Isolation and Characterization of Ribonucleic Acid from Cerebral Cortex of Rat

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SUMMARY

Ribonucleic acid isolated from cerebral cortex of mature male and female rats was fractionated by chromatography on methylated albumin Kieselguhr columns or by sucrose gradient centrifugation. Three principal fractions were obtained with analytical ultracentrifuge $s_{20, w}$ values of 4, 17, and 28. By comparison with ribonucleic acid prepared from purified rat brain ribosomes, the 17 S and 28 S fractions were shown to be predominantly ribosomal ribonucleic acid. Differences in base analyses between the three fractions were small but significant. Compositions of all fractions were markedly complementary with $G = C$ and $A = U$.

Within the past decade experiments by Hydén and Egyházi (1, 2), McConnell (3), and others (4, 5) have suggested possible relations of ribonucleic acid to learning and memory. We are interested in changes which may occur as a consequence of behavioral factors in specific fractions (or kinds) of RNA from mammalian brains. Since any such changes may be extremely small in comparison with a relatively large amount of unchanged RNA, we must have detailed information about the characteristics of brain RNA from control animals.

This paper describes methods for (a) extracting RNA from cerebral cortex of albino rats, (b) fractionating this RNA, and (c) physically and biologically characterizing these fractions. The main portion of the paper describes RNA from male and female rat brain cortex with respect to its fractionation in a sucrose density gradient and on methylated albumin Kieselguhr columns, together with base analyses of each fraction. Data are also given on RNA from purified ribosomes isolated from rat brain.

EXPERIMENTAL PROCEDURES

Rats—Albino rats of Sprague-Dawley strain were obtained from Simonsen Laboratories. Rats used for comparison of male and female brain cortex RNA were matched in age, in one instance 108 and in another 111 days. In the latter case, none of the female rats was in estrus at the time brains were taken. For other work on characterization of cerebral cortex RNA, adult male albino rats from Simonsen, 130 to 150 days old, were used. Male rats were 350 to 400 g and females 250 to 300 g. For comparison with data on protein synthesis systems from rat brain (6, 7) several separations and analyses were done on 20-day-old rats. Limited data so far obtained have not demonstrated significant differences in these RNA fractions as compared with those from mature animals.

Removal of Cortex—Rats were decapitated after ether anesthesia for about 1 min. The skull was rapidly peeled open, exposing the brain. The cerebral cortex was immediately removed and dropped into a Dewar flask containing liquid nitrogen. The removal time from the instant of decapitation was about 2 min.

Isolation of RNA—The procedure was essentially the hot phenol method of Kirby (8). The cortex material was homogenized in a Waring Blendor in 0.14 m NaCl, 0.1% sodium dodecyl sulfate (10 ml per g of cortex), and 0.1% bentonite; this was similar to the extraction used by Georgiev and Mantieva (9). Two 20-min extractions were made with equal volumes of phenol, first at 45° then at 55°. After each extraction, the homogenate was rapidly cooled to 4° in an ice bath and centrifuged in a Spinco model L centrifuge (SW 25 rotor) at 20,000 rpm for 1 min. No appreciable amount of DNA was present in the RNA extract, as determined by analyses by the diphenylamine method (10).

After RNA was extracted, it was precipitated by addition of 2 volumes of absolute ethanol to the aqueous phase and allowed to stand overnight at -20°. The pellet was spun down and washed twice with small volumes of cold 3:1 ethanol. This RNA was used directly on a sucrose gradient. If RNA was to be fractionated on a methylated albumin Kieselguhr column, it was dissolved in 20 ml of 0.002 m NaCl and precipitated with 1 volume of absolute ethanol, 0.02 m magnesium acetate. This procedure yielded the insoluble magnesium salt of RNA, which was centrifuged and transferred to a methylated albumin Kieselguhr column after solution in 0.05 m Tris-acetate-0.3 m NaCl, pH 6.8.

Fractionation on Methylated Albumin Kieselguhr Column—The preparation of this column has been described by Mandell and Hershey (11). We substituted a 0.05 m Tris-acetate solution, pH 6.6, for the original phosphate buffer. This was made by mixing in water 6.05 g of Trizma base (Tris(hydroxymethyl)amino methane, Sigma) and 2.85 ml of glacial acetic acid per liter of solution. It buffers against extremes of pH on either side of pH 6.6. The RNA isolated by the phenol method was dissolved in 100 ml of 0.3 m NaCl, Tris-acetate solution (not in excess of 50 μg of RNA per ml) and loaded onto a methylated
albumin Kieselguhr column under 1.2 to 1.3 atm pressure via a separatory funnel. The loading time was 1.0 to 1.5 hours. A linear concentration gradient of NaCl in 0.05 M Tris-acetate solution was employed to elute RNA from the column, the starting concentration of NaCl being 0.4 M and the final concentration 1.5 M. Two fractions of RNA were eluted by the saline gradient, with the second fraction appearing at approximately 1.1 M NaCl. After the optical density had returned to baseline, a third fraction of RNA (high temperature fraction) was released, it was washed from the column by a continuous flow of 1.5 M buffered saline.

Sucrose Density Fractionation—RNA from rat brain cortex was also fractionated on a linear sucrose density gradient at 0°. A device similar to that of Britten and Roberts (12) was used to build the gradients (25-ml volume) from 5 and 20% sucrose solutions in 0.1 M NaCl-0.01 M acetate buffer, pH 5.2. RNA from three rat brain cortices was dissolved in 0.5 ml of 0.1 M acetate buffer and layered carefully on top of a 25-ml gradient. All operations were carried out in a cold room at 0°. The gradient was spun at 24,000 rpm in the SW 25 rotor of the Spinco model L centrifuge for 12 hours at 0°. After centrifugation, the bottom of the gradient tube was punctured with a needle, and 30 drops were collected in each of 45 to 50 small test tubes. These samples were diluted to 1.0 ml, and optical densities were measured with a Cary model 14 spectrophotometer.

Analytical Ultracentrifugation of RNA—Sedimentation characteristics were determined in a Spinco model E ultracentrifuge with ultraviolet optics at 20°. RNA taken from the two heaviest fractions was dialyzed for 8 hours against 0.1 N NaCl-0.01 M acetate buffer, pH 5.2, in order to remove sucrose. The same procedure was followed with RNA fractions collected from methylated albumin Kieselguhr columns.

Since the sedimentation constants of different RNA fractions were markedly dependent on concentration, they were measured for a series of concentrations (20 to 50 mg per liter) and extrapolated to zero concentration to obtain .S.0,.

Base Analyses—RNA fractions from methylated albumin Kieselguhr columns were precipitated with 1 volume of absolute ethanol containing 0.02 M magnesium acetate and placed in a freezer at -20° for several hours. In precipitating RNA from a sucrose gradient, better results were obtained by addition of solid cetyltrimethylammonium bromide, which formed the insoluble cetyltrimethylammonium RNA salt. Cetyltrimethylammonium bromide was added from a 1% solution to RNA fractions in an ice bath. After centrifugation in a clinical centrifuge, the RNA pellet was hydrolyzed in 0.3 N KOH (0.1 ml per mg of RNA) for 20 hours at 37°. The hydrolysate was neutralized with 70% perchloric acid and centrifuged. The supernatant was applied with a 20-ml pipette onto a Schlieren and Schüll cellulose acetate strip (25 x 300 mm) and subjected to electrophoresis at 4° for 1 hour and 45 min at 400 volts, followed by 90 sec at 1200 volts to tighten the bands. The strips were dried, marked, cut, and eluted in 1.5 ml of 0.05 N HCl. Molar percentages of nucleotides were calculated from optical density measurements. The following molar absorptivities (ε<sub>λ</sub> cm<sup>-1</sup>) were used (13): CMP, 13.2 x 10<sup>4</sup>; AMP, 15.0 x 10<sup>4</sup>; GMP, 12.2 x 10<sup>4</sup>; UMP, 10.0 x 10<sup>4</sup> (at 278, 257, 257, and 262 mμ respectively wave lengths of maximal absorption). The A<sub>280</sub>:A<sub>260</sub> ratios indicated that nucleotide purity was excellent in all fractions. Sections of strip between bands were found to be free of nucleotides. The analytical method was checked against standard synthetic mixtures of nucleotides.

 Apparently random errors in the analyses caused their precision to be less than desirable, but probably as good as most reported data of this kind in the literature. (Recently, however, Yama-gaui, Kawakita, and Naka (14) have quoted base analyses on RNA from guinea pig brain cortex that seem to be of much higher precision.) There was a persistent background absorption of 0.05 to 0.09 optical density units at 240 to 300 mμ, eluted from the cellulose acetate paper. This could not be eliminated by any washing or pretreatment tried, and it may be due to surface oxidation of the paper. Also, the electrophoresis did not distinguish unusual or methylated bases in the nucleosides, so that only fractions designated AMP, GMP, CMP, and UMP are reported.

Preparation of Ribosomes—The method was taken, with few modifications, from a report by Munro, Jackson, and Korner (15) on rat liver ribosomes. The animals were killed by cervical dislocation. The brains were placed in ice-cold Medium M (0.025 M sucrose-0.02 M Tris (pH 7.6)-0.01 M magnesium acetate-0.04 M sodium chloride-0.1 M potassium chloride-0.006 M mercaptoethanol), washed free of blood, and weighed. They were homogenized in a Waring Blender in 2 volumes of Medium M. Nuclei, whole cells, and debris were removed by centrifugation at 900 x g for 10 min (2,700 rpm, Lourdes centrifuge, 23-cm head). The nuclear pellet was discarded, and the crude mitochondrial fraction was removed by centrifugation at 12,000 x g for 10 min, (10,000 rpm, No. 30 rotor, Spinco). Ribosomes were prepared from the postmitochondrial supernatant by treating it with one-ninth its volume of 10% sodium deoxycholate (Mann, Special Enzyme Grade), freshly dissolved in 0.05 M Tris buffer, pH 8.2; this procedure solubilizes lipoprotein membranes of the endoplasmic reticulum, freeing the ribosomes. The sodium deoxycholate was added to the postmitochondrial supernatant with constant stirring for 2 min before centrifugation for 2 hours at 30,000 rpm in the No. 30 rotor. The supernatant was discarded, and the pellet was rinsed several times with Medium M, re-suspended in this medium, and centrifuged for 2 hours to remove all traces of sodium deoxycholate. The final pellet was resuspended to a protein concentration of 20 mg per ml.

Extraction of RNA from Ribosomes—The procedure for the extraction of RNA from rat brain cortex ribosomes was somewhat modified from the standard procedure already described for whole cortex.

The ribosome pellet (from 20 rats) was suspended in 10 ml of 0.14 M NaCl and 1.1% sodium dodecyl sulfate, and homogenized for 30 sec at low speed in a Waring Blender with an equal volume of 90% phenol (preheated to 37°). The homogenate was heated in a water bath at 37° for 5 min, cooled rapidly to 4°, centrifuged for 1 min at 20,000 rpm (Spinco, SW 25.1 rotor). The aqueous layer was removed and combined with 2 volumes of absolute ethanol to precipitate the RNA.

Results and Discussion

Of the RNA extracted from rat brain by the phenol method, up to 80% was actually recovered by the ethanol precipitations, the yield depending primarily on the length of time allowed.
FIG. 1. Chromatograms of RNA from rat cerebral cortex on a column of methylated albumin Kieselguhr. From 1 to 5 mg of RNA are loaded on column in 0.05 M Tris-acetate buffer, pH 6.5, in 0.3 M NaCl. Elution is carried out with a linear gradient from 0.4 to 1.5 M NaCl in buffer at 20° through a Vanguard 1056 ultraviolet analyzer. The high temperature fraction is eluted with 1.5 M NaCl buffer by raising the temperature of the water jacket on the of column gradually from 45° to 65°.

The yield of RNA per cortex (approximately 1.0 g) was approximately 1.25 mg.

RNA Fractions from Methylated Albumin Kieselguhr Column and Sucrose Density Gradient Centrifugation—Fig. 1 shows a typical elution profile of total cortical RNA as fractionated on a methylated albumin Kieselguhr column. Analytical ultracentrifuge measurements on these fractions gave the following results:

- Fraction I (low salt): $s_{20w} = 4$
- Fraction II (high salt): $s_{20w} = 17$
- Fraction III (high temperature): $s_{20w} = 28$

Typical centrifuge runs are shown in Fig. 2, and results given are means of two concordant runs extrapolated to zero concentration.

A typical sucrose density gradient fractionation is shown in Fig. 3. The three fractions of RNA from the sucrose gradient were precipitated with 2 volumes of 0.02 M MgCl₂ in absolute ethanol and passed separately through methylated albumin Kieselguhr columns. These 4 S, 17 S, and 28 S fractions appeared as low salt, high salt, and high temperature peaks, respectively. Thus the methylated albumin Kieselguhr column and sucrose density gradient fractionations achieve essentially the same kind of fractionation of cortical RNA.

Quantitative comparison of sucrose density gradient and methylated albumin Kieselguhr profiles indicated that 4 S, 17 S, and 28 S RNA comprised approximately 18, 28, and 36%, respectively, of total RNA fractionated, the remaining 18% probably being degraded or lost during the fractionation procedures.

On the basis of these data, we could tentatively identify the 4 S peak as predominantly transfer RNA, and the 17 S and 28 S peaks as predominantly ribosomal RNA. Ellem and Sheridan (16) suggested that a similar high temperature RNA peak from L(E929) cells was messenger RNA, but there is no basis for such an identification in our material. Our high temperature fraction would appear to be essentially the same as the 28 S ribosomal RNA peak isolated by Petermann and Pavlovec (17) from rat-liver ribosomes.

Petermann and Pavlovec (17) noted a small peak at 22 S in ultracentrifugal analysis of RNA from rat liver ribosomes, comprising about 10% of the total RNA. These authors also cited other examples of the occurrence of such a species in RNA from mammalian tissues. Excellent separations of 23 S and 16 S RNAs from bacteria have been achieved on methylated albumin Kieselguhr (18). Although we did not see any such fraction when methylated albumin Kieselguhr column eluants were scanned in conjunction with the automatic fraction collector (Vanguard model 1056), we could sometimes detect a small peak between the 17 S and 28 S peaks when the fractions were re-scanned at 260 μm with a Cary model 14 spectrophotometer.

Fractionation of RNA isolated from highly purified ribosomes on methylated albumin Kieselguhr columns yielded patterns such as that in Fig. 4, which provide further evidence that the bulk of the high temperature peak in Fig. 1 must be ribosomal RNA. We know, however, that this ribosomal preparation...
must contain a certain amount of tightly bound messenger RNA, since the purified ribosomes are extremely active in a cell-free protein synthesis system to which no exogenous messenger has been added (7). The small low salt (low molecular weight) fraction from ribosomal RNA is interesting. Its molecular weight would suggest that it is transfer RNA, but we would not expect so much transfer RNA to be tightly bound to the purified ribosomes. It is more likely that this low molecular weight fraction is the transfer-like RNA described by Comb et al. (19). Confirmation of this identification must await tests of amino acid binding and methylation.

The relative amounts of RNA in the high salt and high temperature methylated albumin Kieselguhr fractions varied considerably. For 12 preparations of total cortical RNA, the ratio of high temperature to high salt peak areas ranged from 0.9 to 2.9 with a mean of 1.47 ± 0.36. For 4 preparations of ribosomal RNA, the corresponding ratios were 0.30, 1.44, 0.36, and 0.38. These results suggest a considerable degree of interconvertibility of the 28 S and 17 S ribosomal fractions, depending on isolation conditions as yet under incomplete control. The work of Midgley (20) showed that low Mg"+ and low ribonucleoprotein concentrations during isolation of RNA favor instability of 23 S bacterial ribosomal RNA. Similar factors may have been responsible for the variations in relative amounts of 28 S and 17 S RNA in our preparations.

Analytical Data—Table I gives a comparison of the base ratios of male and female cortical RNA in various fractions eluted from methylated albumin Kieselguhr columns. The sample standard deviations of these analytical figures varied from ± 0.6 to 2.6. Our level of precision in base analyses was not good enough to permit unequivocal conclusions concerning apparent small deviations in analyses between various sample populations. We applied the t test to various pairs of populations and converted the t values to confidence levels for significance of differences between sample means.

In view of the fact that 16 S and 28 S RNA from rat liver are said to be interconvertible to a considerable extent (17), we might expect the same interconvertibility for our 17 S and 28 S rat cortex samples. If the 17 S and 28 S RNA represent different association states of the same material, we should expect their base analyses to be identical. The t tests indicated, however, that the base analyses represented different populations at the 0.98 level of significance. It would be necessary, however, to have only a moderate preponderance in one fraction of a species with different base composition to account for the rather small observed differences, and such a situation would be in accord with a less than 100% interconvertibility of 17 S and 28 S fractions and differences in their rates of synthesis (21) and in their possible inclusion of nonribosomal species.

The statistical tests indicated at the 0.99 significance level that a difference existed in nucleotide composition between the 4 S fractions from male and female cortices. The poor quality of the analytical data require that this result be rechecked before we indulge in any speculation concerning its possible functional significance. A similar conclusion might be made concerning the percentage of uracil in the respective high salt (17 S) fractions, but the generally higher uncertainty of the uracil analyses would lead us to discount this observation. The remaining confidence levels are notably smaller, and thus no sex-linked differences in the nucleotide compositions of the ribosomal fractions have been demonstrated. It is well to remember that the statistical analysis deals with random and not systematic errors. Indications of lack of significance are therefore more convincing than indications of significance.

Reported values (22) of base composition of rat DNA are included in Table I. In contrast with ribosomal RNA of non-mammalian origin, ribosomal RNA fractions from mammalian cells have a composition that is more nearly complementary in the DNA fashion. The present analyses of rat brain ribosomal RNA conform to this pattern to a remarkable extent, with G = C and A = U in both the ribosomal fractions. Evidence from hybridization with DNA indicates that bacterial ribosomal RNA is formed on a single strand of DNA template (23). As would be expected, such RNA does not display base pairing in accord with the G = C and A = U rule. Insufficient data are available to determine whether one or both strands of DNA are transcribed in synthesis of ribosomal RNA in mammalian systems (24, 25). The base pairing observed in rat brain and guinea pig brain (14) ribosomal RNA suggests either that this RNA has been formed on both complementary strands of a DNA double helix, or that, if a segment of a single strand is utilized as template, some mechanism in the course of evolutionary development has caused this segment to follow the base-pairing
Fig. 3. Sucrose density gradient centrifugation of RNA from rat cerebral cortex. About 2 mg of RNA in 0.01 M acetate-0.1 M NaCl buffer at 0°C is layered on top of a previously prepared linear sucrose gradient from 5 to 20 g per 100 ml. Centrifugation is carried out at 24,000 rpm in No. 25.1 rotor of Spinco model L centrifuge for 12 hours. Samples of 0.5 ml per tube are collected by puncturing the bottom of a tube with a 20-gauge needle and diluted to 3 ml, and the optical density is read on a Cary model 14 spectrophotometer at 260 mp.

Fig. 4. Chromatogram of RNA isolated from purified ribosomes from rat brain on methylated albumin Kieselguhr column. Procedure is identical with that outlined in legend of Fig. 1.
These analyses were performed by spectrophotometry of eluants of mononucleotides separated by electrophoresis on cellulose acetate strips. The various RNA fractions were hydrolyzed in KOH solution as described in text. The values quoted are mean molar percentages with sample standard deviations.

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REFERENCES
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