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Title
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Permalink
https://escholarship.org/uc/item/7kr8s77b

Journal
Biochemical Journal, 109(4)

ISSN
0264-6021

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Publication Date
1968-10-01

DOI
10.1042/bj1090533

Peer reviewed

Hybridizable Ribonucleic Acid of Rat Brain

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1. Cerebral RNA of adult and newborn rats was labelled *in vivo* by intracervical injection of [5-^{3}H]uridine or [^{32}P]phosphate. Hepatic RNA of similar animals was labelled by intraperitoneal administration of [6-^{14}C]orotic acid. Nuclear and cytoplasmic fractions were isolated and purified by procedures involving extraction with phenol and repeated precipitation with ethanol. 2. The fraction of pulse-labelled RNA from cerebral nuclei that hybridized to homologous DNA exhibited a wide range of turnover values and was heterogeneous in sucrose density gradients. 3. Base composition of the hybridizable RNA was similar to that of the total pulse-labelled material; both were DNA-like. 4. Pulse-labelled cerebral nuclear RNA hybridized to a greater extent than cytoplasmic RNA for at least a week after administration of labelled precursor. This finding suggested that cerebral nuclei contained a hybridizable component that was not transferred to cytoplasm. 5. The rates of decay of the hybridizable fractions of cerebral nuclei and cytoplasm were faster in the newborn animal than in the adult. Presumably a larger proportion of labile messenger RNA molecules was present in the immature brain. 6. Cerebral nuclear and cytoplasmic RNA fractions from newborn or adult rats, labelled either *in vivo* for periods varying from 4min. to 7 days or *in vitro* by exposure to [^{3}H]-dimethyl sulphate, uniformly hybridized more effectively than the corresponding hepatic preparation. These data suggested that a larger proportion of RNA synthesis was oriented towards messenger RNA formation in brain than in liver.

Cerebral messenger RNA has been the topic of several recent investigations (Herriman & Hunter, 1965; Jacob, Stevenin, Jund, Judes & Mandel, 1966; Yamagami, Fritz & Rappoport, 1966; Bondy & Roberts, 1967; Kimberlin, 1967; Vesco & Giuditta, 1967). Characterization of this fraction in terms of its physical and chemical properties, cytological and regional distribution, developmental alterations and metabolic behaviour is of special interest. Thus it may be assumed that the distinctive biochemistry of the brain depends to a significant extent on the properties of its messenger RNA molecules. Moreover, higher cerebral functions must ultimately be derived in part from the synthesis of specific proteins, directed by messenger RNA.

The present experiments were designed to obtain information on the nature of cerebral messenger RNA by using hybridization techniques based on the immobilization of denatured DNA on nitrocellulose filters (Gillespie & Spiegelman, 1965). As in other tissues, messenger RNA constituted a small proportion of total RNA in both nucleus and cytoplasm of rat cerebral cortex and possessed much higher turnover rates than other cerebral

RNA fractions. These rates were higher in the newborn animal than in the adult. Moreover, messenger RNA species, especially those with high turnover rates, appeared to be present in greater abundance in cerebral cortex than in liver. This finding supports the suggestion that cerebral tissue may contain an unusually large proportion of unstable messenger RNA molecules (Zomzely, Roberts, Gruber & Brown, 1968).

EXPERIMENTAL

Preparation of radioactive nuclear and cytoplasmic RNA. Animals from an inbred strain of Sprague–Dawley rats were used within 18 hr. after birth or were maintained *ad libitum* on Purina laboratory chow until they weighed 140–180g.

Cerebral RNA was labelled by intracervical injection of [5-^{3}H]uridine (25–28·3c/m-mole) or Na$_2$H^{32}PO$_4$ (200mc/m-mole) in animals under light ether anaesthesia. Adult rats were given 1mc of [5-^{3}H]uridine or 2mc of [^{32}P]phosphate in 0·07–0·15ml. of saline (0·14M-NaCl); newborn rats were injected with 300–500μc of [5-^{3}H]uridine in 0·04ml. of saline. Hepatic RNA was labelled by intraperitoneal injection of [6-^{14}C]orotic acid (28–32·6mc/m-mole): 100μc in 1ml. of saline in the adult rat and 25μc in 0·25ml. of saline in the newborn rat. Orotic acid solutions were neutralized with NaOH before injection. Orotic acid was

found to be a more efficient precursor for hepatic RNA than uridine. The radiochemicals were obtained from Schwarz Bioresearch Inc., Orangeburg, New York, N.Y., U.S.A.

Nuclear and cytoplasmic RNA were extracted and purified as follows. At various intervals after injection of radioactive RNA precursor, rats were lightly anaesthetized with ether and exsanguinated from the abdominal aorta. Whole brains and livers were rapidly removed, placed in 12 vol. of cold 0·32 M-sucrose and homogenized by hand with a glass homogenizer and Teflon pestle. The filtrate obtained by passing the homogenate through four layers of cheese-cloth was centrifuged at 750 g at 0° for 10 min. The resulting pellet consisted largely of nuclei (Bondy & Roberts, 1967) and was used for isolation of nuclear RNA. The supernatant was retained for preparation of cytoplasmic RNA (see below).

The crude nuclear pellet from about 1·5 g. of tissue was suspended in 5 ml. of a solution composed of 1% sodium dodecyl sulphate, 3 mM-EDTA and 0·01 M-sodium acetate buffer, pH 5·2. Then 5 ml. of phenol (Mallinckrodt Chemical Works, New York, N.Y., U.S.A.) saturated with this solution was added and the mixture was shaken in a water bath at 65° for 8 min. The suspension was then cooled rapidly and centrifuged in a Sorvall RC-2 centrifuge at 35 000 g at 8° for 10 min. The pellet was discarded. The supernatant was brought to 0·1 M-NaCl with 3 M-NaCl; 2·5 vol. of ethanol was added with constant shaking. This ethanolic solution was held at −10° for 1 hr. and was then centrifuged at 35 000 g for 10 min. The resulting precipitate was taken up in 2 ml. of 3 mM-EDTA, pH 5·2, and 2 ml. of sodium dodecyl sulphate solution was added. This suspen-sion was shaken with 5 ml. of the phenol solution and centrifuged at 1500 g at 10° for 10 min. The supernatant was pipetted off and brought to 0·1 M-NaCl; 2·5 vol. of ethanol was then added. The RNA precipitate was centri-fuged down and resuspended in 1 ml. of 3 mM-EDTA, pH 5·2; 0·5 ml. of 6 M-potassium acetate was added, followed by dropwise addition of ethanol to a final concentration of 20% (v/v). After remaining at −10° for 30 min., the mixture was centrifuged at 1500 g for 15 min. The pre-cipitate was taken up again in 5 ml. of 3 mM-EDTA, pH 5·2, and reprecipitated with potassium acetate and ethanol. This precipitate was suspended in 1 ml. of water and brought to 0·1 M-NaCl; 2·5 vol. of ethanol was added. Finally, a flocculent RNA precipitate was recovered by centrifugation and dried in vacuo.

Cytoplasmic RNA was isolated from the supernatant obtained by centrifugation of the original sucrose homo-genate. The method employed was similar to that used for the preparation of nuclear RNA with the following modifica-tions. The supernatant was shaken for 8 min. with 1 vol. of phenol and 0·5 vol. of sodium dodecyl sulphate solution at room temperature rather than at 65°. Thereafter three or four phenolic extractions of the supernatant were used to deproteinize the cytoplasmic preparation. The potassium acetate–ethanol treatment employed in the preparation of nuclear RNA was omitted. This treatment was replaced by two precipitations of cytoplasmic RNA from 2 ml. of 0·1 M-NaCl solution with 2·5 vol. of ethanol.

The crude nuclear and cytoplasmic RNA preparations were dissolved in water and samples were taken for analysis. The amount of RNA present was estimated from its extinction in the Beckman DU spectrophotometer; 100 μg. of RNA/ml. exhibited E_{260} 2·5. Radioactivity was deter-mined after precipitation of RNA with cold 5% (w/v) trichloroacetic acid. The resulting suspension was passed under vacuum through a Millipore filter (25 mm. diam., 0·8 μ pore size). The precipitate was then washed twice with 10 ml. of cold 5% trichloroacetic acid and twice with 10 ml. of chloroform–ethanol (1:1, v/v). The filter containing the air-dried precipitate was placed in 5 ml. of a toluene scintillator system composed of 0·5% 5-(biphenyl-2-yl)-2-phenyl-1-oxa-3,4-diazole and 0·01% 1,4-bis-(5-phenyl-oxazol-2-yl)benzene. Radioactivity was measured in the Packard Tri-Carb Spectrometer at efficiencies of 53·5% and 13·7% for ^{14}C and ^{3}H respectively.

Preparation and denaturation of DNA. Four livers or 30 brains were homogenized in 400 ml. of 0·32 M-sucrose and filtered through four layers of cheesecloth. The crude nuclear fraction was pelleted by centrifugation of the filtrate at 750 g at 0° for 10 min. DNA was extracted and purified by a method based on the procedure of Massie & Zimm (1965). The pellet was shaken with 50 ml. of a buffer containing 1% sodium dodecyl sulphate, 1 mM-EDTA and 0·1 M-tris–HCl buffer, pH 8·5. The mixture was frozen in ethanol–solid CO_2, thawed and mixed with 50 ml. of phenol saturated with the same buffer. This suspension was shaken for 8 min. at 55° and then centrifuged at 35 000 g at 8° for 10 min. The viscous supernatant was shaken once more with 50 ml. of phenol solution and recentrifuged at 35 000 g. The resulting supernatant was brought to 0·1 M-NaCl with 3 M-NaCl and treated with 2·5 vol. of ethanol. The precipitate that formed was wound out on a glass rod, squeezed dry, homogenized in 9 ml. of water and incubated with Pronase (50 μg./ml.) at 37° for 2 hr. The Pronase had previously been incubated alone for 2 hr. at 37° to destroy nucleases that might be present. The incubation suspension was then shaken with 5 ml. of sodium dodecyl sulphate solution and 5 ml. of phenol. This mixture was centrifuged at 1500 g at 8° for 10 min. The resulting supernatant was re-extracted with 5 ml. of fresh solution and recentrifuged. This supernatant was then brought to 0·1 M-NaCl and 2·5 vol. of ethanol was added. The precipitate obtained was homogenized in 10 ml. of water, shaken with an equal volume of 6 M-potassium acetate buffer, pH 6, and centrifuged at 1500 g for 10 min. Then 2 vol. of ethanol was added to the supernatant. The precipitate that formed was wound out as before, and homogenized in 20 ml. of 1% (0·01×) standard saline–citrate buffer (SSC), composed of 0·15 M-NaCl and 15 mM-sodium citrate at pH 7·4 (Gillespie & Spiegelman, 1965). Denaturation of DNA was carried out by heating this suspension at 100° for 4 min. and then pouring it rapidly into 100 ml. of 0·01 × SSC at 0°. An increase of 29% in E_{260} of the solution indicated that denaturation of DNA was complete. The DNA preparation was shown to be devoid of ribonuclease activity towards [^{14}C]polyuridylic acid by the sensitive method of Barondes & Nirenberg (1962). The amount of DNA present was determined by the diphenyl-amine method (Burton, 1956) with calf thymus DNA as a standard.

RNA–DNA hybridization method. DNA was immobilized on nitrocellulose filters (type B–6, coarse grade; Schleicher and Schuell, Keene, N.H., U.S.A.) as described by Gillespie & Spiegelman (1965). Retention of DNA (25–50 μg.) applied to each filter during the hybridization procedure was in excess of 85%. Incubation conditions were designed to give the highest ratio of specific hybrid-ization of pulse-labelled RNA to homologous DNA com-

pared with the binding of this RNA to bacterial DNA. The filters were preincubated for 4–6 hr. at 60° with Ficoll, polyvinylpyrrolidone and bovine albumin in 1 ml. of 3 × SSC (Denhardt, 1966). Hybridization was carried out at 60° for 16–18 hr. in glass scintillation vials containing radioactive RNA in 1·3 ml. of 6 × SSC. After incubation, the filter was removed, rinsed in a jet of water and placed in a beaker containing 10 ml. of 6 × SSC for 10 min. The filter was then washed on each side with 30 ml. of 6 × SSC by using vacuum filtration, dried at 100° for 10 min. and placed in 5 ml. of a scintillator system in toluene for determination of radioactivity. All assays were performed in duplicate or triplicate.

Sucrose-density-gradient studies. Linear sucrose density gradients were prepared in buffer containing 0·1 M-NaCl, 3 mM-EDTA and 0·01 M-sodium acetate buffer, pH 5·2 (Bondy & Roberts, 1967). A solution of labelled RNA (73–162 μg. in 0·2 ml.) was carefully layered on 4 ml. of the gradient and centrifuged at 70 000g for 18 hr. in the SW 39 rotor of a Spinco preparative ultracentrifuge. The bottom of the tube was punctured and alternate 1-drop and 2-drop fractions were collected for assays of radioactivity and hybridization capacity respectively. Radioactivity in RNA was measured after precipitation with cold 5% trichloroacetic acid as described above. Approximate sedimentation values for the labelled RNA fractions were derived by comparison of their positions with those of the 18s and 28s peaks of cerebral ribosomal RNA in similar gradients.

Radioactive base analyses. The base compositions of the labelled RNA preparations were estimated by a modification of the method of Davidson & Smellie (1952). RNA labelled with [^{32}P]phosphate was hydrolysed for 15–17 hr. at 25° in 0·3 M-KOH. The solution was then adjusted to pH 3–4 with 0·5 M-HClO$_4$, cooled to 0° and centrifuged. The resulting supernatant was stirred with 5 mg. of charcoal (acid-washed Norit) and recentrifuged. Nucleotides were eluted from the charcoal pellet with 0·5 ml. of an aqueous solution containing 70% (v/v) of ethanol and 5% (v/v) of aq. NH$_3$ (sp.gr. 0·88). The charcoal was removed by centrifugation and the supernatant was evaporated down to about 0·1 ml. This material was then applied to Whatman no. 3MM paper. Electrophoretic separation of nucleotides was carried out at 500 v for 3 hr. in 0·05 M-ammonium acetate buffer, pH 3·5, essentially as described by Markham & Smith (1952). Locations of radioactive nucleotides were determined by concurrent electrophoresis of known nucleotides, which were detected under ultraviolet light. The spots were then cut out and placed in 20 ml. of scintillator system for assay of radioactivity.

RNA methylation in vitro. Methylation of the various RNA fractions was accomplished as outlined by Smith, Armstrong & McCarthy (1967). The RNA preparation (0·5 mg.) was incubated at 23° for 2·5 hr. in 0·1 ml. of 0·1 M-sodium phosphate buffer, pH 7·5, containing 500–750 μc of [^{3}H]dimethyl sulphate (105 mc/m-mole). The suspension was diluted with water and the RNA was precipitated four times from 0·1 M-NaCl with 70% ethanol. The final product had a specific radioactivity of 800–5000 counts/μg. of RNA.

RESULTS

Characteristics of the hybridization system. Pulse-labelled RNA from rat cerebral nuclei hybridized

to the same extent with homologous DNA from either liver or brain (see also McCarthy & Hoyer, 1964). The maximum degree of hybridization was achieved after incubation for approx. 14 hr. (Fig. 1). This maximum value (about 15% of the total radioactivity) remained constant for at least 22 hr., but was depressed about 50% after 42 hr. of incubation. These data were similar to those reported earlier for hybrid formation between mammalian DNA and pulse-labelled RNA from other mammalian cells (Birnboim, Pène & Darnell, 1967; Shearer & McCarthy, 1967).

The amount of DNA used per filter in the present experiments was not limiting (Fig. 2). The percentage of RNA bound to the DNA remained constant over a wide range of RNA concentrations up to twice the amount of DNA present (see also
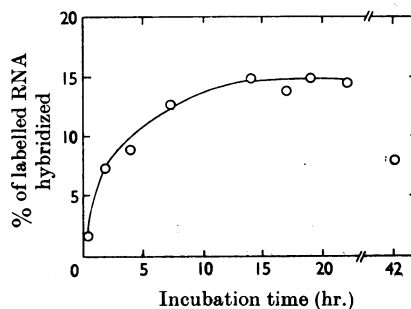

Fig. 1. Time-course of hybridization of labelled RNA. Cerebral nuclear RNA was isolated from adult rats 50 min. after intracervical injection of 1 mc of [5-^{3}H]uridine. This RNA (4·2 μg.) was incubated for the times indicated with 35 μg. of denatured rat liver DNA bound to nitrocellulose filters.
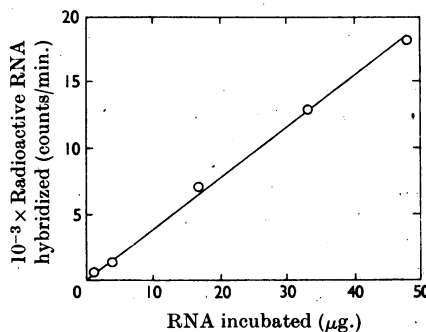

Fig. 2. Effect of various inputs of labelled RNA on extent of hybridization. Cerebral nuclear RNA was isolated from adult rats 50 min. after intracervical injection of 1 mc of [5-^{3}H]uridine. Various amounts of RNA were incubated for 18 hr. with 25 μg. of denatured rat liver DNA.

Table 1. *Hybridization specificity of cerebral nuclear RNA*

Cerebral nuclear RNA was isolated from adult rats 50 min. after intracervical injection of 1 mc of [5-^{3}H]uridine. This RNA (4·2 μg.) was incubated for 18 hr. with the preparations indicated. Results shown are averages of three values.

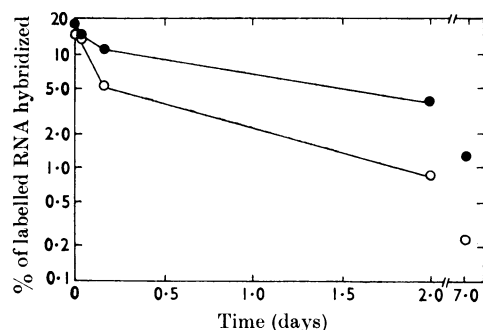|	Radioactive RNA bound (counts/min.)	
Rat liver DNA (50 μg.)	1021	1410
E. coli DNA (50 μg.)	67	89
Blank filter	41	43

Fig. 3. Hybridization of labelled nuclear and cytoplasmic RNA from adult rat brain. RNA fractions were isolated at various intervals after intracervical injection of 1 mc of [5-^{3}H]uridine. Incubation was carried out for 18 hr. with 3·8–10 μg. of RNA and 30–50 μg. of denatured rat liver DNA. ●, Nuclear RNA; ○, cytoplasmic RNA.

Birnboim *et al.* 1967). Species specificity of the DNA–RNA interaction was investigated with filters containing denatured DNA prepared from *Escherichia coli* (Worthington Biochemical Corp., Freehold, N.J., U.S.A.). Pulse-labelled RNA from rat brain was bound to rat liver DNA about 150 times more effectively than to *E. coli* DNA (Table 1). Thus the formation of complexes of cerebral RNA with homologous RNA appeared to involve the formation of specific hybrids.

Hybridization capacities of cerebral and hepatic RNA fractions. Pulse-labelled nuclear and cytoplasmic RNA from cerebral or hepatic tissue of adult rats hybridized less effectively with DNA as the time of autopsy after intracervical administration of [^{3}H]uridine was increased (Fig. 3 and Table 2). The degree of hybridization for pulse-labelled RNA from cerebral nuclei was about 20% at the shortest interval that permitted valid measurements (i.e. about 4 min.). This finding suggested that at least one-fifth of the RNA molecules synthesized by the brain were of the messenger type. However, owing

Table 2. *Hybridization of pulse-labelled cerebral and hepatic RNA from adult rats*

RNA fractions were isolated from brain at various intervals after intracervical injection of 1 mc of [5-^{3}H]uridine and from liver after intraperitoneal administration of 100 μc of [6-^{14}C]orotic acid. Incubation was carried out for 18 hr. with 3·8–32 μg. of RNA and 30–50 μg. of denatured rat liver DNA. Values shown are averages of three to five duplicate determinations.

	Time after pulse label	% of radioactive RNA hybridizing	
		Nuclear RNA	Cytoplasmic RNA
Brain	4 min.	18·5	—
	20 min.	19·5	15·3
	50 min.	14·9	13·3
	2 days	4·1	0·9
Liver	4 min.	10·3	—
	20 min.	10·1	9·4
	50 min.	9·1	8·1
	2 days	1·7	0·7

to rapid decay the concentrations of messenger RNA in cerebral preparations were much lower (see below).

The hybridizable fractions of nuclear and cytoplasmic RNA from brain or liver did not exhibit exponential decay curves, but rather appeared to have a wide range of half-lives. For example, the half-lives of the hybridizable fraction of cytoplasmic RNA from rat brain varied from 2·5 hr. to 5 days. This range was well below the values reported earlier for other cytoplasmic RNA fractions. Bondy (1966) found an average half-life of 12·5 days for cerebral ribosomal and transfer RNA, and Dawson (1967) reported a minimum half-life of 6 days for ribosomal RNA from the same source. In every experiment, pulse-labelled nuclear RNA from brain or liver hybridized to a greater extent than the analogous cytoplasmic RNA from the earliest time at which both fractions could be accurately assayed to at least a week after administration of labelled precursor. In a comparable fashion, Hoyer, McCarthy & Bolton (1963) found that nuclear RNA from mouse liver hybridized to a greater extent than cytoplasmic RNA for periods varying from 30 min. to 3 days after labelling.

Turnover rates of the hybridizable fractions of cerebral nuclear and cytoplasmic RNA were significantly greater in the newborn rat than in the adult (Fig. 4). Thus the proportion of stable messenger RNA synthesized by the brain of the newborn animal appeared to be significantly lower than that of the adult. However, in both age groups, a large proportion of the cerebral RNA that hybridized had relatively short half-lives.

The hybridization properties of cerebral nuclear and cytoplasmic RNA fractions differed markedly from those of the corresponding hepatic preparations in the adult rat (Table 2). After pulse-labelling *in vivo*, the percentage of radioactive cerebral RNA that hybridized to DNA was always greater than that of the corresponding hepatic fraction, even at intervals as short as 4 min. after administration of the radioactive precursor. Valid comparisons may be made of the relative capacities of different RNA preparations to form hybrids with DNA by using RNA labelled *in vivo*. However, the total hybridization capacities of these fractions cannot be measured by this technique. Thus rapidly synthesized molecules tend to be preferentially labelled at early time-periods, whereas at later times only those molecules with long half-lives retain their radioactivity. As an approach to this problem, the technique of labelling RNA preparations *in vitro* with [³H]dimethyl sulphate has been employed (Smith *et al.* 1967). These investigators presented
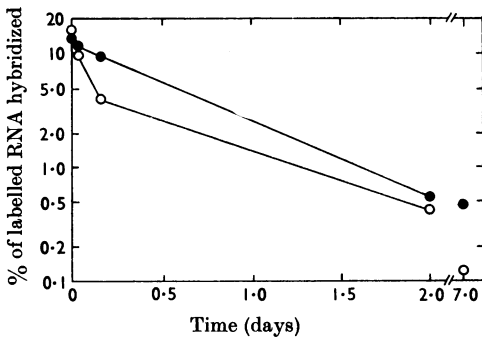

Fig. 4. Hybridization of labelled nuclear and cytoplasmic RNA from newborn rat brain. RNA fractions were isolated at various intervals after intracervical injection of 300–500 μc of [5-³H]uridine. Incubation was carried out for 18 hr. with 6·4–18·1 μg. of RNA and 30–50 μg. of denatured rat liver DNA. ●, Nuclear RNA; ○, cytoplasmic RNA.

evidence that methylated RNA molecules exhibited hybridization properties identical with those of the unmethylated materials and that the methylation procedure labelled all classes of RNA randomly. Consequently hybridization values may be expected to be much lower than those obtained after selective labelling for short periods of time *in vivo*. In the present investigations, although the relative hybridization capacities of methylated cerebral and hepatic RNA preparations were comparable with those noted with pulse-labelled RNA, the absolute values were considerably lower (Table 3). The hybridization capacities of these methylated preparations were of the same order of magnitude as those reported earlier for randomly labelled bacterial RNA (Bolton & McCarthy, 1962). Nuclear RNA fractions from either adult or newborn rat brain hybridized more effectively than the corresponding hepatic fractions. Similar differences were observed in the relative hybridization capacities of cytoplasmic RNA preparations from brain or liver. The content of messenger RNA in the nuclear preparations, as assayed by this technique, was considerably greater than that in the corresponding cytoplasmic fractions. Measurements of template activity in the *E. coli* ribosomal system earlier demonstrated that nuclear RNA preparations from brain and liver contain more messenger RNA than the corresponding cytoplasmic preparations (Barondes, Dingman & Sporn, 1962; Brawerman, Gold & Eisenstadt, 1963; Bondy & Roberts, 1967).

Physicochemical properties of hybridizable cerebral RNA. Base compositions of the radioactive fractions of cerebral nuclear RNA were measured after a short period (50 min.) of pulse-labelling with [³²P]phosphate. The absolute values obtained by this procedure may not precisely reflect the base composition of pulse-labelled RNA as a result of non-uniform labelling of phosphate within the RNA molecules (Spencer, 1962). However, a comparison of two classes of RNA molecules labelled for the same time under identical conditions appears to be

Table 3. *Hybridization of RNA methylated* in vitro

Methylation of RNA fractions was carried out with [³H]dimethyl sulphate as described in the Experimental section. The labelled RNA (8–12 μg.) was incubated for 18 hr. with 25–40 μg. of denatured rat liver DNA. The results of three separate experiments are shown.

	% of radioactive RNA hybridizing					
	Nuclear RNA			Cytoplasmic RNA		
Expt. no.	1	2	3	1	2	3
Adult brain	2·2	1·9	2·2	0·67	0·42	0·86
Adult liver	1·3	0·93	1·3	0·51	0·36	0·58
Newborn brain	1·88	2·03	2·60	0·92	0·83	0·89
Newborn liver	1·38	1·24	1·90	0·48	0·35	0·38

Table 4. *Base composition of pulse-labelled cerebral nuclear RNA*

Cerebral nuclear RNA was isolated from adult rats 50 min. after intracisternal injection of 2 mc of [^{32}P]phosphate. Base analyses were carried out directly on this material and on the hybridizable fraction. Hybridization was effected by incubating 25–45 μg. of pulse-labelled RNA for 18 hr. with 30–50 μg. of denatured rat liver DNA. For base analyses, the bound RNA was released from the filter by hydrolysis in 0·3 M-KOH (see the Experimental section).

	Base composition (%)				GMP+CMP
	CMP	AMP	GMP	UMP	AMP+UMP ratio
Total pulse-labelled RNA	21·2	22·0	28·2	28·6	0·98
	23·2	17·2	24·7	34·9	1·14
	21·5	19·4	25·7	33·4	0·89
	19·7	19·3	25·7	35·2	0·83
	18·8	19·5	27·0	34·7	0·85
	19·4	21·2	30·4	29·1	0·99
Mean	20·6±0·5	19·8±0·7	26·9±0·8	32·7±1·2	0·90±0·05
Hybridizable fraction	22·0	24·2	25·8	28·0	0·92
	17·9	20·2	31·4	30·2	1·00
	26·2	21·6	26·0	26·1	1·09
	20·6	20·6	30·1	28·8	1·02
Mean	21·7±1·7	21·7±0·9	28·3±1·4	28·3±0·9	1·00±0·03

valid. The average (GMP + CMP)/(AMP + UMP) ratio for total radioactive RNA under these conditions was 0·90 (Table 4). This value was similar to that obtained under comparable circumstances by Jacob et al. (1966), who concluded that the rapidly labelled material in rat brain contained a high proportion of DNA-like messenger RNA molecules. Steele, Okamura & Busch (1965) had earlier reported comparable base ratios for nuclear RNA from rat liver after labelling in vivo with [^{32}P]phosphate.

Although only 15% of the radioactively labelled nuclear RNA from rat cerebral cortex hybridized with DNA 50 min. after injection of uridine (Table 2), its base ratio (1·0) appeared to be similar to that of the entire pulse-labelled fraction. This phenomenon might be due to incomplete hybridization of cerebral messenger RNA or to the presence of RNA molecules with DNA-like base composition that were not capable of hybridization. The latter conclusion was supported by the observation that all fractions recovered after sucrose-density-gradient centrifugation of rat brain nuclear RNA that had been pulse-labelled for 30 min. with [^{32}P]phosphate exhibited base ratios varying from 1·04 to 1·22 (Vesco & Giuditta, 1967). Ribosomal RNA had base ratios 1·76 (28 s) and 1·38 (18 s).

The sedimentation profile of cerebral nuclear RNA isolated 50 min. after administration of [^{3}H]uridine exhibited a characteristic heterogeneity (Fig. 5). Similar findings have been reported by Egyházi & Hydén (1966), Jacob et al. (1966), Nievel & Kirby (1966) and Vesco & Giuditta (1967).
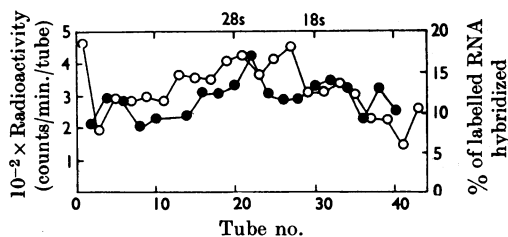

Fig. 5. Sedimentation properties of pulse-labelled nuclear RNA from adult rat brain. Cerebral nuclear RNA was isolated from adult rats 50 min. after intracervical injection of 1 mc of [5-^{3}H]uridine. This RNA (73–102 μg.) was dissolved in 0·2 ml. of water and layered on a linear sucrose gradient (5–20%, w/v) containing 0·1 M-NaCl, 3 mM-EDTA and 0·01 M-sodium acetate buffer, pH 5·2. The gradient was centrifuged at 70 000 g for 18 hr.; alternate 1-drop and 2-drop fractions were collected for determination of radioactivity and hybridization capacity respectively. ●, Radioactivity; ○, % of labelled RNA hybridized.

The hybridization capacity of cerebral nuclear RNA was also found to be widely dispersed in sucrose density gradients. A high proportion of the hybridizable material exhibited sedimentation coefficients that varied from 6 to 28 s.

DISCUSSION

The hybridization technique provides a practical method for the detection and assay of RNA species that are widely represented on the DNA genome,

including messenger RNA. Ribosomal and soluble RNA species, which are relatively homogeneous and have a smaller representation on the genome, hybridize to a much smaller extent with homologous DNA (Attardi, Huang & Kabat, 1965; Ritossa & Spiegelman, 1965; McConkey & Dubin, 1965; Morell, Smith, Dubnau & Marmur, 1967). Additional indices of the presence of messenger RNA, such as sensitivity to degradation by ribonuclease, capacity to stimulate amino acid incorporation in ribosomal systems, DNA-like base composition, heterogeneity of sedimentation values and rapidity of labelling with radioactive precursors, are also exhibited by other RNA species. Thus all techniques used for the detection of messenger RNA are indicative rather than definitive.

In the present experiments about 20% of the cerebral nuclear RNA synthesized in the adult rat during a short period of pulse labelling (4–20min.) was capable of hybridizing with rat DNA. The corresponding value for hepatic nuclear RNA was only 10%. These may represent minimum values for cerebral synthesis of messenger RNA. Thus the pulse-labelled RNA from cerebral nuclei that did not hybridize, as well as the hybridizable fraction, exhibited a DNA-like base composition. However, evidence has been presented for the presence of DNA-like RNA in mammalian cells that is devoid of template activity (Hadjivassiliou & Brawerman, 1965). Several investigators have estimated that 23–60% of the total RNA synthesis in the mammalian nucleus may be directed towards the formation of messenger RNA (Perry, Srinivasan & Kelley, 1964; Greenman, Wicks & Kenney,1965; Roberts, 1965; Soeiro, Birnboim & Darnell, 1966).

The values obtained for hybridization of radioactive RNA from cerebral and hepatic cytoplasm were lower than those for the analogous nuclear fractions at all time-periods after pulse-labelling. Moreover, randomly labelled cytoplasmic RNA preparations also hybridized to a smaller extent than the corresponding materials from nuclei. Although cytoplasm may contain a higher proportion of relatively small messenger RNA molecules than nuclei from the same source (Samarina, 1964; Attardi, Parnas, Hwang & Attardi, 1966; Scherrer, Marcaud, Zajdela, London & Gros, 1966; Soeiro et al. 1966), there is no evidence that hybridization properties of the two preparations differ. The data suggest that cerebral nuclei may contain long-lived RNA species that are not readily transferred to cytoplasm. Thus the specific radioactivity of pulse-labelled nuclear RNA from rat brain never fell below that of cytoplasmic RNA. It is unlikely that reutilization of radioactive precursors was responsible for this phenomenon, in view of the rapid loss of radioactivity from the cerebral nucleotide pool after intracisternal administration of labelled nucleic acid precursors (see, e.g. Bondy, 1966). Evidence for the occurrence of unique RNA molecules that are restricted to the nucleus has been presented earlier (Attardi et al. 1966; Houssais & Attardi, 1966; Bonner & Widholm, 1967; Levis, Krsmanovic & Errera, 1967; Shearer & McCarthy, 1967; Drews, Brawerman & Morris, 1968).

Hybridizable RNA constituted only about 2% of the total RNA in randomly labelled cerebral nuclear preparations from the adult rat. The corresponding value for hybridizable RNA from cerebral cytoplasm was 0·65%. Thus the hybridizable fraction of cerebral nuclei was labelled about ten times more rapidly than non-hybridizable RNA from this source. The comparable ratio for cerebral cytoplasmic RNA was 24:1. Since the average half-lives of nuclear and cytoplasmic RNA in adult rat brain were both about 12·5 days (Bondy, 1966), it may be calculated that the hybridizable fractions possessed average half-lives of 31hr. in cerebral nuclei and 12hr. in cerebral cytoplasm. This result supports the conclusion that certain long-lived RNA species may be restricted to the nucleus. Employing other techniques, several investigators have reported half-lives in the range of 1hr. to more than 20hr. for most species in brain and other mammalian tissues that exhibited properties of messenger RNA (Trakatellis, Axelrod & Montjar, 1964; Goodwin & Sizer, 1965; Greenman et al. 1965; Marchis-Mouren & Cozzone, 1966; Appel, 1967; Orrego & Lipmann, 1967). In the present investigations, the range of half-life times calculated for hybridizable RNA in adult brain was 2·5hr. to 5 days. This wide range of turnover times suggested that cerebral messenger RNA species may vary markedly in stability. A heterogeneous distribution of half-lives and the occurrence of long-lived messenger RNA species in mammalian tissues have been suggested earlier (Seed & Goldberg, 1963; Revel & Hiatt, 1964; Marchis-Mouren & Cozzone, 1966; Ney, Davis & Garren, 1966; Wilson & Hoagland, 1967).

It appears, from the hybridization capacity of randomly labelled RNA, that a relatively high proportion of RNA synthesis in brain may be oriented towards formation of messenger RNA. Thus the percentage of radioactive RNA in cerebral nuclei and cytoplasm that was capable of hybridizing with homologous DNA was greater than that of the corresponding hepatic fraction under all experimental conditions. This finding was consistent with the observation that nuclear RNA from adult rat brain exhibited greater template activity in an E. coli amino acid-incorporating system than the analogous hepatic preparation (Bondy & Roberts, 1967). The proportions of hybridizable RNA in either nuclear or cytoplasmic preparations from brain appeared to be similar in newborn and

adult rats. However, the rates of renewal of these fractions were greater in the young animal. Moreover, Yamagami *et al.* (1966) reported that cerebral nuclear RNA from newborn rats exhibited greater template activity in a homologous ribosomal system than similar preparations from adult animals. These alterations in turnover and activity of cerebral hybridizable RNA during development suggested a decrease in the proportion of short-lived messenger molecules that may be highly active in protein synthesis. This conclusion was supported by the observations that protein synthesis was more active in newborn brain than in adult brain and that polyribosomal preparations contained a higher proportion of large aggregates in the former instance (Murthy, 1966). In addition, the hybridization data suggest that short-lived messenger RNA molecules are replaced during development by longer-lived species. The latter may be responsible for the appearance of proteins with relatively long half-lives (Lajtha, Furst, Gerstein & Waelsch, 1957) whose activities are related to the mature functions of the central nervous system. In any event, a high proportion of the hybridizable RNA molecules in either newborn or adult brain exhibited relatively short half-lives. These findings suggest that cerebral cells may have the potential for adapting rapidly to changing physiological conditions by alterations in protein synthesis made possible by the existence of a relatively large population of unstable messenger RNA molecules (see also Zomzely, Roberts, Brown & Provost 1966; Roberts & Zomzely, 1966). This phenomenon may be of great significance to the specialized biological functions of the brain.

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