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The Role of Ribosomal Ribonucleic Acid in the Structure and Function of Mammalian Brain Ribosomes

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In order to resolve the functional role of intact rRNA in polypeptide chain elongation mouse brain ribosomes were treated with dilute pancreatic or T_1 RNAase (ribonuclease). After RNAase treatment, several physical–chemical properties as well as the functional activity of the ribosomes were measured. RNAase treatment resulted in the extensive hydrolysis of both 18S and 28S rRNA; however, the sedimentation properties of monoribosomes were unaltered and more than 90% of the relatively low-molecular-weight RNA fragments remained associated with ribosome particles. Analysis of the ability of RNAase-treated ribosomes to participate in cell-free protein synthesis showed that ribosomes with less than 2% intact rRNA retained more than 85% of their activity in polyphenylalanine incorporation. Proof that the incorporation of phenylalanine by ribosomes with hydrolysed rRNA actually represented active translocation was obtained by the effective inhibition of incorporation by diphtheria toxin. In addition, the oligopeptide products of protein synthesis could be identified by BD (benzoylated diethylaminoethyl)-cellulose column chromatography. Analysis of the size distribution of oligopeptides synthesized by normal and RNAase-treated ribosomes showed no significant differences which indicated that there was no change in the proportion of ribosomes engaged in protein synthesis. Thus strong RNA–protein and protein–protein interactions must serve to maintain the functional integrity of ribosomes even when the rRNA is extensively degraded. The ability of the enzyme-treated ribosomes to efficiently incorporate amino acids clearly demonstrated that 'intact' rRNA is not required for protein-synthetic activity.

Although many intrinsic operations required for protein synthesis have been ascribed to ribosomes, the precise functional roles of each of the various ribosomal components have yet to be fully elucidated. Extensive efforts have been undertaken to determine the structure of rRNA species (Cox, 1969; Nomura, 1973; Spirin & Gavrilova, 1969) in relation to the proteins which comprise the ribonucleoprotein complexes. In addition to information defining the structural arrangement of rRNA and proteins within ribosomal particles, data have begun to accumulate about the specific ribosomal components required for the various steps in polypeptide chain elongation. The isolation of bacterial mutants which are resistant to the action of antibiotics affecting protein synthesis at the ribosomal level has been particularly useful in identifying the ribosomal components which are essential to translational events. In almost all cases the altered functional activity has been attributed to an alteration in a ribosomal protein (Davies & Nomura, 1972). However, in three instances variations in the degree of methylation of rRNA have been implicated as the cause for antibiotic resistance, e.g. kasugomycin (Helser *et al.*, 1972), erythromycin (Lai & Weisblum, 1971) and lincomycin (Lai *et al.*, 1973).

Since the antibiotics mentioned above primarily affect rRNA methylation, the alteration in subsequent ribosomal activity may actually be a reflexion of abnormal ribosome assembly. A considerable amount of controversy has arisen as to the actual role, if any, of rRNA in peptide chain elongation by mature ribosomes. Although evidence has been reported to support both sides of this dispute, a complete study that has actually characterized the functional properties of ribosomes with extensively hydrolysed RNA has not been carried out.

In order to resolve the possible function of rRNA in polypeptide chain elongation, particularly in eukaryotic ribosomes, our experiments were designed to determine the effect of hydrolysis of rRNA on the ability of mouse brain ribosomes to participate in protein synthesis. Pancreatic RNAase* has been used extensively as a tool to resolve the configuration of rRNA within the ribosomal particle and the physical–chemical interactions of rRNA with ribosomal proteins (Cox, 1969; Spirin & Gavrilova, 1969; Hartman *et al.*, 1970; Santer & Székely, 1971). In the present study we utilized pancreatic and T_1

* Abbreviations: RNAase, ribonuclease; BD-cellulose, benzoylated diethylaminoethyl-cellulose.

RNAase to hydrolyse the rRNA of mouse brain ribosomes and, subsequently, tested the ability of the RNAase-treated ribosomes to incorporate phenylalanine into polypeptides.

Materials and Methods

Materials

RNAase-free sucrose was purchased from Schwarz/Mann, Orangeburg, N.Y., U.S.A., pancreatic RNAase A and T_1 RNAase was from Sigma Chemical Co., St. Louis, Mo., U.S.A. *E. coli* K12 tRNA and BD-cellulose were from Gallard–Schlesinger Chemical Manufacturing Corp., Carle Place, N.Y., U.S.A. and formamide was from Fisher Scientific Co., Fair Lawn, N.J., U.S.A.

Isolation of mouse brain ribosomes

The isolation of ribosomes from mouse brain tissue has been described in detail by Grove *et al.* (1974). Briefly, brains were washed in ice-cold RSB buffer [10mM-Tris–HCl (pH7.4)–10mM-KCl–1.5mM-$MgCl_2$] and homogenized in the presence of 1.5mg of bentonite per ml of homogenate. The crude homogenate was centrifuged at 7000*g* for 10min at 4°C to pellet the nuclei, mitochondria and synaptosomes. The resulting supernatant fraction was then centrifuged at 200000*g* for 45min at 4°C to pellet the microsomal fraction. Ribosomes were obtained by treating the resuspended microsomal pellet with sodium deoxycholate to a final concentration of 0.5% and pelleting them by ultracentrifugation.

Treatment of ribosomes with RNAase

Ribosomal RNA in intact ribosomes was treated with either pancreatic or T_1 RNAase by incubation of solutions (100μl) which contained 5mM-Tris–HCl, pH7.4, 5mM-KCl, 0.75mM-$MgCl_2$, 1.0E_{260} unit of ribosomes and the appropriate concentrations of RNAase (Grove & Johnson, 1973). Periods of treatment and the temperatures of incubation are described in the legends of the appropriate Figures and Tables. Control ribosome preparations were exposed to identical incubation conditions except that the RNAase was omitted.

Polyacrylamide-gel electrophoresis of rRNA

Ribosomes were prepared for electrophoresis by the addition of 50μl of threefold-concentrated electrophoresis buffer to a final concentration of 40mM-Tris–HCl, pH7.2, 20mM-sodium acetate, 1mM-EDTA and 0.2% sodium dodecyl sulphate. The concentration of sodium dodecyl sulphate present was sufficient to terminate the action of RNAase (Hartman *et al.*, 1970; Grove & Johnson, 1973). Crystalline RNAase-free sucrose was then added to each sample to facilitate loading and 50μl portions were electrophoresed on either 3 or 3–10% (w/v) tandem polyacrylamide gels at room temperature for 90min at 5mA/gel (Bishop *et al.*, 1967; Johnson, 1973). A Gilford, model 2400, spectrophotometer adapted with a linear transport was used to analyse the RNA at 260nm immediately after the electrophoresis run.

Sucrose-gradient analysis of ribosomal particles

Ribosome-sedimentation properties were analysed by centrifuging 5.0E_{260} units of brain ribosomes on a 1.6ml (5–20%, w/v) linear sucrose–RBS gradient at 180000*g* for 120min at 4°C. After centrifugation, the gradient was collected from the top with a Buchler Densi-Flow apparatus and the E_{254} was continuously monitored.

Polyphenylalanine synthesis

The functional assay for poly(U)-directed phenylalanine incorporation has been previously reported (Gilbert *et al.*, 1972). The source of enzymes for protein synthesis was a brain microsomal supernatant fraction which had been dialysed against 10mM-Tris–HCl (pH7.8)–10mM-$MgCl_2$–6mM-2-mercaptoethanol overnight at 4°C. Reaction mixtures (200μl) contained 10mM-Tris–HCl (pH7.4), 50mM-KCl, 10mM-$MgCl_2$, 6mM-2-mercaptoethanol, 0.8mM-GTP, 10mM-phosphoenolpyruvate, 8mg of pyruvate kinase, 40μg of poly(U), 0.26mg of dialysed post-microsomal supernatant fraction, approx. 0.4E_{260} unit of ribosomes and 5pmol (4200d.p.m.) of [^{14}C]phenylalanyl-tRNA (*Escherichia coli* K12). Incubation was at 37°C for 10min after which time 1ml of ice-cold buffer[10mM-Tris–HCl (pH7.4)–50mM-KCl–10mM-$MgCl_2$–6mM-2-mercaptoethanol] was added to terminate the reaction. The reaction products were then collected on Millipore filters (0.45μm) and washed with 15ml of the above buffer solution. This washing procedure was shown to be adequate. The filters were dried with an i.r. lamp and the amount of radioactivity retained was determined (Gilbert *et al.*, 1972). All calculations were corrected for non-specific radioactivity bound to filters.

Analysis of polyphenylalanine products by BD-cellulose chromatography

The distribution of oligopeptide products in the cell-free reactions was measured by BD-cellulose chromatography essentially as described by Gilbert *et al.* (1972) by the method of Pestka (1971). Each reaction was terminated by the addition of 2 drops of 1M-KOH and left at room temperature (22°C) for 5min. The mixture was neutralized with 1 drop of 1M-HCl, and potassium acetate buffer (0.05M, pH5.7) was added to bring the total volume to 1ml. After

addition of the samples, the BD-cellulose columns were washed in succession with 2ml of 0.05M-potassium acetate buffer, pH5.7; 3ml of formamide–ethanol–water (32:30:38, by vol.) and 2ml of 0.5M-KOH in 95% ethanol to obtain fractions of mono-, di- and oligo-phenylalanine respectively. The eluates were collected in 1ml fractions and the radioactivity in 0.2ml samples was determined. Also the resin from each column was added directly to scintillation fluid to count any radioactivity remaining after the final elution step (Gilbert *et al.*, 1972).

Heat inactivation of brain ribosomes

Thermal denaturation of brain ribosomal activity was achieved by preincubating approx. 4.0E_{260} units of ribosomes at 37°C in 0.4ml of 5mM-Tris–HCl (pH7.4)–5mM-KCl–0.75mM $MgCl_2$ (Grove *et al.*, 1974). Portions 40 μl of ribosomes were added to polyphenylalanine-synthesizing reaction mixtures after various times of preincubation.

Results

In order to demonstrate the sensitivity of rRNA to pancreatic RNAase, ribosomes were treated with 0.02μg of RNAase/ml for 10min at 0°, 22° and 37°C, and the rRNA was examined by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis. The RNA proved to be very sensitive to the dilute concentrations of RNAase with which the ribosomes were treated. The enzymic nature of the reaction was evident by the increased hydrolysis of rRNA at elevated temperatures (Fig. 1). It is also apparent from the gel scans that the 18S rRNA from the small subunit is more sensitive to the action of RNAase than is the 28S rRNA from the large subunit. This is consistent with an earlier observation in our laboratory (Johnson, 1973; Grove & Johnson, 1973).

One possible complication of assaying the activity of ribosomes after RNAase treatment was that the exposure of ribosomes to the temperatures used for RNAase treatment had an effect on the ability of ribosomes to participate in protein synthesis. For example, ribosomes preincubated at 37°C for 10min lose 40–50% of their original activity when tested in a polyphenylalanine-synthesizing system (Grove *et al.*, 1974). Thus to minimize the thermolability of ribosomal activity, which is independent of the state of the rRNA (Grove *et al.*, 1974), 22°C was selected as the temperature of all subsequent RNAase treatments. RNAase treatment of ribosomes under these circumstances resulted in rRNA hydrolysis but the effects of heat on subsequent ribosomal activity were minimized.

It was decided to see if RNAase treatment had affected the sedimentation properties of the ribosomes in which both the 18S and the 28S rRNA species were extensively degraded. In comparison with non-RNAase-treated control ribosomes, the sucrose-gradient profile of ribosomes exposed to RNAase for 10min at 22°C showed an increase in the proportion of monoribosomes (Fig. 2) as would be expected from the cleavage of mRNA by the RNAase. Yet there was little evidence of ribosome damage indicating that ribosomes appeared to retain their structural integrity even though their RNA had been extensively cleaved.

In both this report (Fig. 1) and a previous one (Johnson, 1973), we have shown that RNAase treatment of ribosomes results in the degradation of rRNA. However, we did not know the fate of the products of this enzymic digestion. To determine the amount, if any, of rRNA that was released from the RNAase-treated ribosomes, ribosomes were treated at 22°C for 30min in the presence and absence of RNAase. Subsequently, the ribosomes were pelleted by ultracentrifugation and compared for both RNA and protein content (Table 1). In spite of the fact that under these circumstances 98% of the rRNA exhibits some hydrolysis (Grove & Johnson, 1973), RNAase-treated ribosomes lost only 6% of their RNA content. Concomitant with the loss in RNA, 12% of the ribosomal proteins were released.

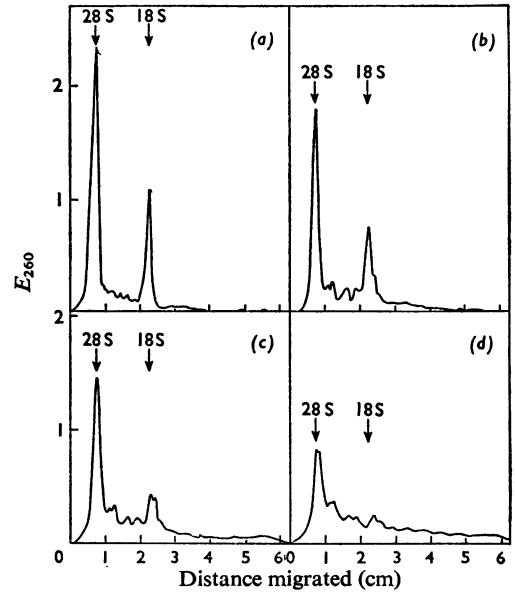

Fig. 1. *Enzymic hydrolysis of rRNA by pancreatic RNAase*

Approximately 1.0E_{260} unit of ribosomes was retained as native non-treated ribosomes (*a*) or preincubated in the presence of RNAase for 10min at 0°C (*b*), 22°C (*c*) or 37°C (*d*), and the RNA was analysed by polyacrylamide-gel electrophoresis. The direction of migration was from left to right and the arrows indicate the normal migration of 28S and 18S RNA.

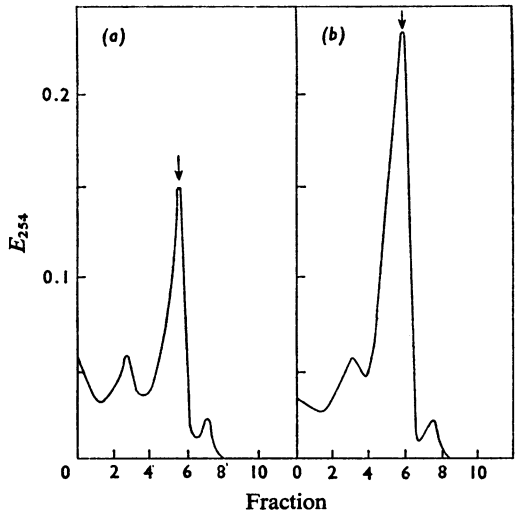

Fig. 2. *Effect of RNAase treatment on ribosomal sucrose-gradient profiles*

Native ribosomes (*a*) or ribosomes treated with RNAase for 10min at 22°C (*b*) were centrifuged through 5–20% (w/v) linear sucrose gradients and the E_{254} was recorded. The direction of sedimentation was from right to left and the arrow indicates the position of the 80S monoribosomes (Lerner & Johnson, 1970).

Table 1. *Effect of RNAase treatment on ribosomal components*

Approx. 13 E_{260} units of ribosomes/ml were incubated for 30min at 22°C in the presence or absence of 0.02μg of RNAase/ml as described in the Materials and Methods section. The ribosomes were immediately diluted with ice-cold 5% sucrose in RSB buffer, and pelleted by ultracentrifugation at 105000*g* for 2h at 4°C. The ribosomal pellet was resuspended in RSB buffer. The E_{260} was read to determine the amount of RNA associated with the pellet and a Lowry (1951) assay was performed to measure the protein content. The values are means of four determinations ±S.E.M.

	Ribosomal component	
Treatment	RNA (mg)	Protein (mg)
None (control)	0.50±0.01	0.60±0.03
RNAase	0.47±0.01	0.53±0.02

Thus it appears that the RNA–protein and protein–protein interactions within ribosomes are sufficient to maintain a major portion of the structural integrity of the ribosomes.

Since there was very little rRNA released from ribosomes treated with RNAase and the ribosomes retained their structural integrity on sucrose gradients, it was decided to ascertain the extent of hydrolysis and

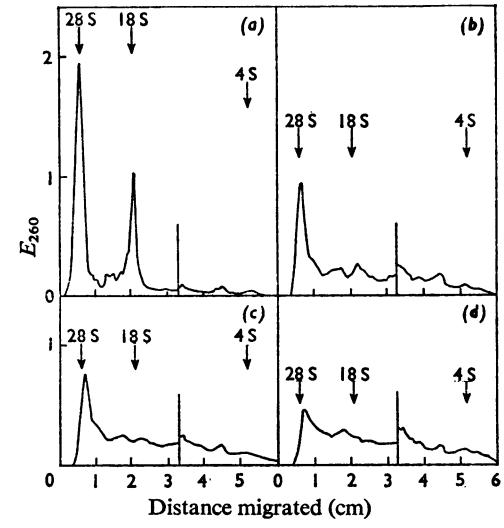

Fig. 3. *Resolution of rRNA from native and RNAase-treated ribosomes on discontinuous* 3–10% (*w/v*) *tandem polyacrylamide gels*

Ribosomes were exposed to RNAase at 22°C for 10(*b*), 20(*c*) and 30(*d*) min before the rRNA was resolved by 3–10% (w/v) tandem-gel electrophoresis. The direction of migration was from left to right with the 3% (w/v) acrylamide above the 10% (w/v) acrylamide. The centre line represents the junction of the two gel components. The arrows indicate the normal migration of 28S and 18S native (non-treated) brain rRNA (*a*) and *E. coli* tRNA that was used as the 4S RNA marker.

the sizes of the fragments produced. Discontinuous tandem gels composed of 3 and 10% (w/v) polyacrylamide were utilized for electrophoresis of rRNA from RNAase-treated ribosomes to retain the smaller fragments. After 20 and 30min of RNAase treatment at 22°C, the ribosomes contained little intact 18S and 28S RNA (Fig. 3). The concentration of 28S RNA remaining after RNAase treatment, exclusive of any hydrolysed fragments, was calculated with the aid of a compensating polar planimeter. By 20min of RNAase treatment less than 2% of the 28S RNA remained intact. This value for the percentage of 28S remaining is significant because it represents the maximum number of ribosomes containing intact rRNA. Moreover, in contrast with the discrete RNA fragments resulting from RNAase treatment of *E. coli* subunits observed by Hartman *et al.* (1970), RNAase treatment of eukaryotic ribosomes yielded a heterodisperse assortment of RNA fragments. Most of these heterogeneous fragments have relatively low molecular weights as indicated by the position of the 4S tRNA marker. This finding concurs with that of Delihas (1970) who also obtained a high percentage of small pieces of RNA from RNAase-digested *E. coli*

ribosomes by urea–Sephadex column chromatography.

Since these experiments were also designed to test whether or not intact rRNA was required for protein synthesis, it was important to know that the hydrolysed rRNA observed on polyacrylamide gels was actually cleaved by RNAase during the time of treatment. At this point the RNAase-treated ribosomes would be added to a polyphenylalanine-synthesis reaction mixture. Thus we have demonstrated that the addition of 0.2% sodium dodecyl sulphate before electrophoresis terminated any detectable enzymic action (Johnson, 1973). Also, to show the RNAase dependence of rRNA hydrolysis, ribosomes were incubated for 30min at either 22° or 37°C in the absence of exogenous RNAase. These conditions did not result in any detectable hydrolysis of rRNA (Grove *et al.*, 1974). To assure ourselves that heating the ribosomes did not result in 'hidden breaks' in the rRNA, reaction mixtures were adjusted to pH3, maintained at 0°C for 2min and then the sodium dodecyl sulphate was added (Cox, 1969). When the RNA was resolved by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis, the pH3-treated rRNA profiles from both 0°C control ribosomes and ribosomes incubated at 37°C for 30min were identical.

To ascertain the impact of rRNA degradation on ribosomal function, RNAase-treated ribosomes were assayed for their ability to participate in polypeptide chain elongation. After 10, 20 or 30min of exposure to RNAase at 22°C, ribosomes were added to a poly(U)-directed protein-synthesizing reaction mixture. Control ribosomes incubated at 22°C in the absence of RNAase were tested at each time-period so that any effects resulting from RNAase action could be distinguished from those due to heat inactivation. Even incubation at 22°C for 30min resulted in a loss of 22% of the original protein-synthetic activity (Table 2). Thus when ribosomes incubated with RNAase for 30min at 22°C are compared with the respective control ribosomes, they still retain 88% of their capacity to synthesize polyphenylalanine (Table 2). These results suggest that only a loss in activity of 12% could possibly be attributed to the hydrolysis of rRNA.

Since 0.8ng of RNAase was added to the protein-synthesis reaction mixtures along with the RNAase treated ribosomes, the possibility existed that RNAase may have inhibited protein synthesis by hydrolysis of the synthetic mRNA and tRNA. However, addition of such minute quantities of RNAase directly to reaction mixtures had no significant effect on protein synthesis (Table 2).

To substantiate the conclusion that ribosomes with hydrolysed rRNA are not functionally defective, another RNAase, T_1, was used to hydrolyse rRNA. T_1 RNAase was selected because it has a different specificity from pancreatic RNAase. Whereas pancreatic RNAase cleaves at pyrimidine nucleotides, T_1 RNAase cleaves at guanylate residues. The specificity of T_1 RNAase also insures that the poly(U)-template will not be degraded in the protein-synthetic reactions. Ribosomes were incubated with various concentrations of T_1 RNAase and the extent of rRNA hydrolysis and the subsequent ability of the ribosomes to synthesize proteins was measured. Even after exposure of ribosomes to 100 units of T_1 RNAase/ml for 20min at 22°C, when virtually no 28S or 18S RNA remained intact, the ribosomes were still 97% as active as control ribosomes (Table 3).

Table 2. *Effect of RNAase treatment on the ability of ribosomes to incorporate [^{14}C]phenylalanine into polypeptides*

Ribosomes were preincubated at 22°C in the presence or absence of 0.02μg of RNAase/ml. Samples were added to 0.2ml of cell-free protein-synthesizing reaction mixtures (described in the Materials and Methods section) which were then incubated at 37°C for 10min before determining the amount of [^{14}C]phenylalanine incorporated. Incorporation is expressed as a percentage of the control (no preincubation and without RNAase) ±S.E.M. Each value represents the mean of seven independent experiments. Approx. 4.3pmol/E_{260} unit of ribosomes were incorporated in these control reactions.

Time of preincubation (min)	[^{14}C]Phenylalanine incorporated	
	−RNAase (%)	+RNAase (%)
0 (control)	100	101±2
10	86±2	87±4
20	91±4	77±2
30	78±5	69±3

Table 3. *Effect of T_1 RNAase treatment on rRNA and on subsequent ribosomal activity*

Approx. 1.0E_{260} unit of ribosomes was incubated for 20min at 22°C in the absence or presence of the concentrations of T_1 RNAase indicated. Subsequently, the percentage of intact 28S rRNA was determined on sodium dodecyl sulphate–polyacrylamide-gel electrophoresis of the RNA. Ribosomes treated identically were also assayed in polypeptide-synthesizing reaction mixtures. Each value for pmol of phenylalanine incorporated represents the average of two experimental assays.

Activity of T_1 RNAase (units/ml)	Intact rRNA (%)	Phenylalanine incorporation (pmol/E_{260} unit)
0 (control)	100	6.56
10	56	6.84
20	33	7.14
50	8	7.02
100	0	6.49

Even though it appeared that prior RNAase treatment had little effect on the protein-synthetic activity of ribosomes, it was conceivable that the radioactive phenylalanine collected on the filters was not incorporated into peptides but was simply a reflexion of an increase in aminoacyl-tRNA binding to ribosomes as a consequence of RNAase treatment. Diphtheria toxin, an inhibitor of peptidyl translocase in eukaryotic cells, was used to investigate this possibility. The addition of diphtheria toxin to protein-synthetic reaction mixtures which contained ribosomes treated for 10, 20 or 30min with RNAase significantly decreased the phenylalanine that was apparently incorporated (Table 4). This diphtheria toxin-resistance level is comparable with the binding of phenylalanine to ribosomes as [^{14}C]phenylalanyl-tRNA (Gilbert *et al.*, 1972). Thus the incorporation previously measured with RNAase-treated ribosomes was clearly the result of active translocation and independent of any appreciable alterations in aminoacyl-tRNA binding.

Although RNAase treatment of ribosomes had little effect on the total pmol of phenylalanine incorporated, RNAase treatment might have altered the proportion of ribosomes actively participating in protein synthesis. It was possible to approach this problem by analysing the size distribution of the peptide products by BD-cellulose column chromatography. The results (Table 5) suggested only a slight overall decrease in the number of polypeptides being synthesized by ribosomes incubated with and without RNAase at 22°C for 30min compared with the synthesis by control ribosomes. However, there were no significant shifts in the distribution of peptides which

Table 4. *Effect of diphtheria toxin on [^{14}C]phenylalanine incorporation by RNAase-treated ribosomes*

After preincubation with RNAase (see the Materials and Methods section) for the lengths of time indicated, samples of ribosomes were added to either a normal protein-synthesis reaction mixture or one containing in addition 0.65μg of diphtheria toxin and 1.0mg of NAD$^+$. Incorporation of [^{14}C]phenylalanine was determined after incubation for 10min at 37°C.

Time of preincubation (min)	Presence of diphtheria toxin	[^{14}C]Phenylalanine incorporation (pmol/E_{260} unit)
10	−	4.44
	+	0.58
20	−	3.46
	+	0.77
30	−	3.32
	+	0.50

Table 5. *Analysis of protein-synthesis reaction products by BD-cellulose chromatography*

Approx. 1.0 E_{260} unit of ribosomes was preincubated under various conditions. Preincubated and RNAase-treated ribosomes were those exposed to 22°C for 30min in the absence and presence of RNAase, and native ribosomes were the non-treated controls. Double protein-synthetic reaction mixtures were incubated and the products were analysed. Of the 7900c.p.m. applied to each BD-cellulose column, more than 90% of the radioactivity was recovered from each reaction mixture. The results obtained from duplicate reaction mixtures are given.

	Ribosomes		
Products	Native (%)	Preincubated (%)	RNAase-treated (%)
$(Phe)_1$	49	53	55
	51	54	57
$(Phe)_2$	5	5	4
	3	5	3
$(Phe)_3$	12	11	12
	11	12	11
$(Phe)_n$	34	31	29
	35	29	29

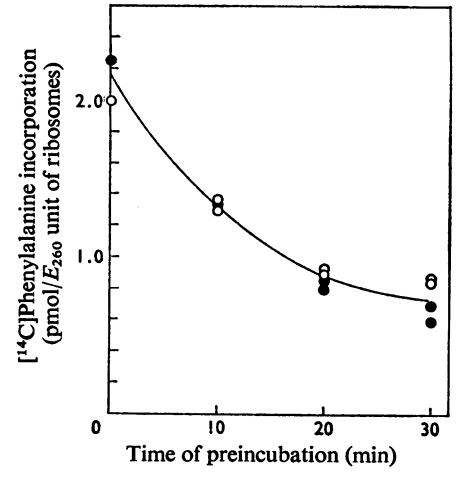

Fig. 4. *Thermostability of RNAase-treated ribosomes*

Ribosomes were treated with RNAase for 30min at 22°C and pelleted by ultracentrifugation (Table 1). The heat stability of RNAase-treated ribosomes (○) and control ribosomes (●) treated in the absence of RNAase was tested by preincubation of the ribosomes at 37°C for the times indicated as described in the Materials and Methods section. The ability of the heat-inactivated ribosomes to incorporate [^{14}C]phenylalanine was assayed by cell-free protein synthesis.

could be separated, indicating no major changes in the proportion of ribosomes engaged in protein synthesis.

One possible explanation which could account for the slight loss of protein-synthetic activity attributed to the degradation of rRNA is not that rRNA has a functional role in protein synthesis but that there is a structural function in the maintenance of the structural integrity of ribosomes necessary for protein synthesis. Since ribosomal activity has been shown to be relatively thermolabile (Grove *et al.*, 1974), RNAase-treated ribosomes were preincubated at 37°C for 10, 20 and 30min before assaying their ability to participate in protein synthesis. The conditions of this experiment necessitated the removal of the RNAase so that rRNA hydrolysis would not be continuing during the incubation at 37°C. Ribosomes treated at 22°C in the presence and absence of RNAase were immediately diluted with 9ml of ice-cold 5% (w/v) sucrose in RSB buffer, pelleted by ultracentrifugation, and then tested for their sensitivity to thermal denaturation. Not only did pelleting the RNAase-treated ribosomes not have an adverse effect on their activity, there was no apparent change in their thermal stability (Fig. 4).

Discussion

It is quite clear that the presence of all three species of rRNA is essential for the assembly and maturation of active ribonucleoprotein particles (Nomura, 1973). However, the necessity for intact rRNA subsequent to the formation of ribosomal subunits is still in question. The precise function(s), if any, of rRNA in polypeptide chain elongation has been recently under intensive investigation in several laboratories. In the present report we have exposed mouse brain ribosomes to dilute concentrations of pancreatic RNAase, which resulted in an extensive hydrolysis of rRNA, and measured the ability of the enzyme-treated ribonucleoprotein particles to participate in active protein synthesis. Although RNAase has been frequently used to determine the physical–chemical properties of ribosomal structure and to permit the identification of rRNA segments protected by proteins (Cox, 1969; Delihas, 1967), little information is available on the capacity of RNAase-treated ribosomes to participate in the various steps involved in protein synthesis.

There have been reports that suggested that intact rRNA is not a requisite for ribosomal function (Delihas, 1970; Szer, 1969; Kuechler *et al.*, 1972). However, other investigators have concluded that hydrolysis of rRNA does lead to a marked decrease in ribosomal activity (Cox, 1969; Spirin & Gavrilova, 1969; Hüvös *et al.*, 1970). The reasons for these differences may have been the result of technical problems and/or an incomplete analysis of the experimental data which have been discussed in a previous publication (Grove & Johnson, 1973).

We have found that although less than 1% of rRNA remains intact after exposure to RNAase, more than 85% of ribosomal-directed protein synthesis remained. These measured amounts of ribosomal activity were shown to be independent of any changes in aminoacyl-tRNA binding and clearly the result of active translocation. Possible alterations in aminoacyl-tRNA binding, as a result of RNAase treatment, were ruled out by the use of diphtheria toxin which specifically inhibits peptidyl-tRNA translocation while having little, if any, effect on aminoacyl-tRNA binding (Table 4). In addition, our finding that the size distribution of oligopeptides from both native and RNAase-treated ribosomes are similar (Table 5) clearly illustrated the ability of ribosomes, with extensively hydrolysed rRNA, to synthesize polyphenylalanine.

Examination of the structural integrity of RNAase-treated ribosomes showed that the monomers remained intact. A comparison of the sucrose-gradient-centrifugation profiles of native and RNAase-treated ribosomes showed an expected increase in the proportion of monoribosomes by exposure to dilute RNAase, but no apparent increase in either ribosomal subunits or soluble RNA–protein complexes (Fig. 2). This observation is consistent with previous studies that have shown that the structural integrity of RNAase-treated ribosomes is retained (Cox, 1969; Spirin & Gavrilova, 1969). When the RNA and protein content of RNAase-treated ribosomes was measured a maximum loss of 6 and 12% respectively was detectable (Table 2). Delihas (1970) and Kuechler *et al.* (1972) have observed a 5% loss in rRNA after exposure of bacterial ribosomes to RNAase, and Cox (1969) has found only a 20–30% loss of rRNA after extensive exposure of reticulocyte ribosomes to RNAase. Thus almost all of the extensively hydrolysed heterodisperse RNA products of RNAase digestion remain physically associated with ribosomal particles.

The maintenance of ribosomal activity and rRNA after extensive hydrolysis of the nucleic acid suggests that the protein–RNA and protein–protein interactions are sufficient to retain both ribosomal structure and their ability to participate in amino acid incorporation. In fact, we could not detect any increased lability of RNAase-treated ribosomes to either repeated centrifugation or heat inactivation.

Although we have clearly shown that 'intact' rRNA is not required for ribosomal-directed protein synthesis, the possibility that rRNA or the fragments may play a role in peptide chain elongation cannot be ruled out. Several lines of evidence have implicated the participation of one or more species of rRNA. For instance, the action of three antibiotics in bacteria (Helser *et al.*, 1972; Lai & Weisblum, 1971; Lai *et al.*,

1973) appears to be dependent on their interaction with rRNA. Mutations which lead to altered methylation patterns of the 16S or 23S rRNA provide resistance to the action of these antibiotics. However, such mutations in rRNA may alter the binding pattern or conformation of ribosomal proteins during ribosome assembly, which are ultimately the functional components of polypeptide chain elongation in mature ribosomal particles. Another indication of a functional role for rRNA comes from the inactivation of 30S subunits from *E. coli* treated with colicin E3 (Bowman *et al.*, 1971; Senior & Holland, 1971). The proposed mechanism of colicin E3 action involves the activation of a highly specific nuclease which cleaves the 16S RNA 50 nucleotides from its 3′ terminus.

Treatment of ribosomes with kethoxal, a chemical reagent shown to bind specifically to guanine residues of RNA, has been reported to result in a marked decrease in their ability to bind aminoacyl-tRNA (Noller & Chaires, 1972). Reconstitution experiments with the rRNA and proteins of normal and kethoxal-inactivated ribosomes suggested that the inactivation was primarily the result of a modified rRNA molecule. However, this evidence needs further investigation since more recent findings (Delihas *et al.*, 1973) have indicated that kethoxal also forms stable complexes with several 30S and 50S proteins.

Avadhani & Buetow (1973) have shown that although RNAase hydrolyses all species of rRNA no loss in *E. coli* ribosomal activity was observed until the 5S RNA was cleaved. However, the loss in activity when the 5S RNA was hydrolysed may have been circumstantial since the prolonged exposure that was required to permit the degradation of the 5S RNA molecules may have resulted in significant structural alterations in the ribonucleoprotein particles. Erdmann *et al.* (1973) have suggested that the 5S moiety of rRNA may play a role in aminoacyl-tRNA binding, although the tRNA binding affinity was higher in the presence of a 5S ribosomal protein complex. Unfortunately, the resolving power of the techniques used in the present study was not sufficient to measure the possible role of the 5S rRNA in brain cell ribosomal activity (Grove & Johnson, 1973).

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