Radioactive Labelling of Ribosomal Proteins with Reductive Alkylation and its Use in Studying Ribosome–Cytosol Interactions

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Mouse brain ribosomes were radioactively labelled by a cell-free reductive alkylation reaction with NaBH_4 and $[^{14}\text{C}]$ formaldehyde. The radioactivity was largely associated with ribosomal proteins, but little, if any, of the rRNA was radioactive after the alkylation procedure. Both ribosomal structural proteins and loosely associated components were successfully labelled by this procedure. The sedimentation properties of the ribosomes were unaltered and their ability to carry out poly(U)-directed protein synthesis, although decreased, was largely retained. Incubation of ^{14}C -labelled ribosomes with brain cytosol resulted in a 17% loss of radioactivity, although treatment of the ribosomes with 1.0M-KCl to remove the loosely associated factors rendered the ribonucleoprotein particles resistant to cytosol effects. The ribosome–cytosol interactions did not appear to be related to an exchange process, since the released radioactivity was largely degraded to acid-soluble material. In addition, the incubation of native ribosomes with brain cytosol resulted in an almost complete loss in the ability of the ribosomes to participate in cell-free protein synthesis.

It has been shown that the synthesis of ribosomal proteins in mouse L cells can take place in the absence of rRNA synthesis (Craig & Perry, 1971), but that the concomitant synthesis of rRNA is necessary for the incorporation of these proteins into mature ribosomes in both HeLa and L cells (Maisel & McConkey, 1971; Craig, 1971). The simultaneous synthesis of rRNA and structural ribosomal proteins in liver has been demonstrated *in vivo* (Tsurugi *et al.*, 1972). Hirsch & Hiatt (1966) reported that the half-lives of rRNA and structural proteins are similar, indicating that liver ribosomes are metabolized as a single functional unit. However, Dice & Schimke (1972) reported that, once liver ribosomes were formed, at least some of the proteins appeared to exchange rapidly with cytoplasmic pools of free ribosomal proteins. An active exchange of proteins between the ribonucleoprotein particles and the constituents of the cytoplasm could be of major importance in alterations of ribosomal activity that are related to development and cellular aging in several tissues (Lerner & Johnson, 1970; Henshaw *et al.*, 1971; Palacios & Sanchez de Jiménez, 1975).

A variety of cytoplasmic proteins can combine with the structural ribosomal particle and are termed apparent ribosomal proteins (Warner & Pene, 1966). Some investigators have reported that the removal of these loosely bound proteins results in a loss of any cytosol-mediated exchange of ribosomal proteins (Tsurugi *et al.*, 1974; Terao *et al.*, 1974). In the present

report we describe a procedure for the reductive alkylation of mouse brain ribosomes with $[^{14}\text{C}]$ -formaldehyde, which provides ribosomes of a high specific radioactivity. These radioactive ribosomes were used to measure the effects of cytoplasm on both structural and loosely associated ribosomal proteins. In addition to the exchange of ribosomal proteins, the effects of the alkylation procedure and the incubation of ribosomes with cytosol were assessed with regard to the activity of ribosomal-directed protein synthesis.

Materials and Methods

Materials

SCH:ARS (ICR), non-inbred Swiss albino mice were purchased from ARS/Sprague–Dawley, Madison, WI, U.S.A. $[^{14}\text{C}]$ Formaldehyde (56–85 mCi/mmol) and $[^3\text{H}]$ phenylalanine (16.1 Ci/mmol) were purchased from New England Nuclear, Boston, MA, U.S.A. RNAase*-free sucrose was purchased from Schwarz/Mann, Orangeburg, NY, U.S.A. All other chemicals were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.

Isolation of ribosomes and cytosol

Ribosomes were prepared from mouse brain tissue by the method of Lerner & Johnson (1970). Whole

* Abbreviation: RNAase, ribonuclease.

mouse brains were removed, washed with ice-cold RSB medium (10mM-KCl, 1.5mM-MgCl₂, 10mM-Tris/HCl, pH7.4) and homogenized with a glass/Teflon homogenizer. The homogenate was centrifuged at 8400g for 15min at 4°C to remove mitochondria, synaptosomes and cellular debris. The resulting post-mitochondrial supernatant fluid was then centrifuged at 140000g for 90min to pellet the microsomal fraction. The microsomal pellet was resuspended in 5% (w/v) sucrose/RSB medium, sodium deoxycholate was added to a final concentration of 0.25%, and the ribosomes were pelleted by centrifugation at 140000g for 2h at 4°C. The ribosomal pellet was then resuspended in RSB medium, the insoluble material was removed by low-speed centrifugation at 3500g for 10min, and the soluble material was carefully layered over 8ml of 5% sucrose/RSB medium and the ribosomes were pelleted at 200000g for 45min at 4°C. The resulting ribosomal pellets were stored at -70°C for 1-3 days.

The initial post-mitochondrial fraction (approx. 20ml) was dialysed overnight at 4°C against 200vol. of 10mM-Tris/HCl buffer (pH7.5) containing 10mM-2-mercaptoethanol and 10mM-MgCl₂ as described by Johnson *et al.* (1974). Batches of the dialysed cytosol, containing 4-5mg of protein/ml, were stored at -70°C for up to 3 months.

Protein concentrations were determined by the spectrophotometric method of Lowry *et al.* (1951), with bovine serum albumin as the protein standard.

Reductive alkylation of ribosomes

The radioactive labelling procedure used in this study was a modification of the technique reported by McMillen & Consigli (1974). Approx. 300 *E*₂₆₀ units of ribosomes were suspended in 1-2ml of 0.1M-sodium borate buffer (pH9.0). NaBH₄ was then added to a final concentration of 160µg/mg for ribosomal protein, immediately followed by the addition of 250µCi of [¹⁴C]formaldehyde (4.25µmol). The ribosomes were incubated at 0°C for 30min, after which they were partially purified by gel filtration through a column (1.5cm × 15cm) of Sephadex G-25 at 4°C with TKM buffer (50mM-Tris/HCl, pH7.2, 25mM-KCl, 5mM-MgCl₂). Approx. 1ml fractions were collected, those containing ribosomes were pooled and, to remove any remaining exogenous [¹⁴C]formaldehyde, the ribosomes were dialysed against TKM buffer at 4°C until no measurable radioactivity could be detected in the dialysis buffer. Portions (0.5-1.0ml) of the ¹⁴C-labelled ribosomes were layered over 8ml of 5% sucrose/TKM buffer and centrifuged at 200000g for 1h. The supernatant fluid was carefully decanted and the ribosomal pellets were stored at -70°C for 2-3 months without an appreciable loss of radioactivity or activity in cell-free protein synthesis.

To obtain ribosome preparations free of loosely

associated proteins, the final centrifugation step described above was carried out with 5% sucrose/TKM buffer made 1M with respect to KCl (Garrison *et al.*, 1972).

Protein synthesis in vitro

The pH5 enzyme was isolated and protein synthesis was measured essentially as described by Lerner & Johnson (1970). Each reaction mixture (0.55ml) contained 25µmol of Tris/HCl (pH7.4), 25µmol of KCl, 5µmol of MgCl₂, 20µg of pyruvate kinase, 7µmol of 2-mercaptoethanol, 5µmol of phosphoenolpyruvate, 2µmol of ATP, 0.2µmol of GTP, 100µg of poly(U), 4*E*₂₆₀ units of ribosomes, 1.5-2.0mg of pH5 enzyme and 1.0µCi of [³H]phenylalanine (0.19nmol). The reaction mixtures were incubated at 37°C for 30min and then reactions were terminated by precipitation of proteins by the addition of 0.5ml of ice-cold 10% (v/v) trichloroacetic acid. The proteins were repeatedly precipitated with 5% trichloroacetic acid (Lerner & Johnson, 1970), and the final precipitate was solubilized in 0.5ml of 0.15M-NaCl with 2 drops of 1M-NaOH. The amount of radioactivity in the acid-insoluble material was determined in a Beckman LS-100 liquid-scintillation system as described by Johnson (1968). The amount of [³H]-phenylalanine incorporated into acid-insoluble proteins was corrected for control samples which were incubated at 0°C for 30min and processed as described above.

Incubation of ribosomes with cytosol

Brain ribosomes were incubated with cytosol by a modification of the procedure described by Garrison *et al.* (1972). Ribosomes were resuspended in TKM buffer, and 2.0*E*₂₆₀ units were incubated with 4mg of brain cytosol in a total volume of 1.0ml at 22°C for 30min. After incubation, the reaction mixtures were layered on 8ml of 5% sucrose/TKM buffer and centrifuged at 200000g for 1h. Fractions (1ml) were collected from the sucrose solution and the total and acid-insoluble radioactivity in each fraction was determined as described above. The ribosomal pellet was resuspended in 1ml of TKM buffer, the *E*₂₆₀ was measured, and the ribosomal proteins were precipitated with 10% (w/v) trichloroacetic acid. The amount of acid-insoluble radioactivity was measured after collecting the resulting precipitates on Whatman GF/C glass-fibre filters.

Sucrose-gradient-centrifugation analysis

Ribosomes were resuspended in TKM buffer and layered on 3.8ml linear (0.7-1.5M) sucrose/TKM buffer gradients which were then centrifuged at 280000g for 72min in an International centrifuge, model SB 405 rotor, at 4°C as described by Taub & Johnson (1975). After centrifugation, the gradient was collected from the top with a peristaltic pump

through a Buchler Densiflow apparatus and the E_{254} was monitored continuously with an Isco model UA-2 u.v. analyser.

Polyacrylamide-gel electrophoresis

The rRNA was analysed on polyacrylamide gels as described by Grove & Johnson (1974). Ribosomes were resuspended in electrophoresis buffer (40mM-Tris/HCl, pH 7.2, 20mM-sodium acetate, 1.0mM-EDTA, 0.2% sodium dodecyl sulphate). Crystalline RNAase-free sucrose was then added to each sample to facilitate loading and 0.1 ml portions were electrophoresed on 3.15% polyacrylamide gels at 5 mA/gel for 90 min at room temperature (22°C). After electrophoresis, the gels were scanned at 260 nm with a Gilford model 2400 spectrophotometer equipped with a linear transport. After the positions of the RNA peaks were identified, the radioactivity in these areas of the gels was determined as described by Mathews *et al.* (1976).

Results

Proteins associated with ribosomes comprise two major classes: structural ribosomal proteins and loosely associated proteins. Loosely associated proteins are those that are dissociated from the ribosomes by 1.0M-KCl and include ribosomal-associated factors that participate in polypeptide synthesis as well as non-specifically bound proteins (Garrison *et al.*, 1972). Since our study was concerned with the efficiency of the labelling of ribosomal proteins in intact ribosomes with [^{14}C]formaldehyde *in vitro*, it was first necessary to ascertain the feasibility of labelling both classes of proteins. Brain ribosomes were incubated in sodium borate/ NaBH_4 buffer and [^{14}C]formaldehyde as described in the Materials and Methods section and, after gel filtration and dialysis, samples from the same alkylation reaction were pelleted by centrifugation. Labelled ribosomes were centrifuged at 200 000g for 1 h through either 5% sucrose/TKM buffer (native ribosomes) or through 5% sucrose/TKM buffer made 1.0M with respect to KCl (salt-washed ribosomes). After centrifugation the ratio of radioactivity to the E_{260} of the ribosomal pellets was measured. Native ribosomes had 234 pmol of [^{14}C]formaldehyde/ E_{260} unit and treatment of ribosomes with 1.0M-KCl resulted in an approx. 17% loss of radioactivity (194 pmol of [^{14}C]formaldehyde/ E_{260} unit). This is consistent with the proportion of ribosomal protein that has been reported as ribosomal-associated factors (Warner & Pene, 1966; Terao *et al.*, 1974). The retention of over 80% of the radioactivity by the KCl-washed ribosomes indicated that the structural proteins of the ribonucleoprotein particles had also been labelled with the [^{14}C]formaldehyde. Evidently both classes

of ribosomal proteins are efficiently labelled by this reductive alkylation procedure.

The possibility that at least a portion of the radioactive label was associated with rRNA, as well as with the ribosomal proteins, was also considered. To determine the amount of radioactivity associated with rRNA, [^{14}C]formaldehyde-labelled ribosomes were dissociated with sodium dodecyl sulphate and electrophoresed on 3.15% polyacrylamide gels as described in the Materials and Methods section. Under these electrophoretic conditions the ribosomal proteins would migrate off the gels (Johnson, 1973). Reductive alkylation of the brain ribosomes with [^{14}C]formaldehyde did not alter the electrophoretic mobility of the rRNA or cause any measurable breaks in the nucleic acids, and less than 0.2% of the total ribosomal radioactivity was associated with the 28 S and 18 S rRNA molecules (Fig. 1). Therefore the

Fig. 1. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of rRNA

Ribosomes were alkylated with [^{14}C]formaldehyde as described in the Materials and Methods section and approx. 12000 c.p.m. of radioactive ribosomes were electrophoresed on sodium dodecyl sulphate/polyacrylamide gels in duplicate. After electrophoresis the gels were scanned at 260 nm, the gels were sliced into 1 mm sections, and the radioactivity in rRNA was determined. Less than 10 c.p.m. of ^{14}C was found in each of the areas indicated by brackets.

ribosomal components primarily labelled by the alkylation procedure appeared to be the loosely associated and structural ribosomal proteins.

It was possible that the reaction conditions necessary for efficient reductive alkylation (i.e. pH 9.0), or the alkylation itself, may have altered the structure or integrity of the ribosomal particles. To assess this possibility, ribosomes that had been treated with formaldehyde were analysed by sucrose-gradient centrifugation. Samples of brain ribosomes were alkylated as described above, except that an equimolar concentration of [^{12}C]formaldehyde was added to the reaction mixtures in the place of the radioactive substrate. Control ribosomes were incubated for the same length of time in TKM buffer. After incubation the ribosomes were isolated by gel filtration and dialysis and the sedimentation profiles were examined on linear sucrose/TKM buffer gradients as described in the Materials and Methods section. Gradient profiles of both control ribosomes and ribosomes treated with formaldehyde under alkylating conditions were similar and consisted of a relatively large peak at the 80 S region of the gradient, as reported for brain ribosomes isolated by these methods (Lerner & Johnson, 1970). These results indicated that the reductive alkylation did not significantly affect the structural integrity of the ribosomal particles.

^{14}C -labelled ribosomes were then used to determine the effect of cytosol on the stability of the ribonucleoprotein particles and to ascertain the possibility that an exchange of cytoplasmic and ribosomal proteins could occur. ^{14}C -labelled ribosomes were either maintained in TKM buffer (native ribosomes)

or treated with 1.0 M-KCl (salt-washed ribosomes) before their incubation with brain cell cytosol. The ribosomes were incubated with 4 mg of cytosol protein at 22°C for 30 min, after which the entire reaction mixture was layered over 8 ml of 5% sucrose/TKM buffer, and the ribosomes were pelleted by centrifugation at 200 000 g for 1 h. Fractions (1 ml) were then carefully removed from the top with a Pasteur pipette and the acid-soluble and acid-insoluble radioactivity in each fraction and the ribosomal pellet were measured and compared with native and salt-washed ribosomes that were incubated in the absence of brain cell cytosol. The incubation of the salt-washed ribosomes with brain cytosol resulted in an approx. 5% loss of radioactivity compared with salt-washed ribosomes incubated with TKM buffer alone (Fig. 2a). However, the incubation of native ribosomes with cytosol resulted in an approx. 20% loss of radioactivity (Fig. 2b). These data suggested that the ribosomal proteins most sensitive to interactions with brain cytosol were the loosely associated factors. More importantly, when the top 1 ml fractions were precipitated with 10% trichloroacetic acid, in the presence of 0.5 mg of bovine albumin as a carrier, less than 5% of the released radioactivity was acid-insoluble. This was in marked contrast with the intact ribosomes, where at least 95% of the radioactivity was acid-insoluble. These results suggested that the radioactive proteins released from the ribosomes by cytosol were not the result of a cytosol-ribosome exchange, but rather the consequence of proteolysis.

To determine if this apparent proteolysis would be

Fig. 2. Distribution of radioactivity after the incubation of ^{14}C -labelled ribosomes with cytosol

Radioactively labelled salt-washed (a) and native (b) ribosomes were incubated with brain cytosol as described in the Materials and Methods section. The entire incubation mixtures were then layered over 8 ml of 5% sucrose in TKM buffer and the ribosomes pelleted by centrifugation. The radioactivity was determined for the pellets and each 1 ml fraction collected. Results are expressed as the percentage of the total recovered radioactivity (efficiency of recovery, 97%): ■, incubated without cytosol; ▨, incubated with cytosol.

Table 1. *Effect of reductive alkylation and incubation with brain cytosol on brain ribosome activity*

Native ribosomes were reductively alkylated as described in the Materials and Methods section. The results are expressed as the means of the experiments $\pm$ S.E.M. with the numbers of independent experiments indicated in parentheses.

Ribosomes	[³ H]Phenylalanine incorporated (pmol/ <i>E</i> ₂₆₀ unit)	Relative activity (%)
Native (control)	0.95 $\pm$ 0.08 (5)	100
Alkylated	0.54 $\pm$ 0.06 (6)	57
Alkylated + cytosol	0.16 $\pm$ 0.02 (5)	17

reflected by a decreased ribosomal activity in protein synthesis, formaldehyde-treated ribosomes were incubated with brain cytosol and then added to poly(U)-directed protein synthesis reaction mixtures. Formaldehyde treatment alone resulted in some loss of ribosomal activity (Table 1). This decrease in activity may have been the result of the incubation conditions necessary for maximum alkylation, but not necessarily the result of formaldehyde treatment. In subsequent studies we were able to show that ribosomes reduced with NaBH₄ at pH 9.0, without the addition of formaldehyde, underwent a similar decrease in protein-synthetic activity (approx. 40%; results not shown). However, ribosomes incubated with cytosol were almost completely inactivated (Table 1). Evidently the proteolytic activity resulted in a considerable alteration of ribosomal activity, although the structural integrity of the ribonucleoprotein particles may have been little altered.

Discussion

Although ribosomal structure and function have been studied widely, many investigations have been hindered by the difficulty in obtaining radioactively labelled ribosomes with a high specific radioactivity. To this end, we have adapted a procedure for the alkylation of brain ribosomal proteins *in vitro* with NaBH₄ and [¹⁴C]formaldehyde, as described by Means & Feeney (1968), which has been used to radioactively label viral proteins (McMillen & Consigli, 1974) and bacterial ribosomes (Moore & Crichton, 1973, 1974). This technique provides a rapid and relatively simple method for obtaining ribosomes with a relatively high specific radioactivity. The amount of radioactivity per ribosomal unit was greater than that reported by investigators who utilized techniques for labelling *in vivo* (Dice & Schimke, 1972; Menzies & Gold, 1972; Terao *et al.*, 1974). This alkylation procedure labelled both the structural and loosely associated ribosomal proteins,

although little, if any, of the [¹⁴C]formaldehyde was associated with the major rRNA molecules. This is consistent with the observations of Moore & Crichton (1973), who used a similar procedure to radioactively label *Escherichia coli* ribosomal proteins. Analyses of the alkylated ribosomes by sucrose-gradient centrifugation illustrated that the structural integrity of the ribosomes was not significantly altered by their incubation with formaldehyde and ribosomal-directed protein synthesis was only partially inhibited. In fact, the decrease in ribosomal protein synthesis was not the result of incubation with formaldehyde, but rather of the incubation conditions in sodium borate buffer and NaBH₄ at pH 9.0, which is necessary for efficient alkylation (Means & Feeney, 1968). We also considered the possibility that the incubation of ribosomes with formaldehyde resulted in a fixation of the ribosomal proteins. However, Cox (1969) has shown that these concentrations of formaldehyde at 0°C do not result in ribosomal-protein fixation. In the light of these observations, the alkylation procedure may prove to be applicable to numerous types of studies concerning both functional and structural properties of ribosomes from both prokaryotic and eukaryotic cells.

When radioactively labelled brain ribosomes were incubated with brain cytosol preparations, a significant proportion of the radioactivity was released (Fig. 2). However, the radioactivity that was released from native ribosomes was acid-soluble and no longer associated with macromolecules (Fig. 2), which suggested that the release of radioactive components from the brain ribosomes was most likely related to proteolysis. When cytosol-treated ribosomes were tested for protein synthesis in cell-free reaction mixtures, most of their activity had been lost. These results are interesting in light of the suggestion by Dice & Schimke (1972) that the incubation of liver ribosomes with cytosol led to a significant loss of radioactivity from the ribosomes. However, they concluded that their observations were the result of an exchange of ribosomal proteins with pools of these ribosomal proteins in the cell cytoplasm. It appears likely that at least some of the exchanged ribosomal proteins may have been the result of proteolytic activity of the liver cytosol.

When ribosomes were treated with 1.0M-KCl, to remove the loosely associated proteins, before their incubation with brain cytosol, the ribonucleoprotein particles appeared to be relatively resistant to any loss of radioactivity (Fig. 2). This suggested that the loss of radioactive protein that was mediated by brain cytosol was primarily related to the loosely associated factors and not the structural components. These observations are consistent with the report of Östner & Hultin (1968), who showed that these proteins are relatively sensitive to proteolytic attack and that proteolysis results in a loss of ribosomal

activity. However, at this time we cannot rule out the possibility that even structural proteins underwent proteolysis and that the resultant peptides remained associated with the ribonucleoprotein particles by the strong RNA-protein interactions that have been shown to confer structural stability to brain ribosomes (Grove & Johnson, 1974).

Our results are compatible with those of Terao *et al.* (1974) and Subramanian (1974), who reported a lack of exchange of ribosomal proteins with cytoplasmic preparations from both prokaryotic and eukaryotic cells. Wool & Stöffler (1974), using immunoprecipitation with antibodies prepared to ribosomal proteins, also failed to detect significant pools of free ribosomal proteins in cell cytoplasm. Of course, it was possible that our results reflected a proteolytic degradation of the ribosomal-associated proteins soon after their dissociation from the ribosomal particles. However, a true exchange would not be expected to alter the ribosomal activity since the ribosomal components that were lost should have been replaced by proteins from the cytoplasm. It is clear that the loss of ribosomal activity after the incubation of native ribosomes with cytosol indicates that at least some of these proteins are important in ribosomal protein synthesis. These ribosomal-associated proteins, which include several initiation and elongation factors (Garrison *et al.*, 1972; Levin *et al.*, 1973; Gilbert & Johnson, 1973), have been suggested to be critical in the expression of ribosomal activity in mammalian cells (Henshaw *et al.*, 1971; Rowley *et al.*, 1971). An instability of ribosomal-associated factors to cytoplasmic components may be related to alterations in ribosomal activity during various stages of development and aging (Lerner & Johnson, 1971; Rowley *et al.*, 1971; Menzies & Gold, 1972; Palacios & Sanchez de Jiménez, 1975). A maturation-dependent change in either ribosomal proteins and/or cytoplasmic components could lead to an alteration in ribosomal capacity to participate in polypeptide metabolism.

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