration of [^{32}P]P_i. This radioactivity was also completely solubilized by heating the trichloroacetic acid precipitates at 90°C for 15min (Table 2), and this result confirms the electrophoretic analysis in Fig. 2(*b*) showing complete removal of the labelled non-nucleic acid contaminant by LiCl precipitation, which can thus be used for the preparation of labelled purified high-molecular-weight brain RNA.



Electrophoretic analyses of high-molecular-weight RNA in the developing rat brain after intracranial administration of [^{32}*P*]P_i

High-molecular-weight RNA prepared from the forebrains of developing rats, killed at various times after the intracranial administration of [^{32}P]P_i, was then analysed electrophoretically on gels. These experiments were designed to determine whether there were any changes in the incorporation of radioactivity into the rRNA component of high-molecular-weight RNA during development, since it is known that rRNA continues to accumulate in the brain up to the third week after birth. In the 3-day-old

Fig. 1. *Electrophoretic analysis of high-molecular-weight RNA prepared by LiCl precipitation*

Rats (10 days old) were injected intracranially with 1mCi of [^{32}P]P_i/g of brain and killed 10h later. Nucleic acids were phenol-extracted from the forebrains and precipitated with 75% (v/v) ethanol. The ethanol precipitate was dissolved in 10mM-Tris/HCl, pH7.6, containing 2% sodium dodecyl sulphate and a portion (1 E_{260} unit) was analysed (total nucleic acid extract; *a*). High-molecular-weight RNA was precipitated with 2.0M-LiCl, dissolved in the Tris/sodium dodecyl sulphate buffer and a portion (1.5 E_{260} units) was analysed (high-molecular-weight RNA; *b*). The radioactive RNA in the LiCl-soluble fraction was reprecipitated with 75% (v/v) ethanol, dissolved in the Tris/sodium dodecyl sulphate buffer and a portion (1 E_{260} unit) was analysed (LiCl-soluble RNA; *c*). Electrophoresis was performed on 2.2% (w/v) polyacrylamide gels at 5mA for 3h. Details of the LiCl procedure and measurements of radioactivity in the gels are described in the text. For clarity in this and subsequent Figures the scales for E_{260}, which gives only relative measurements of the RNA, are omitted: ——, E_{260}; ----, radioactivity.

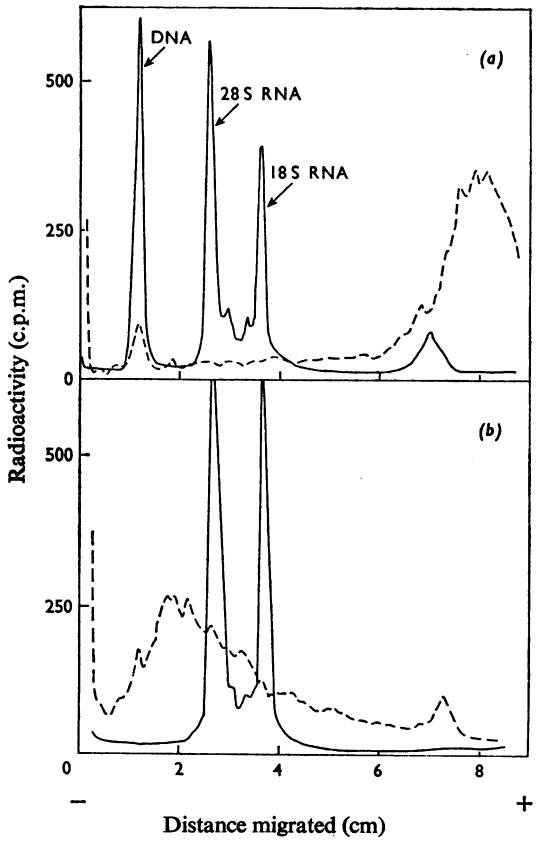


Fig. 2. *Detection of rapidly labelled high-molecular-weight RNA after LiCl precipitation*

Rats (10 days old) were killed 1h after the intracranial administration of a standard dose of [^{32}P]P_i. (*a*) Phenol-extracted ethanol-precipitated nucleic acids and (*b*) high-molecular-weight RNA prepared by LiCl precipitation of this nucleic acid extract of forebrains were subjected to electrophoretic analyses as described in Fig. 1. ——, E_{260}; ----, radioactivity.

rat brain the high-molecular-weight RNA labelled 1h after administration of [^{32}P]P_i was heterogeneous in size, with most of the labelled RNA being considerably larger than 28S rRNA. The presence of discrete rRNA species could not be detected (Fig. 3*a*). After 5h of labelling rRNA was found to be highly radioactive. Some radioactivity could also be detected as a shoulder on the heavy side of the peak of labelled 28S RNA. The mRNA present was presumably in the region underlying the rRNA, i.e. about 16–30S in size. After 24h of labelling, radioactivity in the high-molecular-weight RNA was almost exclusively in rRNA, as seen by coincidence of the radioactivity with absorbance in the gels.

Table 1. *Precipitates obtained with trichloroacetic acid from solutions of ^{32}P-labelled ethanol precipitates of brain phenol extracts*

Rats (10 days old) were injected intracranially with 400μCi of [^{32}P]P_i each. Two or three rats were killed at each time and the forebrains were subjected to phenol extractions for isolation of nucleic acids without the LiCl precipitation step (see the Materials and Methods section). The nucleic acid precipitated in 75% (v/v) ethanol was washed twice in 75% ethanol and dissolved in 10mM-Tris/HCl, pH7.6, containing sodium dodecyl sulphate. Samples were removed and the ^{32}P-labelled precipitates in 10% (w/v) trichloroacetic acid were assayed for radioactivity. Recovery was calculated on the basis of 100% for the total ethanol-precipitate radioactivity. The results shown here and in Table 2 are the averages of triplicate measurements and are representative of at least four separate experiments.

Period of labelling (h)	Proportion of radioactivity in trichloroacetic acid precipitates (%)
3	4.7
6	10.6
12	24.0
48	57.8

Table 2. *Polyribonucleotide nature of LiCl precipitates of phenol extracts of ^{32}P-labelled brain preparations*

Rats (8 days old) were injected intracranially with 1mCi of [^{32}P]P_i/g of brain. Phenol-extracted RNA was purified by precipitation with 2.0M-LiCl. The details for the assay of radioactivity in ethanol precipitates, as well as in the trichloroacetic acid precipitates of high-molecular-weight RNA prepared by LiCl precipitation, are given in the Materials and Methods section. Recovery of radioactivity is based on the original radioactivity of ^{32}P-labelled RNA precipitated by 75% (v/v) ethanol after first undergoing a LiCl precipitation-purification step.

Period of labelling (h)	10^{-3}×Total radioactivity (c.p.m.)	Proportion of radioactivity recovered in precipitate (%): Insoluble in trichloroacetic acid at 4°C	Insoluble after heating at 90°C
2.5	25	99.4	0.6
5.0	45	99.1	0.4
24.0	160	98.9	0.1
48.0	90	99.0	0.2

In the 26-day-old rat, chosen because cellular division as well as accumulation of rRNA has ceased and because differentiation, e.g. myelination, is well under way, the brain high-molecular-weight RNA labelled within 1h consisted not only of the large RNA species, which are considerably heavier than

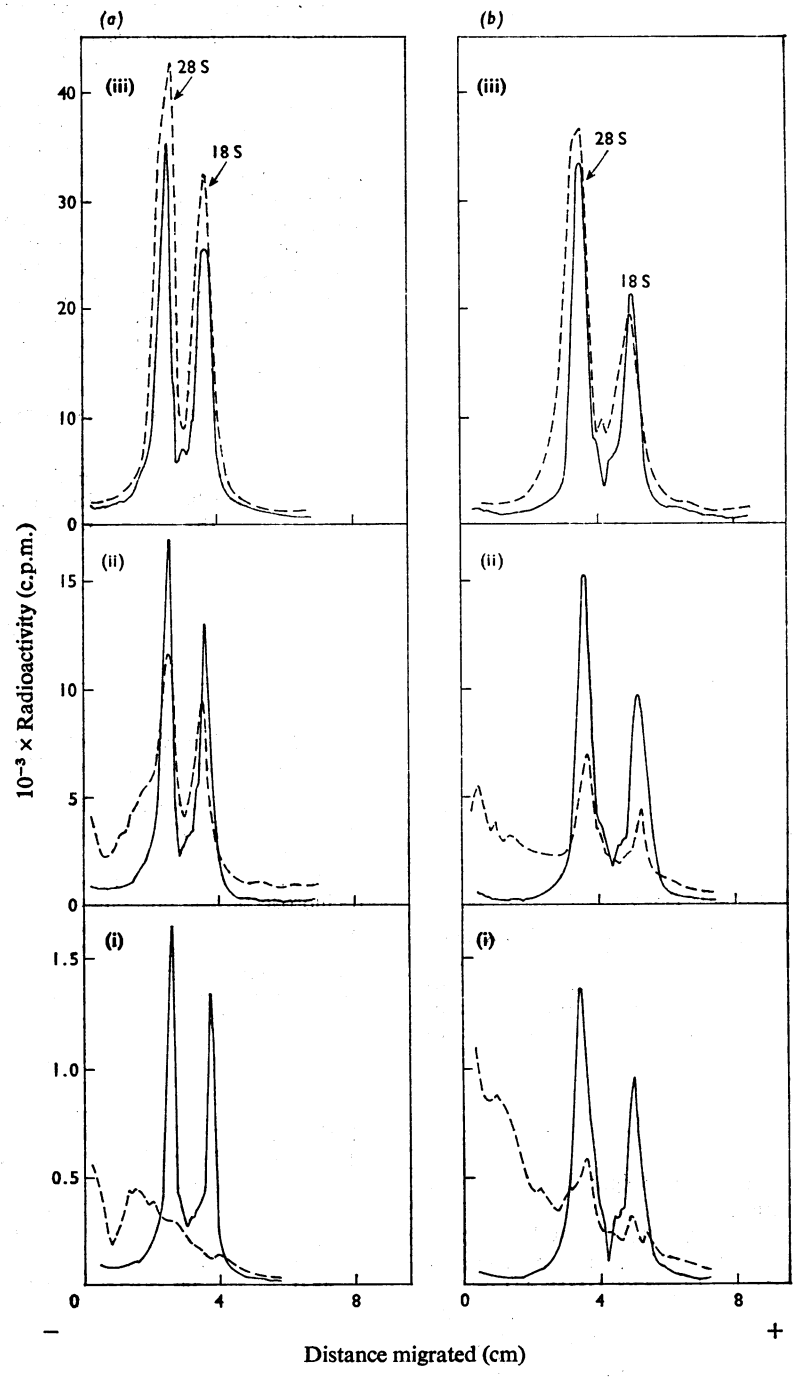


Fig. 3. *Characterization of ^{32}P-labelled brain high-molecular-weight RNA from (a) neonatal and (b) 26–27-day-old rats at different times after administration of precursor*

Rats (*a*) 3 days old and (*b*) 26–27 days old were injected intracranially with the standard dose of 1 mCi of [^{32}P]P_i/g of brain. They were killed either (i) 1 h, (ii) 5 h or (iii) 24 h later. High-molecular-weight RNA prepared from the forebrains was analysed by electrophoresis as described in Fig. 1. ——, E_{260}; – – – –, radioactivity.

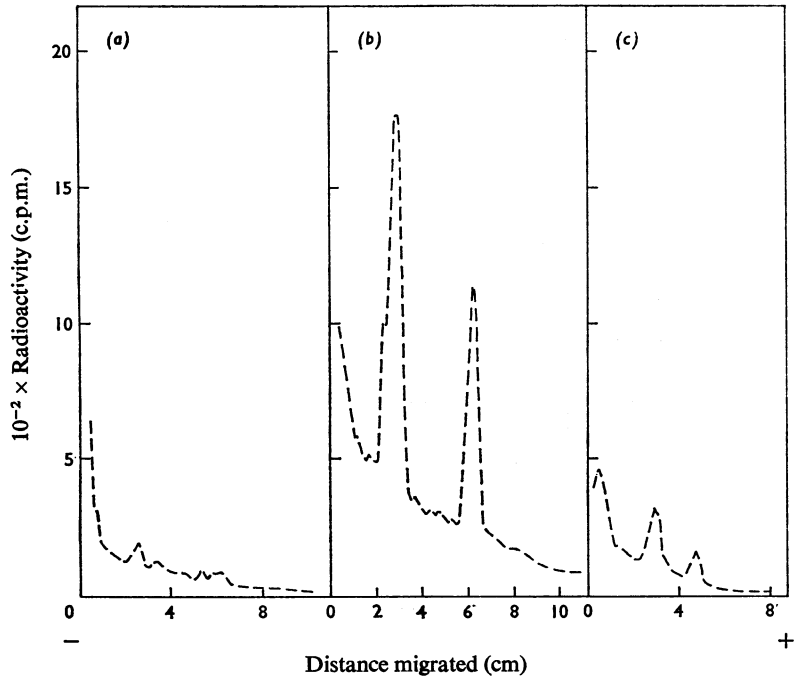


Fig. 4. *Electrophoretic analyses of rapidly labelled high-molecular-weight RNA from 18-day-old and adult rat forebrains*

High-molecular-weight RNA was prepared from forebrains of 18-day-old rats (*a*) 1h and (*b*) 5h after the intracranial administration of 1mCi of [^{32}P]P_i/g of brain. Electrophoretic analysis of the RNA was as described in Fig. 1. (*c*) represents an analysis of high-molecular-weight RNA from adult rat forebrain extracted 1h after [^{32}P]P_i administration. The corresponding adult brain high-molecular-weight RNA labelled for 5h is essentially similar to (*b*) and is not shown.

28S RNA, but also of distinct rRNA species (28S and 18S RNA), with radioactivity also present in the 32S rRNA precursor (Fig. 3). In these older animals, the high-molecular-weight RNA labelled after 5h, in contrast with the 3-day-old animals, contained a considerable proportion of radioactivity in the larger forms of RNA besides that in the rRNA, although these latter form the bulk of the labelled RNA. Radioactivity in high-molecular-weight RNA labelled after 24h was mostly in rRNA, although traces of the larger forms were present as small amounts of radioactivity in the upper portion of the gel. This pattern of early distinct labelling of rRNA in the brain of older animals and persistence of radioactivity in the RNA larger than 28S even after 5h of labelling was also shown by corresponding preparations from 18-day-old rats as well as adults (Fig. 4).

Isolation of polyadenylated RNA

High-molecular-weight RNA can be further fractionated into polyadenylated RNA, representing mRNA and precursors, and RNA without polyadenylate, which consists largely of rRNA and precursor forms, although this fraction will also include unpolyadenylated mRNA and/or precursor. The quantitative elution of RNA not containing poly(A), as well as the amount of radioactivity in the polyadenylated RNA fraction binding to oligo(dT)-cellulose chromatography columns, was monitored by Čerenkov radiation (Fig. 5*a*). Before fractionation labelled high-molecular-weight RNA consisted of both 28S and 18S RNA as well as the larger species (Fig. 5*b*). After the fractionation on oligo(dT)-cellulose, the first effluent, i.e. RNA containing no poly(A) residues, consisted mostly of rRNA, although traces of the larger species could be detected as labelled shoulders on the heavy side of the 28S RNA peak. The polyadenylated RNA eluted next clearly contained no rRNA and was heterogeneous in size, although generally being larger than 28S RNA.

Differential metabolism of RNA with or without poly(A) residues in the developing rat brain

The ability to isolate polyadenylated RNA permitted us to determine whether changes in the metabolism of polyadenylated RNA, relative to that of other forms of RNA, occurred during the develop-

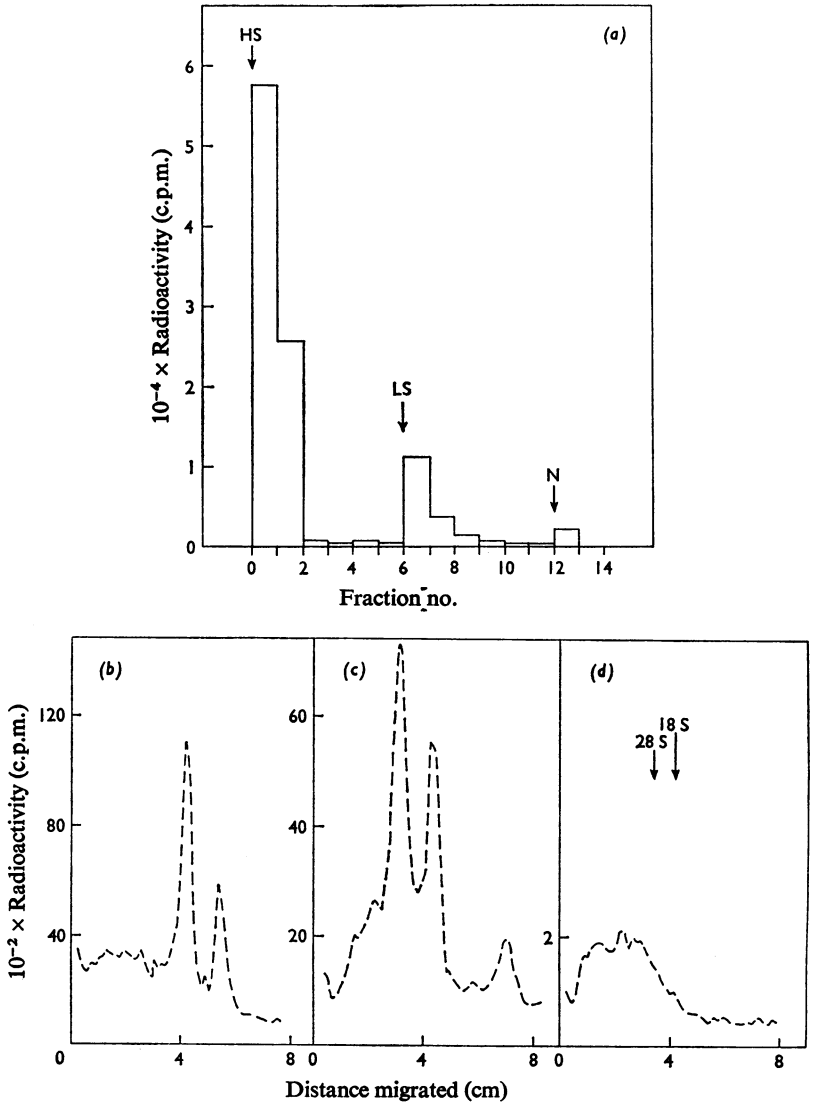


Fig. 5. *Isolation and characterization of ^{32}P-labelled polyadenylated RNA*

(*a*) High-molecular-weight RNA was isolated from forebrains of 8-day-old rats, 5h after the intracranial injection of 1mCi of [^{32}P]P_i/g of brain. The RNA (80×10^3c.p.m. in 6 E_{260} units) was applied in high-salt buffer containing 0.4M-NaCl, 10mM-Tris/HCl, pH7.4, 0.2% sodium dodecyl sulphate, on to a column of 200mg of oligo(dT)-cellulose. The RNA not containing poly(A) was eluted in 2ml fractions with high-salt buffer (HS). Polyadenylated RNA was then eluted with 10mM-Tris/HCl, pH7.4, containing 0.2% sodium dodecyl sulphate (low-salt buffer; LS), again in 2ml fractions. ^{32}P radioactivity was monitored by Cerenkov radiation. Residual radioactivity in the column was removed with 0.1M-NaOH (N). Unfractionated high-molecular-weight RNA (*b*), RNA not containing poly(A) (*c*) and polyadenylated RNA (*d*) were electrophoresed on polyacrylamide gels as described in Fig. 1. Radioactivity measurements are shown.

ment of the brain that would correlate with changes from a proliferating phase to a differentiated and largely non-dividing state. Initial studies were undertaken with samples prepared from the cortex and from the brain stem of rats at different stages of development, after a short-term labelling of 5h and a long-term labelling of 72h (Table 3). In the young brain, in both the cortex and brain stem, the specific

Table 3. *Differential incorporation of [^{32}P]P_i into polyadenylated RNA in the cerebrum and brain stem of newborn and adult rats*

Rats (4 or 25 days old) were each injected with 200μCi and 1mCi of [^{32}P]P_i respectively. At the different times indicated two or three rats were killed, the cortex and brain stem dissected out and high-molecular-weight RNA was isolated from these brain preparations. The proportion of radioactivity in the polyadenylated RNA constituent was measured after chromatography on oligo(dT)-cellulose (see the Materials and Methods section). The last column here and in Table 4 represents the percentage difference between the values of (A) and (B) relative to the value of (B). The results shown here and in Table 4 are representative of at least three separate sets of experiments.

Age (days)	Period of labelling (h)	Brain region	Sp. radioactivity of RNA (c.p.m./E_{260} unit)	Radioactivity in polyadenylated RNA (%)	Relative difference [(A−B)/B]×100 (%)
4	5	Cortex	135000	(A) 31.8	+56.5
		Brain stem	122000	(B) 20.4	
	72	Cortex	158000	4.0	
		Brain stem	174000	6.7	
25	5	Cortex	21500	(A) 26.1	−27.4
		Brain stem	44900	(B) 35.9	
	72	Cortex	88700	7.7	
		Brain stem	94500	5.9	

Table 4. *Developmental changes in the incorporation of [^{32}P]P_i into polyadenylated RNA in cortex and brain stem*

Rats of the different ages shown were injected intracranially with 1mCi of [^{32}P]P_i/g of brain. They were all killed 5h later and the proportion of radioactivity in the polyadenylated RNA component of high-molecular-weight RNA was measured as described in the Materials and Methods section.

Age (days)	Brain region	Radioactivity in polyadenylated RNA	Relative difference [(A−B)/B]×100 (%)
3	Cortex (A)	34.5	+29.7
	Brain Stem (B)	26.7	
5	Cortex (A)	34.3	+16.7
	Brain stem (B)	29.4	
8	Cortex (A)	31.9	+7.8
	Brain stem (B)	29.4	
40	Cortex (A)	22.2	−22.2
	Brain stem (B)	28.6	
150	Cortex (A)	23.5	−22.9
	Brain stem (B)	30.6	

radioactivity of the high-molecular-weight RNA after 72h was not substantially increased over the 5h values. The proportion of ^{32}P-labelled polyadenylated RNA fell in both brain regions from values of over 20% at 5h to about 5% after 72h. In the 25-day-old rats, the specific radioactivity of the RNA in both brain regions at 72h was substantially increased over the corresponding values after 5h of labelling. The proportion of ^{32}P-labelled polyadenylated RNA in both brain regions fell sharply within this time, as with the younger rats.

Changes in the proportion of labelled polyadenylated RNA in the cortex relative to the brain stem during brain development

In the initial studies on the labelling of polyadenylated RNA (Table 3), we noticed that there were differences in the proportion of radioactivity in the polyadenylated RNA in the cortex, relative to the brain stem, after 5h of labelling. Since the brain stem develops earlier than the cortex during differentiation of the brain (Fish & Winick, 1969), the differential labelling of the polyadenylated RNA was investigated throughout the early part of development, the 5h period being chosen as an arbitrary period for comparison. The choice of this time is explained in the Discussion section. The proportion of the polyadenylated RNA labelled at 5h in the brain stem was about 27–30%. The corresponding values for the cortex fell from 35% in the 3-day-old rat to around 22–23% in the 40-day-old and adult rats (Table 4). The data are also expressed as percentage changes in the proportion of the polyadenylated RNA labelled in the cortex relative to the brain stem and plotted in Fig. 6 to illustrate changes in the labelling of polyadenylated RNA during development. The proportion of labelled polyadenylated RNA in the cortex relative to that of brain stem was found to decrease progressively with increasing age of the rat until about 40 days after birth, when this ratio remains constant.

Discussion

Developmental differences in the incorporation of [^{32}P]P_i into high-molecular-weight RNA

We investigated the metabolism of only the high-molecular-weight RNA components of rat brain in order to obtain selective information on the relation-

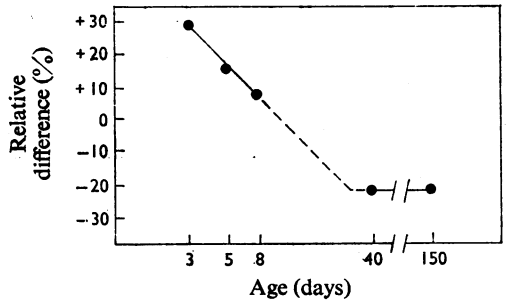


Fig. 6. *Developmental changes in the incorporation of* [^{32}P]P_i *into polyadenylated RNA in (A) cerebral cortex and (B) brain stem*

Data from Table 4 (last column) are plotted as the relative percentage {[(A−B)/B]×100} versus age of the animals.

ship between the synthesis of mRNA and rRNA during brain development. The LiCl precipitation procedures for the isolation of high-molecular weight RNA (Baltimore & Girard, 1966) proved the most convenient and effective method for eliminating radioactivity from DNA and tRNA as well as in non-nucleic acid contaminants present in the unpurified phenol extracts of nucleic acids. The LiCl precipitation procedure has also been used by Jelinek *et al.* (1973) for the preparation of heterogeneous nuclear RNA containing polyadenylated RNA from HeLa cells, as well as by Judes & Jacob (1973) for isolating rRNA from chick embryo brain.

Synthesis of 28 S and 18 S rRNA

In rats of all ages, the incorporation of the radioactive precursor into brain high-molecular-weight RNA could be detected 1 h after the intracranial injection and in sufficient amounts to permit characterization of the labelled macromolecules by electrophoresis on polyacrylamide gels. We found distinct differences in the population of the rapidly labelled RNA in brain of neonatal rats compared with older rats (Figs. 3 and 4). Thus in the neonatal rat, the labelling of discrete 28 S and 18 S RNA could not be detected as clearly as in the adult. This inability to distinguish rRNA clearly in the rapidly labelled population of high-molecular-weight RNA in young brain suggests the possibility that the processing of the ribosomal precursor to yield rRNA is relatively slower in brains of neonatal rats than in old. Our subsequent investigations on the characterization of both nuclear and cytoplasmic high-molecular-weight RNA support this contention (Berthold & Lim, 1976). The other possibility, of increased emphasis on polyadenylated RNA production, relative to rRNA in the brains of neonatal rats, resulting in masking of the radioactivity in rRNA, was considered unlikely, since in the young about 32% of the initial transcript is polyadenylated whereas in the adult animals the corresponding value is 28%. This small difference in polyadenylated RNA production cannot be responsible for the large changes in the labelling pattern of the high-molecular-weight RNA.

Metabolism of polyadenylated RNA

One of the main constituents of high-molecular-weight RNA is polyadenylated RNA, the cytoplasmic component of which clearly corresponds to mRNA in brain as well as in other tissues. We have previously demonstrated that brain microsomal polyadenylated RNA has the ability to direct the synthesis of the brain-specific myelin encephalitogenic protein in the *Xenopus* oocyte (Lim *et al.*, 1974). There is good evidence that at least some of the polyadenylated RNA in the nucleus is the precursor to the cytoplasmic form (Brawerman, 1974).

In comparing the changes in the metabolism of brain polyadenylated RNA as a whole, we have chosen a 5 h labelling period as a measure of the synthesis of the polyadenylated RNA for the following reasons. At early periods after administration of the radioactive precursor (e.g. 1 h) the proportion of radioactivity in the polyadenylated RNA may not accurately depict the relative transcription rate of RNA molecules that ultimately are polyadenylated, since terminal addition of labelled adenylic acid residues to pre-formed RNA may lead to an anomalous high value. Current estimates on the rate of transcription suggest that synthesis *de novo* of an RNA molecule of about 3000 nucleotides (mol.wt. 2×10^6) should take about 30–40 s. The delay in the terminal addition of poly(A) to transcribed sequences is of the order of 15 min (Perry *et al.*, 1974). At later periods of labelling this terminal addition of poly(A) would not be unduly represented in estimates of the synthesis of polyadenylated RNA. The 5 h labelling period resulted in a substantial amount of radioactive precursor being incorporated into RNA and had the distinct advantage in that the values for the proportion of radioactive polyadenylated RNA present in the radioactive high-molecular-weight RNA closely approached the nuclear values (Berthold & Lim, 1976). There is therefore no need for an additional nuclear fractionation step to eliminate the contribution of the cytoplasmic high-molecular-weight RNA in estimating the relative synthesis of polyadenylated RNA.

It is well established that the vertebrate brain

develops primarily in a caudal–rostral direction (Schade & Ford, 1973). Thus at birth the phylogenetically older regions of the rat brain (e.g. the brain stem, which in our preparation included the medulla and pons) is more developed than the cerebrum. In the brain stem the rate of cell division is extremely slow from 6–12 days, with minimal increase in DNA content, in contrast with the cerebral cortex and cerebellum, where in the corresponding period DNA content increases severalfold (Fish & Winick, 1969). In the adult our results show that there was a constant difference between the brain stem and cortex in the proportion of polyadenylated RNA that was synthesized. In the graph (Fig. 4) this difference is expressed as a ratio and shows that in the newborn the synthesis of polyadenylated RNA is relatively higher in the cortex than in the brain stem (+30%). This ratio reaches parity when the rats are 10 days old, i.e. both regions now have equal proportions of radioactive polyadenylated RNA being synthesized. The ratio changes in favour of the brain stem thereafter until it reaches a constant value (−30%) at around 25–40 days after birth. Therefore in the cerebrum, relative to the brain stem, the synthesis of polyadenylated RNA decreases progressively during development until it reaches a constant adult value.

In the brain stem, there was little change in the synthesis of polyadenylated RNA from the third day onward, which is consistent with the accepted view that this region of the brain matures early. Although estimates of the synthesis of polyadenylated RNA in the cerebrum are made relative to the brain stem for comparative purposes, it must be emphasized that actual values for the synthesis of polyadenylated RNA in the cerebrum also decrease. Thus these results can be construed as a decrease in the synthesis of mRNA in the developing cortex until it reaches a final adult value, when it appears that more mRNA is synthesized in the brain stem than in the cortex.

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