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Developmental changes in the composition of polyadenylated RNA isolated from free and membrane-bound polyribosomes of the rat forebrain, analysed by translation *in vitro*

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Free and membrane-bound polyribosomes were isolated from the rat forebrain during its development. Polyadenylated RNA [poly(A)$^+$ RNA] was isolated from both fractions, by using oligo(dT)–cellulose chromatography, and its composition studied by translating the poly(A)$^+$ RNA *in vitro* in reticulocyte lysates. Electrophoretic analysis of the translation products showed that both free and membrane-bound polyribosomal poly(A)$^+$ RNA gave many common components, but that there were also distinct differences in the protein composition of the products of the two fractions. Several proteins, of mol.wts. 39000, 37000, 31000, 27000 and 17000, appeared to be products predominantly of free polyribosomal poly(A)$^+$ RNA, whereas others, of mol.wt. 47000, 33000, 24000 and 21000, were specific to the membrane-bound polyribosomal poly(A)$^+$ RNA fraction. More developmental changes were observed in the translational products of the membrane-bound poly(A)$^+$ RNA fraction. Proteins of mol.wts. 33000 and 21000, which were predominant components of the translational products of this fraction when isolated from 10-day and older rats, were not present in translational products derived from preparations isolated from 3-day-old rats. The developmental appearance of these proteins as translational products of the membrane-bound poly(A)$^+$ RNA suggests the appearance of new mRNA species. These transcriptional changes are discussed in relation to processes involved in brain differentiation, including myelination.

During the postnatal growth and development of the rat brain, there is a period of glial-cell proliferation, cessation of which is accompanied by the process of myelination. Other developmental events also occur, including the formation of synapses. These events follow an ordered sequence which ultimately results in the establishment of differentiated nerve-cell function (Davison & Dobbing, 1968). We have previously reported changes in the metabolism of RNA during this developmental period (Berthold & Lim, 1976*a*,*b*; Lim, 1977; Hall & Lim, 1978). In the transition from the replicative developmental phase to the non-replicative fully differentiated state, there were decreases both in the nucleo-cytoplasmic transport of RNA and in the synthesis of poly(A)$^+$ RNA relative to rRNA. The co-ordinated changes in RNA metabolism appear to correlate with the decline in the rate of protein synthesis with brain development (Dunlop *et al.*, 1977). We have also reported an increased complexity in the protein composition of brain polyribosomal mRNA–protein particles which may be related to translational control mechanisms (Elliott *et al.*, 1980). The brain mRNA population also increases in complexity with age (Grouse *et al.*, 1972), and this probably reflects the differentiation of specific cell types. It is also well established that there is a greater transcriptional diversity in the adult brain than in any other tissue (Ryffel & McCarthy, 1975; Bantle & Hahn, 1976; Grouse *et al.*, 1978).

In the present study we have investigated the changes in the poly(A)$^+$ RNA population with development by analysis of the translation products *in vitro* and compared the poly(A)$^+$ RNA isolated from free and membrane-bound polyribosomes. Most mRNA species are polyadenylated (Lim & Canellakis, 1970; Lim *et al.*, 1970; Brawerman, 1974); however, there is also reported to be a separate non-polyadenylated population of mRNA (Milcarek *et al.*, 1974), which has more recently been found in brain (Van Ness *et al.*, 1979; Chikaraishi, 1979).

Abbreviations used: SDS, sodium dodecyl sulphate; poly(A)$^+$ RNA, polyadenylated RNA; TKMD buffer, 50mM-Tris/HCl(pH 7.6)/75mM-KCl/5mM-$MgCl_2$/1mM-dithiothreitol.

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The translation *in vitro* and in *Xenopus* oocytes of several brain-specific mRNA species has previously been reported. These include mRNA for proteins 14-3-2 (neuron-specific enolase) and S-100 (Zomzely-Neurath *et al.*, 1973; Mahony *et al.*, 1976), myelin basic protein (Lim *et al.*, 1974), brain tubulin and actin (Gozes *et al.*, 1975, 1980) and hypothalamic neurophysins (Guidice & Chaiken, 1979). Although some of these, e.g. proteins S-100 and 14-3-2 (Zomzely-Neurath *et al.*, 1973), have been reported to be synthesized on free polyribosomes, little is known of the proteins synthesized by membrane-bound polyribosomes in the brain. In other tissues membrane proteins and proteins for secretion/export are synthesized on membrane-bound polyribosomes (Palade, 1975). A comparative study of the membrane-bound and free polyribosomal mRNA population may help to elucidate the nature of cellular interactions in the brain and to clarify further the mechanisms of myelin-sheath formation.

Materials and methods

Materials

Oligo(dT)-cellulose type T3 was from Collaborative Research (supplied by Uniscience, Cambridge, U.K.). The reticulocyte-lysate translational system was obtained from New England Nuclear, Southampton, U.K., and was supplied together with either L-[3,4,5-^{3}H]leucine (2mCi/ml; sp. radioactivity 117Ci/mmol) or L-[^{35}S]methionine (6.6mCi/ml; sp. radioactivity 932.5Ci/mmol). [^{14}C]Methylated protein mixture (molecular-weight markers) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Proteinase K was from Boehringer Corp., Lewes, East Sussex, U.K. Sucrose grade 1 (nuclease-free) was obtained from Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K. All other chemicals were A.R. grade. Glassware was treated with 0.05M-NaOH to destroy ribonuclease activity and/or was heat-sterilized. All solutions were prepared in sterile deionized distilled water.

Animals

Female Wistar rats (Cob strain) bred in our own laboratory were used at various ages between 3 and 55 days. Animals were decapitated and the forebrains dissected out and homogenized by hand in 3 vol. (v/w) of 0.25M-sucrose/TKMD buffer at 0–4°C in a Teflon–glass homogenizer (Tri-R Instruments Inc., Camlab, Cambridge, U.K.).

Preparation of polyribosomes

Polyribosomes were isolated by the method of Ramsey & Steele (1976) with minor modifications. All operations were performed at 0–4°C except where stated. The homogenate was centrifuged in a MSE 3×25 SW aluminium rotor initially at 740g for 2min and subsequently at 90000g for 17min. The supernatant was kept for the purification of free polyribosomes. The pellet was resuspended in 3 vol. (relative to the original weight of tissue) of 0.25M-sucrose/50mM-Tris/HCl (pH 7.6)/250mM-KCl/5mM-$MgCl_2$/1mM-dithiothreitol. Triton X-100 was added to a final concentration of 1% (w/v) and the suspensions were homogenized as before with three strokes. After centrifugation at 1470g for 5min to sediment nuclei, the supernatant was decanted and $\frac{1}{9}$ vol. of 13% (w/w) sodium deoxycholate was added dropwise, with mixing, to release bound polyribosomes from membranous material. Free and membrane-bound polyribosomes were purified from the two supernatant fractions by centrifugation through discontinuous sucrose gradients composed of 1.5M- and 2.0M-sucrose in TKMD buffer at 150000g for 3.5h. Polyribosomal pellets were either stored at −70°C or extracted with phenol.

Analysis of polyribosome size distribution

Polyribosome pellets were resuspended in TKMD buffer and samples layered over linear 15–35% (w/v) sucrose gradients prepared in the same buffer. Centrifugation was at 150000g for 1.25h in an MSE 6×4.2 Ti rotor and A_{260} was monitored with an ISCO UA5 continuous absorbance monitor.

RNA extraction and isolation of poly(A)$^+$ RNA

Polyribosomal or microsomal pellets were resuspended in 10mM-Tris/HCl (pH 8.5)/0.1M-NaCl/0.5% (w/v) SDS/5mM-EDTA and nucleic acids were extracted with phenol/chloroform/3-methylbutan-1-ol (25:24:1, by vol.) (Strair *et al.*, 1977). Ethanol-precipitated RNA was dissolved in 10mM-Tris/HCl (pH 7.6)/0.2% (w/v) SDS, heated to 65°C for 5min and rapidly cooled by the addition of an equal volume of ice-cold 1M-NaCl/10mM-Tris/HCl (pH 7.6) immediately before application to an oligo(dT)-cellulose column. Chromatography was as previously described (Aviv & Leder, 1972; Lim *et al.*, 1974), except that NaCl replaced KCl and 0.1% (w/v) SDS and 1mM-EDTA were added to the high-salt (0.5M) and intermediate-salt (0.1M) buffers.

Translation of poly(A)$^+$ RNA and analysis of products

The reticulocyte-lysate system was already micrococcal-nuclease-treated (Pelham & Jackson, 1976) when supplied. The amount of incorporation of radioactivity into protein was measured by precipitation with 10% (w/v) trichloroacetic acid as described by Pelham & Jackson (1976). Radioactivity was determined by using Bio-solv BBS-3/toluene scintillant (White *et al.*, 1978).

SDS/polyacrylamide-gel-electrophoretic analysis of translation products was as described by Laemmli (1970), by using 7cm × 14cm × 2.7mm vertical slab gels with a linear acrylamide gradient of 5–15% (w/v) or 5–25% (w/v). Electrophoresis was at 125V for 5h. Gels were either stained with Coomassie Blue or fixed in 10% (v/v) acetic acid/40% (v/v) methanol and subsequently fluorographed as described by Bonner & Laskey (1974) by using Kodak XRP5 X-ray film.

Size estimation of poly(A)+ RNA

Poly(A)+ RNA (1–2 A_{260} units) in 10mM-Tris/HCl (pH 7.6)/0.1M-NaCl/1mM-EDTA/0.2% (w/v) SDS was heated to 65°C for 5min and rapidly cooled before loading on 15–30% (w/v) sucrose gradients prepared in the same buffer (Ryffel & McCarthy, 1975; Auffray & Rougeon, 1980). Centrifugation was at 325000g for 4h at 23°C in an MSE 6 × 4.2 Ti rotor. Sized markers (28S, 18S and 4S RNA) were centrifuged in parallel gradients. The gradients were monitored at 260nm with an ISCO UA5 instrument with an ISCO 640 fraction collector. Serial fractions (0.2ml) were collected, adjusted to contain 0.2M-sodium acetate (pH 5.5), and precipitated with ethanol. All fractions were dissolved in 10 μl of sterile water and 1 μl portions assayed for translational activity in reticulocyte lysate.

Results and discussion

Isolation of free and membrane-bound polyribosomes

Brain polyribosomes were isolated by a modification of the method of Ramsey & Steele (1976, 1977), since other methods, utilizing the postmitochondrial supernatant, result in a selective loss of polyribosomes associated with heavier fragments of the rough endoplasmic reticulum, which sediment with the nuclear fraction (Blobel & Potter, 1967; Venkatesan & Steele, 1972). There was a progressive decline during development in the content of both free and membrane-bound polyribosomal RNA when expressed per g wet wt. of tissue (Table 1). This decline has been previously reported for both rat and mouse brain polyribosomes (Zomzely *et al.*, 1971; Campagnoni & Harris, 1977) and parallels the decrease in total brain RNA per g of tissue (Adams, 1966; Table 1). The population of polyribosomes selected would exclude monoribosomes, which do not sediment through 2M-sucrose (Wettstein *et al.*, 1963; Johnson, 1979; cf. Fig. 1). There is evidence that monoribosomes are found as translationally inactive precursors which provide subunits for the formation of initiation complexes (Henshaw *et al.*, 1973). The proportion of monoribosomes has been shown to increase with development in the rat brain (Zomzely *et al.*, 1971), and this may be a contributory factor in the decreased recovery of polyribosomes with development. This may also explain the decrease in the protein-synthetic capacity in the developing rat brain (Dunlop *et al.*, 1977).

When analysed on sucrose density gradients, the resuspended polyribosomes from both neonatal and older animals were found to be undegraded and large in size. The analysis of free and membrane-bound polyribosomes from 25-day-old animals is shown in Fig. 1.

The polyribosomes were also active in stimulating amino acid incorporation into protein in the reticulocyte-lysate system; at a concentration of 70 μg/ml there was a 6–13-fold stimulation of [3H]leucine incorporation into proteins. These proteins formed a heterogeneous population, ranging in molecular weight from over 200000 to under 14000

Table 1. *RNA content of free and membrane-bound polyribosomes*

Polyribosomes were prepared from forebrains of 3-, 10-, 25- and 45-day-old animals as described in the Materials and methods section and RNA was extracted with phenol, precipitated with ethanol and washed. Total high-molecular-weight RNA was isolated by phenol extraction of unfractionated homogenates, followed by 2M-LiCl precipitation (Baltimore & Girard, 1966; Berthold & Lim, 1976*a*). Ethanol precipitates were dissolved in 10mM-Tris/HCl (pH 7.6)/0.2% (w/v) SDS and A_{260} was determined (A_{260} for 1mg of RNA/ml = 22.6).

Age (days)	Content of total high-molecular-weight RNA (mg/g wet wt.)	Content of RNA (mg/g wet wt.) (% distribution in parenthesis): Free polyribosomes	Membrane-bound polyribosomes	Proportion of total high-molecular-weight RNA in polyribosomes (%)	Proportion of poly(A)+ RNA (%): Free polyribosomes	Membrane-bound polyribosomes
3	1.39	0.45 (50)	0.45 (50)	64	1.6	2.9
10	1.34	0.39 (56)	0.30 (44)	52	1.3	1.8
25	1.00	0.25 (60)	0.17 (40)	42	1.1	2.4
45	0.83	0.20 (64)	0.11 (36)	31	1.7	3.2

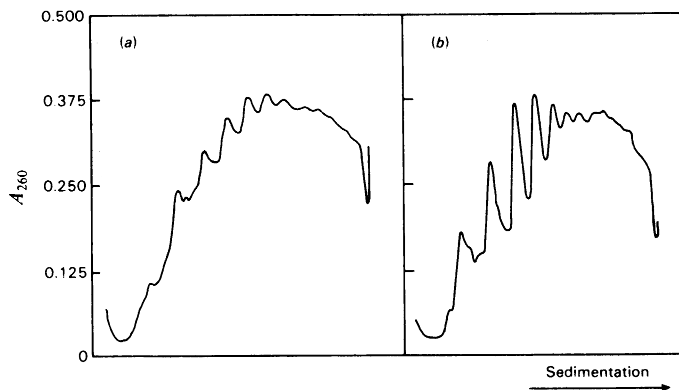

Fig. 1. *Size distribution of polyribosomes: (a) free and (b) membrane-bound*
Free and membrane-bound polyribosomes, purified through discontinuous sucrose gradients, were resuspended in TKMD buffer and centrifuged on 15–35% (w/v) sucrose gradients prepared in TKMD buffer as described in the Materials and methods section

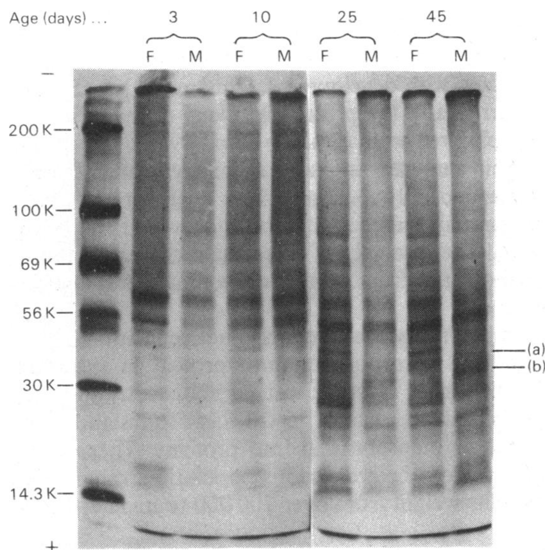

Fig. 2. *SDS/polyacrylamide-gel-electrophoretic analysis of the translation products of polyribosomes*
Free and membrane-bound polyribosomes, prepared from the forebrains of 3-, 10-, 25- and 45-day-old animals were added to reticulocyte lysate (25 μl, containing 680 μCi of [^{3}H]leucine/ml) at a concentration of approx. 70 μg/ml and incubated at 37°C for 60 min. Electrophoresis and fluorography of the [^{3}H]leucine-labelled products in 5–15% (w/v) polyacrylamide gels were as described in the Materials and methods section. Key: F, products of free polyribosomes; M, products of membrane-bound polyribosomes. Left-hand lane shows molecular-weight markers (K = 1000 daltons).

(Fig. 2). In preparations from 25- and 45-day-old animals, the compositions differed and several labelled proteins appeared to be confined primarily to one or the other polyribosomal fraction [e.g. band (a) in free polyribosomes, band (b) in bound polyribosomes; Fig. 2].

Translation of poly(A)$^{+}$ RNA in reticulocyte lysates

The average content of poly(A)$^{+}$ RNA from free and membrane-bound polyribosomal RNA was respectively 1.5 and 2.5% of the total RNA (Table 1). The poly(A)$^{+}$ RNA directed the incorporation of [^{3}H]leucine into protein in the reticulocyte lysate for at least 60 min (Fig. 3*a*). The optimal K^{+} and Mg^{2+} concentrations (including endogenous lysate cations) were 126 mM and 2.7 mM respectively. The saturating concentration of brain poly(A)$^{+}$ RNA under these conditions was approx. 20 μg/ml for all preparations examined (Fig. 3*b*). For poly(A)$^{+}$ RNA isolated from free polyribosomes, maximal stimulation was attained at a concentration of 18 μg/ml, with inhibition of translation at higher mRNA concentrations. The stimulatory activity of different poly(A)$^{+}$ RNA preparations was variable (10–44-fold); the maximal value obtained was a 65-fold stimulation, with poly(A)$^{+}$ RNA purified on sucrose density gradients (with [^{35}S]methionine as the radioactive precursor). The stimulatory activity of the poly(A)$^{+}$ RNA of free polyribosomes was greater than that of membrane-bound polyribosomes, although the reason for this is as yet unclear. The stimulatory activity of the poly(A)$^{+}$ RNA preparations and the pattern of the translation products were not altered by the use of additional purification procedures, which included either proteinase K treatment (MacNaughton *et al.*, 1974) of phenol-extracted, ethanol-precipitated RNA or 2.0 M-LiCl precipitation of RNA (Baltimore & Girard, 1966; Berthold & Lim, 1976*a*) before oligo(dT)-cellulose chromatography.

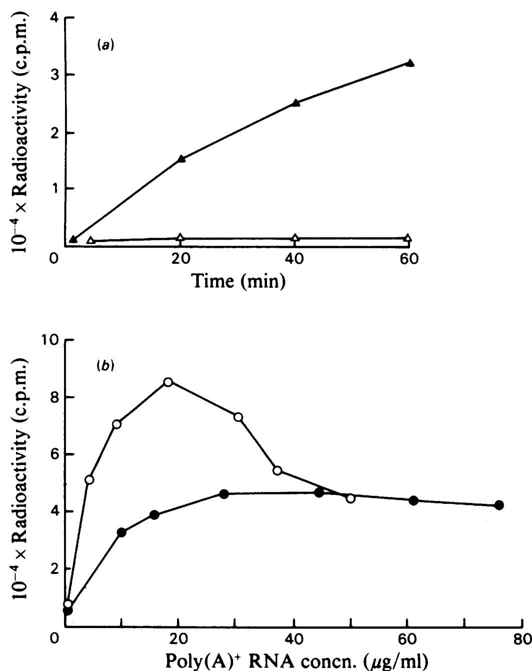

Fig. 3. *Translation of poly(A)+ RNA in reticulocyte lysate: (a) time course; (b) RNA-concentration curve*
Poly(A)+ RNA eluted from oligo(dT)-cellulose in 10mM-Tris/HCl (pH 7.6) was adjusted to contain 0.2M-sodium acetate (pH 5.5), precipitated with ethanol and washed and dissolved in sterile water. Portions (1–2 µl) were added to 25 µl of the reticulocyte-lysate system and incubated at 37°C. Duplicate samples (2 µl) were removed at 20 min intervals (*a*) or after 60 min (*b*), and trichloroacetic acid-precipitable [^{3}H]leucine was assayed as described in the Materials and methods section. The poly(A)+ RNA concentration was 35 µg/ml (*a*) or as stated in (*b*). ▲, poly(A)+ RNA isolated from microsomal fraction; △, control (−mRNA); ○, poly(A)+ RNA isolated from free polyribosomes; ●, poly(A)+ RNA isolated from membrane-bound polyribosomes.

Estimation of size of poly(A)+ RNA

After one cycle of binding to oligo(dT)-cellulose, poly(A)+ RNA can contain up to 50% of contaminating rRNA (Bantle *et al.*, 1976). This made it difficult to determine by absorbance measurements the size of the poly(A)+ RNA underlying contaminating 18S and 28S rRNA (Figs. 4*a* and 4*b*). However, by assaying the translocational activity of the RNA in the different gradient fractions, the average size of the poly(A)+ RNA could be determined [assuming that the various size classes of brain poly(A)+ RNA are translated with the same efficiency]. Maximal stimulatory activity of the poly(A)+ RNA isolated from both free and membrane-bound polyribosomes was associated with RNA of 16–18S; a secondary peak of activity was derived from RNA of 12S. This is in agreement with other reports of the size of brain poly(A)+ RNA (Bantle & Hahn, 1976) and our own previous studies (Berthold & Lim, 1976*b*; Elliott *et al.*, 1980). The compositions of translation products from the serial gradient fractions are also shown in Figs. 4(*c*) and 4(*d*). Ryffel & McCarthy (1975) have found that a prior heating at 65°C (see the Materials and methods section) results in a similar size distribution of poly(A)+ RNA to that obtained under denaturing conditions with formamide. However, some aggregation of brain RNA was apparent, since there was an additional peak of translational activity at the bottom of the gradient, coincident with 28S rRNA. Proteins of low molecular weight were also found as translational products of the fractions of larger RNA. The aggregation observed probably involves only a small proportion of the total RNA, which does not substantially influence the overall size distribution of poly(A)+ RNA in the gradient, since the size observed corresponds to that previously found under denaturing conditions with 85% (v/v) formamide (Elliott *et al.*, 1980).

Translation products of poly(A)+ RNA: developmental changes

The composition of proteins synthesized by poly(A)+ RNA isolated from 3-, 10-, 25- and 45-day-old animals and labelled with either [^{3}H]-leucine or [^{35}S]methionine is shown in Figs. 5 and 6. The predominant protein products from both free and membrane-bound poly(A)+ RNA ranged in molecular weight from approx. 10000 to 90000 (Fig. 5), although minor components of higher molecular weight were also detectable. Within this molecular-weight range, the protein products closely resembled those from translation of the intact polyribosomes (cf. Fig. 2). The absence of the higher-molecular-weight proteins from the translation products of poly(A)+ RNA probably reflected the loss of large poly(A)+ RNA molecules during oligo(dT)-cellulose chromatography (Deeley *et al.*, 1977). RNA from the intermediate-salt fraction from the oligo(dT)-cellulose column, which includes predominantly rRNA as well as a fraction of poly(A)+ RNA, was found to direct the synthesis of a larger proportion of high-molecular-weight proteins than did poly(A)+ RNA from the low-salt fraction (result not shown). On repeated translation of each poly(A)+ RNA preparation, the composition of the protein products was constant. This suggests that the composition was representative of the relative concentrations of the individual mRNA species, although there may also be some differences in the initiation rates of individual mRNA molecules.

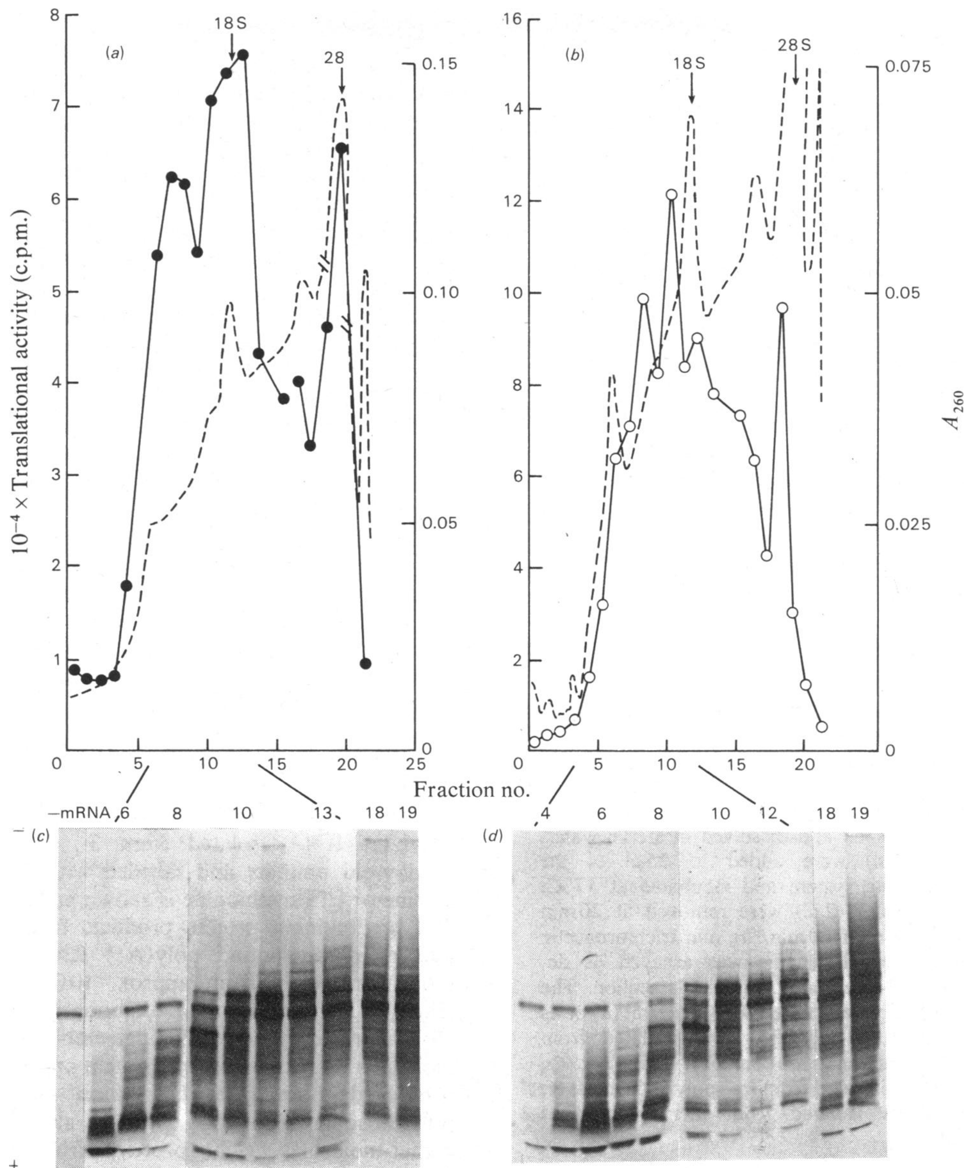

Fig. 4. *Sucrose-density-gradient analysis of poly(A)$^+$ RNA isolated from free and membrane-bound polyribosomes*
Poly(A)$^+$ RNA isolated from membrane-bound (*a*) and free (*b*) polyribosomes from forebrains of 10-day-old animals was centrifuged on linear 15–30% (w/v) sucrose density gradients and RNA in the serial gradient fractions was precipitated with ethanol and dissolved as described in the Materials and methods section. Portions (1 μl) of RNA were added to 12 μl portions of the reticulocyte-lysate system containing [^{35}S]methionine (1.3 mCi/ml) and incubated for 1 h for 37°C. The translation products of the fractions [*c*, membrane-bound polyribosomal poly(A)$^+$ RNA; *d*, free polyribosomal poly(A)$^+$ RNA] were analysed on 5–15% (w/v) polyacrylamide gels. Fluorography was at −70°C for 24 h. ----, A_{260}; ○, translational activity of poly(A)$^+$ RNA from free polyribosomes; ●, translational activity of poly(A)$^+$ RNA from membrane-bound polyribosomes.

Proteins of mol.wt. 55000 (probably tubulin) and 46000 (actin; Gozes *et al.*, 1977; J. Guest, S. Whatley, C. Hall & L. Lim, unpublished work) were present as major components of the translation products of both polyribosomal fractions. These proteins were more abundant in the poly(A)$^+$ RNA products from brains of 3-day-old animals than in those derived from 25- and 45-day-old animals (Fig.

5). Tubulin and actin have previously been reported to comprise approx. 15% of the translation products of neonatal-rat brain RNA (Schmitt *et al.*, 1977). There was an increasing complexity with age in the translation products migrating in the region of mol.wts. 53000–56000 and 46000 in both polyribosomal fractions.

Although there were similarities in the composition of the translation products of the two poly(A)+ RNA fractions, clear qualitative differences could be observed. A comparison of the translation products of poly(A)+ RNA derived from 45-day-old animals (Fig. 5, lanes 7 and 8) showed the presence of protein bands of mol.wts. 33000, 21000 and 24000 only in products of translation of membrane-bound polyribosomal poly(A)+ RNA. Proteins of mol.wts. 39000, 37000, 31000, 27000 and 17000 appeared to be products predominantly of free-polyribosomal poly(A)+ RNA. The distribution of several protein bands, e.g. of mol.wts. 31000 (free-polyribosomal RNA) and 33000 (membrane-bound polyribosomal RNA), as translation products of only one or the other fraction suggests that cross-contamination of free and membrane-bound polyribosomal fractions must be very small. Further characterization of the two classes of translation products by two-dimensional electrophoresis is required. During development, there was an increase (between 3 and 10 days) in the amount of the 15000-mol.wt. band relative to the 17000-mol.wt. band in the translation products of poly(A)+ RNA from the free-polyribosomal fraction (Fig. 5). The major developmental differences, however, were found in the translation products of the membrane-bound-polyribosomal poly(A)+ RNA. The 33000- and 21000-mol.wt. polypeptides, which were well represented in the translation products derived from 10-day and older animals, were not detectable, or were present in only trace amounts, in those derived from 3-day-old animals. This 33000-mol.wt. membrane-derived polypeptide was not found as a translational product of liver polyribosomal poly(A)+ RNA (result not shown). Neither was it present in the translational products of poly(A)+ RNA prepared from a conventional microsomal fraction from brain (Fig. 6, cf. lanes 8–10). This

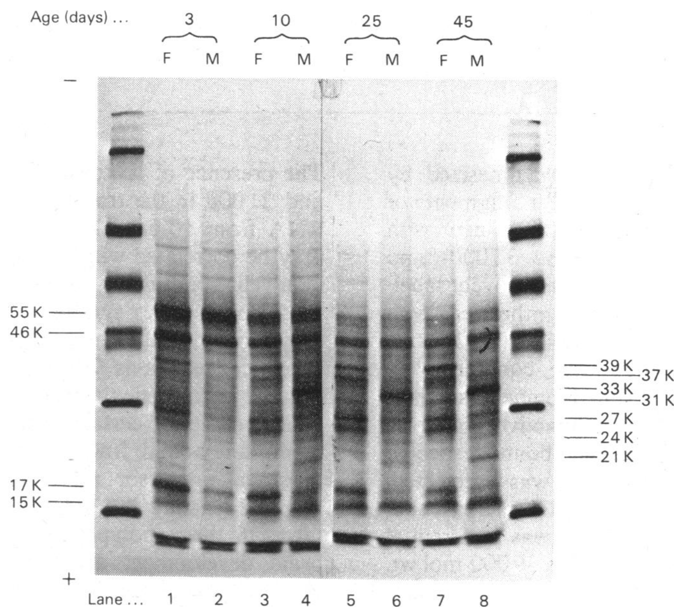

Fig. 5. *Developmental changes in the* [^{3}H]*leucine-labelled translation products of poly(A)+ RNA isolated from free and membrane-bound polyribosomes*

Free and membrane-bound poly(A)+ RNA preparations (approx. 20μg/ml) from 3-, 10-, 25- and 45-day-old animals were translated in 25μl portions of the reticulocyte lysate containing [^{3}H]leucine (680μCi/ml). The translation products of poly(A)+ RNA from free (F; lanes 1, 3, 5, 7) and membrane-bound (M; lanes 2, 4, 6, 8) polyribosomal fractions were analysed by SDS/polyacrylamide-gel electrophoresis [on 5–15% (w/v) acrylamide gels] and fluorography as described in the Materials and methods section. Samples, containing equivalent amounts of trichloroacetic acid-precipitable radioactivity (2×10^5 c.p.m.), were analysed on two gels, with [^{14}C]methylated markers [a mixture of myosin (200K), phospholipase *b* (100K) and (92.5K), bovine serum albumin (69K), ovalbumin (46K), carbonic anhydrase (30K) and lysozyme (14.3K)] on one lane in each gel. Fluorography exposure was at −70°C for 7 days. Values of 15K, 17K etc. refer to mol.wts. of 15000, 17000 etc.

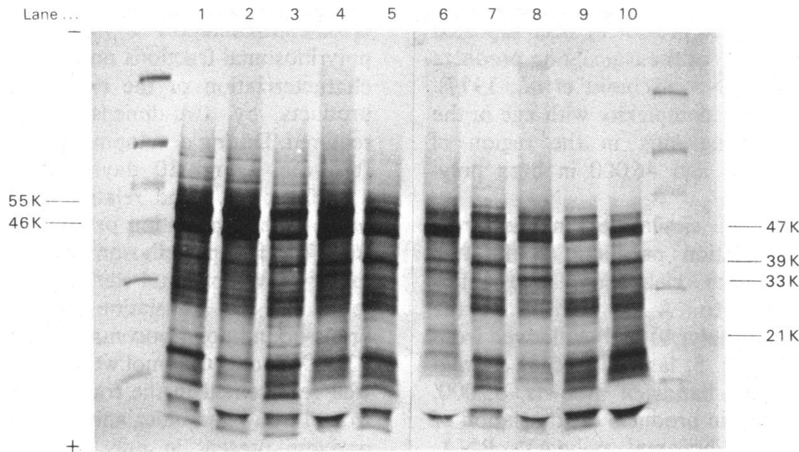

Fig. 6. *Electrophoretic analysis of [^{35}S]methionine-labelled translation products of poly(A)$^+$ RNA isolated from free and membrane-bound polyribosomes: developmental changes*

Free and membrane-bound polyribosomal poly(A)$^+$ RNA and microsomal poly(A)$^+$ RNA preparations were translated in reticulocyte lysate with [^{35}S]methionine as the radioactive precursor, as described in Figs. 4 and 5, and analysed on 5–25% (w/v) polyacrylamide gels as described in the Materials and methods section. Fluorography exposure was for 24h. Lanes 1, 3, 5 and 7, translational products of free-polyribosomal poly(A)$^+$ RNA from preparations from 3-, 10-, 25- and 45-day-old animals respectively; lanes 2, 4, 6 and 8, translational products of membrane-bound-polyribosomal poly(A)$^+$ RNA from preparations from 3-, 10-, 25- and 45-day-old animals, respectively; lane 9, translational products of microsomal poly(A)$^+$ RNA isolated from the hypothalamus of 55-day-old animals; lane 10, translational products of microsomal poly(A)$^+$ RNA isolated from the cortex of 55-day-old animals. Lanes with molecular-weight markers are shown on either side of lanes 1–10; values of 55 K, 46 K etc. refer to mol.wts. of 55000, 46000 etc.

suggests that this protein may be synthesized by polyribosomes associated with heavier fragments of rough endoplasmic reticulum, which sediment with nuclei and mitochondria. Both the 33000- and 21000-mol.wt. proteins were more effectively labelled (relative to the other proteins synthesized) with [^{3}H]leucine than with [^{35}S]methionine (cf. Figs. 5 and 6), suggesting that they were comparatively methionine-poor. When [^{35}S]methionine was used instead of [^{3}H]leucine as the radioactive precursor (Fig. 6), a further membrane-bound specific polypeptide (47000 mol.wt.) was detected, which also showed a developmental increase between 3 and 10 days. Similarly there was preferential labelling with [^{35}S]methionine of a 39000-mol.wt. protein found in the translation products of membrane-bound-polyribosomal poly(A)$^+$ RNA (of all ages investigated), suggesting that this is not the same protein(s) as the 39000-mol.wt. component of the translation products of free-polyribosomal poly(A)$^+$ RNA, which was equally well labelled with either precursor (cf. lanes 7 and 8 in Figs. 5 and 6).

Conclusion

Clear developmental changes in the translational products of poly(A)$^+$ RNA were seen in the membrane-bound-polyribosome-derived fraction. The presence of proteins of mol.wts. 47000, 33000 and 21000 in the translation products of poly(A)$^+$ RNA from 10-day-old, but not 3-day-old, animals may be correlated with the developmental processes of myelination and synaptogenesis. In other tissues, most polypeptides synthesized on membrane-bound polyribosomes have an additional *N*-terminal leader sequence (see Davis & Tai, 1980), which may not be cleaved in the reticulocyte-lysate system. The molecular weights of certain translation products of the membrane-bound fraction may therefore represent those of precursor molecules and may not correspond exactly to brain proteins synthesized *in vivo*. If these polypeptides which appear during brain development are tissue-specific, their molecular weights suggest that they may be related to the myelin proteins. Proteolipid protein and protein DM 20 have mol.wts. of 30000 and 25000, Wolfgram protein has mol.wt. 49000–54000 (Chan & Lees, 1974) and pre-large basic protein has mol.wt. 21500 (Barbarese *et al.*, 1977). We have previously reported the synthesis of rat large myelin basic protein (mol.wt. 18500; Eylar *et al.*, 1971) in response to brain microsomal poly(A)$^+$ RNA in *Xenopus* oocytes, in which post-translational modifications are known to occur (Lim *et al.*, 1974). The localization of this mRNA to either free or

membrane-bound polyribosomes is as yet uncertain.

Alternatively, these membrane-bound polypeptides may be involved in synaptogenesis. Of the well-characterized proteins identified in synapses, synaptin, proteins D_1 and D_2 have subunit molecular weights greatly in excess of these values, but protein D_3 has a smaller subunit structure (mol.wts. 14000, 23500, 34400; Jorgensen, 1979). Further experiments are required to establish the identity of these protein products of translation of membrane-bound polyribosomal poly(A)+ RNA. Many of the translation products appeared to be common to both polyribosomal fractions. These may include proteins with a major structural role, such as tubulin, actin and neurofilament subunit proteins, which function in axonal elongation and synapse formation as well as axoplasmic transport (Shelanski & Feit, 1972). There was a relative decrease with development in the proportional synthesis *in vitro* of tubulin and actin, confirming the report of Schmitt *et al.* (1977), which parallels the decrease *in vivo* in rat brain (Gozes *et al.*, 1975; Schmitt *et al.*, 1977) and in both neuronal and glial cells (Gozes *et al.*, 1977). This suggests that there is indeed a decline in the relative amounts of the respective mRNA species. In other tissues, proteins that are exported from the cell are synthesized on membrane-bound polyribosomes (e.g. albumin in rat liver; Yap *et al.*, 1977). In brain, membrane-bound polyribosomes may also have a role in the synthesis of proteins for axoplasmic transport to the cell terminals (Rambourg & Droz, 1980). There may be other differences in the translational products of the two poly(A)+ RNA fractions, since our analysis did not differentiate between different proteins of the same molecular weight. Further, the translational products detected by gel electrophoresis and fluorography represent products of only the most abundant classes of brain poly(A)+ RNA (Chikaraishi, 1979), and there may be differences among the less abundant poly(A)+ RNA sequences which remain undetected. We have recently synthesized complementary DNA species, which have been found to correspond in length to the poly(A)+ RNA. (C. Hall & L. Lim, unpublished work), for further characterization of the poly(A)+ RNA sequences of free and membrane-bound polyribosomes.

Identification of these translation products by using specific antisera and purification of the mRNA species is required. The purification of specific mRNA species which appear during brain development should facilitate a study of the cell regulatory mechanisms that control transcription in the brain.

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