

A Detergent-Free Method for Isolation of Viable Ribosomes from Rat Brain

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A detergent-free method based on sucrose density gradient centrifugations was developed for isolation of free ribosomes from rat brain. The purity of the preparations was checked by RNA/protein ratio, electron microscopy and marker enzymes. They were uncontaminated by other cell organelles. The incorporation of leucine, isoleucine and valine into acid-insoluble fraction by ribosomes conformed to the Hill equation $v/V = s^n / (K + s^n)$. Branched-chain amino acids mutually inhibited each other's incorporation by ribosomes, but the inhibition was abolished with detergents frequently used in isolation procedures.

The most common methods of ribosome isolation from brain are based on deoxycholate treatment liberating membrane-bound polysomes.¹⁻¹⁰ The method is also applicable for micro-scale preparations.¹¹ Ribosomes can be released from membrane protein also by salt-washing.^{12,13} Diethyl pyrocarbonate, bentonite and heparin^{3,14,15} have been used to inhibit ribonucleases, preferably in combinations.¹⁶

Very few detergent-free methods based on density gradient centrifugations are reported.^{17,18} Bloemendal *et al.*¹⁹ have developed a detergent-free method for isolation of membrane-bound and free ribosomes from rat liver. The method is applicable for isolation of polysomes also from other tissues, but ribonuclease inhibitor, a glycoprotein from rat liver, should be added to the preparations. The method of Andrews and Tata,¹⁷ based on density gradient centrifugations, is a detergent-free method without the liver extract. This was initially used but we obtained a fairly poor yield of ribosomes with this method.

Substantial modifications of the method were found necessary. A detergent-free means of isolating ribosomes has to be used in some of our experiments on amino acid incorporation into brain protein. Branched-chain amino acids have been shown to inhibit each other's incorporation into acid-insoluble fraction in brain homogenates.^{20,21} This inhibition is discernible also in cell-free systems, but detergents abolish the phenomenon.

MATERIAL AND METHODS

Preparation of ribosomes The major modifications to the method of Andrews and Tata¹⁷ were as follows: The Mg^{2+} concentrations were higher in our method, pH was 7.4 throughout the procedure, the sucrose gradients were altered, filtering through a sieve was added and centrifugal forces increased. The final procedure was the following: The rats were decapitated and the brains prepared and homogenized in a motor-driven Potter-Elvehjem homogenizer with 3.5 vol. of ice-cold Tris-HCl buffer, pH 7.4, containing 50 mM Tris-HCl, 36 mM $MgCl_2$, 0.1 M KCl and 0.25 M sucrose. The Teflon pestle was pressed down three times at 800 rev./min. The homogenate was filtered through a 53 μm nylon sieve and centrifuged twice at 10,000 g_{av} for 10 min. Seven ml of the supernatant was layered on 3.25 ml of 0.7 M sucrose containing 25 mM KCl, 30 mM $MgCl_2$ and 50 mM Tris-HCl buffer, pH 7.4. The gradients were kept at 277 K for 1 h and centrifuged at 105,000 g_{av} for 2 h. The pellets were dissolved in 20 ml of homogenizing buffer and layered on 0.9 M sucrose solution (5 ml of

ribosome solution and 11 ml of sucrose) containing 0.1 M KCl, 36 mM MgCl₂ and 50 mM Tris-HCl buffer, pH 7.4. After centrifuging the gradients at 65,000 g_{av} for 16 h the final pellets were dissolved in 2 ml of homogenizing medium and used for amino acid incorporation experiments.

In some isolation procedures 1 mM 1,4-dithio-DL-threitol and 10 g/l Triton X-100 were added to the homogenizing medium and the post-mitochondrial supernatant mixed with 10 g/l deoxycholate for 15 min. In these experiments the MgCl₂ concentration was reduced to 5 mM. The method of Hall and Lim² was also used for purification of ribosomes.

Inhibition of ribonucleases. Method 1: 1 g/l diethylpyrocarbonate (DEP) was added to the homogenizing medium. Method 2: All glassware was autoclaved and polyethylene tubes were treated with 1 g/l DEP in 310 K for 12 h. DEP was completely removed from tubes by heating prior to the experiments. Method 3: Bentonite was added to the homogenizing medium.

Marker enzymes. Lactate: NAD⁺ oxidoreductase (EC 1.1.1.27) was used as an enzymatic marker for cytoplasm, citrate oxaloacetate lyase (EC 4.1.3.7) for mitochondria,²² NADPH: ferricytochrome oxidoreductase (EC 1.6.2.4) for microsomes²³ and acetylcholine acetylhydrolase (EC 3.1.1.7) for synaptic membranes.²⁴

Electron microscopy. The ribosomes were fixed with glutaraldehyde and embedded in DMP-Epon according to Whittaker and Baker.²⁵ Sections were cut with an LKB-8800 Ultratome® III using glass knives and stained with uranyl acetate. Stained preparations were examined with a Jeol Jem-100 C electron microscope.

Protein determination. Protein was determined by the method of Lowry *et al.*²⁶ using bovine serum albumin as reference. It was assumed that an albumin solution of 10 g/l gives an absorbance of 6.6 at 280 nm in 1 cm path length.

RNA determination. The RNA content was calculated from the phosphorus measurements according to Ames,²⁷ using Na₂HPO₄ · 12H₂O as standard and presuming that RNA contains 9.13 % phosphorus.²⁸ RNA was routinely determined according to Ragnotti,²⁹ but the low recovery of RNA in this method, about 53 %, was corrected.

Absorption spectrum. The absorption spectrum of ribosome preparation was measured with a

Unicam SP 1800 spectrophotometer at wavelengths of 200–300 nm.

Cell sap. The cell sap was prepared by the method of Andrews and Tata.¹⁷

Incorporation of amino acids. The incubation mixture contained in 1 ml 50 μmol Tris-HCl, 100 μmol KCl, 36 μmol MgCl₂, 10 μmol 2-mercaptoethanol, 3 μmol ATP, 1 μmol GTP, 5 μmol creatine phosphate, 10 μg creatine kinase (EC 2.7.3.2.), 50 nmol each of the protein amino acids, 74 kBq tritium-labelled valine, leucine or isoleucine and cell sap containing 2 mg protein. The reaction was started by adding ribosomes (150 μg RNA) to prewarmed (310 K) incubation medium. Some incubations were done without ribosomes and some with unlabelled branched-chain amino acids in excess and/or 10 g/l Triton X-100 and 0.5 g/l deoxycholate. After 30 min, ice-cold trichloroacetic acid (50 g/l), containing 0.5 g/l unlabelled valine, leucine or isoleucine, was added to the mixture. In control zero samples the acid was added before the ribosomes. The precipitate was washed once with warm (353 K), once with cool (298 K) trichloroacetic acid, ethanolether (1/1, v/v) and ether. The precipitates were dried overnight in a vacuum desiccator at room temperature. The protein samples were dissolved in 0.5 ml of 1 M NaOH, 45 μl of concentrated HCl and 4.5 ml of liquid scintillation cocktail being added. The radioactivity was determined with an LKB-Wallac 81000 liquid scintillation counter. For sample standardization the method of external standardization with channels ratio was used.

The incorporation rates (v) at varying concentrations (s) of the precursor amino acids in the incubation medium were analyzed with a digital computer Honeywell 1644, assuming that the complex mechanism of protein synthesis approximately conforms to the Hill equation $v/V = s^n / (K + s^n)$. Parameters V , n and K were determined with unweighted experimental data using Marquardt's algorithm ('maximum neighbourhood method').³⁰

RESULTS

Purification of ribosomes. The yield of RNA in different isolation procedures varied between 540–890 mg/kg brain fresh weight. The mean of 17 isolations was 666 ± 98 (S.D.) mg RNA/kg.

Table 1. The relative activities of marker enzymes during ribosome isolation.

Preparation	Lactate:NAD ⁺ oxido- reductase	Citrate oxalo- acetate- lyase	NADPH:ferri- cytochrome oxido- reductase	Acetyl- choline acetyl- hydrolase
Homogenate	100.0	100.0	100.0	100.0
Precipitate of 10 000 g	54.1	63.9	63.8	90.0
Supernatant of 10 000 g	43.8	3.6	25.8	9.4
Pellets on 0.9 M sucrose	2.9	0.6	2.8	2.0
Ribosomes	0.7	0.1	1.4	0.4

The RNA/protein ratio was 1.0. The ultraviolet absorption maxima were at 213 and 260 nm and the absorption minimum at 240 nm. The A_{260} to A_{240} ratio was 1.2 and A_{260} to A_{280} ratio 1.6.

Table 1 indicates the activities of the marker enzymes during purification. The activities of microsomal, synaptic and evidently also of mitochondrial enzymes were removed to a large extent during the first two centrifugations. During this step the mitochondrial enzyme was partly inactivated. The cytoplasmic and microsomal enzymes were also largely removed after the first sucrose gradient. Of the initial activity of the microsomal marker 1.4 per cent remained in the ribosomal fraction. Electron microscopy showed the pure ribosomal fraction to be free of other cell organelles (Fig. 1).

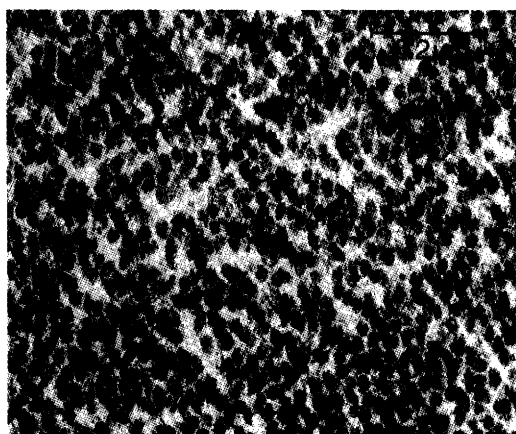


Fig. 1. Electron micrograph of isolated ribosomes. Calibration bar 0.2 μ m.

Attempts to inhibit ribonucleases by different methods were not satisfactory. The yield of ribosomes by the inhibition method 1 was high, but the incorporation of amino acids into the acid insoluble fraction by these ribosomes was hampered. Methods 2 and 3 failed likewise to increase the ribosomal yield.

The yield of free ribosomes isolated after the Triton X-100 and deoxycholate treatments was considerably smaller (about 180 mg RNA/kg brain fresh weight) than that by our method. The method of Hall and Lim² gave a good yield of ribosomes, but the incorporation of branched-chain amino acids into the acid insoluble fraction was inhibited and the mutual inhibition among them nearly abolished (data not shown).

Incorporation of leucine, isoleucine and valine. The incorporation of branched-chain amino acids into the acid-insoluble fraction by ribosomes

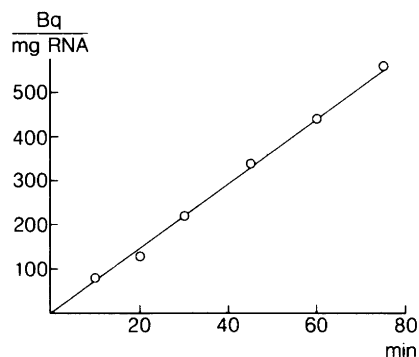


Fig. 2. Time course of incorporation of 7.5 μ M of leucine into the acid insoluble fraction by brain ribosomal preparations.

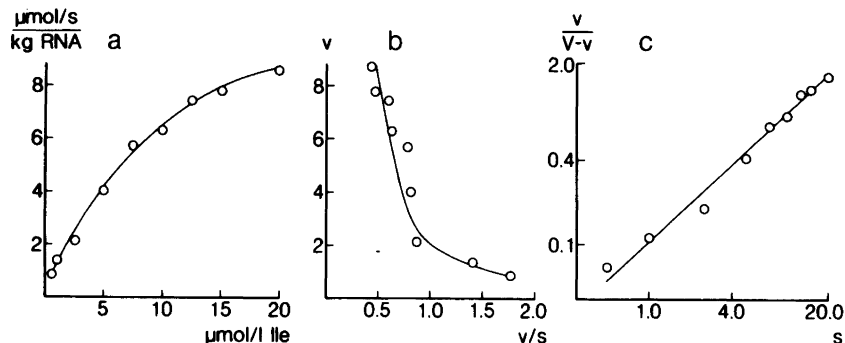


Fig. 3. (a) Incorporation of isoleucine into the acid insoluble fraction by brain ribosomal preparations. (b) Eadie-Hofstee and (c) Hill plots of the incorporation data.

isolated by our method was constant for at least 75 min, as shown for leucine in Fig. 2. The incorporation (Fig. 3a) did not obey Michaelis-Menten kinetics (Fig. 3b) but the rather more complex Hill equation (Fig. 3c), as demonstrated with isoleucine, for example. The parameters of the Hill equation for each branched-chain amino acid are compiled in Table 2. The maximal incorporation rates were about 9 nmol/s per kg RNA (Table 2). Isoleucine and leucine in excess mutually inhibited each other's incorporation by decreasing V . Valine did not significantly affect the incorporation of either leucine or isoleucine except for increases in constant n . The incorporation of valine was inhibited both by leucine and by isoleucine, but no statistically significant changes could be demonstrated in the calculated kinetic parameters under the present test condi-

tions. Triton X-100 and deoxycholate in incubation medium markedly inhibited the incorporation of leucine, isoleucine and valine into the acid insoluble fraction and diminished or entirely abolished the mutual inhibition in their incorporation (Table 3). If the detergents were added at the beginning of the purification procedure their effect on amino acid incorporation was less. Cell sap alone had a minor incorporation capacity, while no mutual inhibition was seen then among the branched-chain amino acids (data not shown).

DISCUSSION

Purification of ribosomes. Our method is easy and inexpensive but time-consuming and requires

Table 2. Hill parameters of the incorporation of leucine, isoleucine and valine into acid insoluble fraction in control experiments and in the presence of 750 μM of another branched-chain amino acid.

Amino acid incorporated	Amino acid in excess	V nmol/s per kg RNA	n	K μM
leucine	none (5 expts) ^a	9.6±1.1	0.98±0.04	5.8±1.2
leucine	isoleucine	4.9	1.23	6.7
leucine	valine	9.1	1.26	5.4
isoleucine	none (6 expts) ^a	8.8±1.5	0.96±0.04	21.4±7.0
isoleucine	leucine	4.6	0.92	23.9
isoleucine	valine	7.9	1.16	26.4
valine	none (4 expts) ^a	9.2±1.9	0.95±0.03	33.3±8.3
valine	leucine	6.3	1.08	44.8
valine	isoleucine	5.9	1.04	8.7

^a Means±SEM.

Table 3. Inhibition of amino acid incorporation into acid insoluble fraction by Triton X-100 and deoxycholate and by an excess of another unlabelled branched-chain amino acid.

Amino acid in excess	Relative incorporation without/with detergents ^a					
	Valine		Leucine		Isoleucine	
	without	with	without	with	without	with
none	100	69	100	73±1	100±12	83±4
leucine	80±8	83±11			71±5	68±3
isoleucine			50±1	68±11		

^a Means of two or three experiments±SEM. Concentrations: Amino acid incorporated 20 μM (valine and isoleucine) or 8 μM (leucine), amino acid in excess 9.75 mM, Triton X-100 10 g/l and deoxycholate 0.5 g/l.

fast processing during the first stages. It is suitable for amino acid incorporation studies since no detergents are used that could be inhibitory or destroy any of the regulatory systems in protein synthesis. Triton X-100 and deoxycholate are widely used in the isolation of polysomes, but in our incorporation assays they were harmful.

Ribosomes are very sensitive to ribonucleases, but our yield of ribosomes was reasonably good in spite of the absence of ribonuclease inhibitors. The most common inhibitors of RNases are bentonite and DEP. Bentonite adsorbs protein such as RNase but it also adsorbs RNA. It is not very efficient at the physiological pH used in our experiment.¹⁵ The half-life of DEP in Tris-HCl buffer is very short at physiological pH, but evidently long enough to destroy the amino acid incorporation apparatus. Free ribosomes make a smaller proportion of total ribosomes.¹⁸ Even though the amount of membrane-bound ribosomes decreases and that of free ribosomes increases during development,² in the adult brain only 40 % of the polysomes are found in the postmitochondrial supernatant fraction.³¹ Eukaryotic ribosomes contain almost equal amounts of protein and RNA.²⁸ Thus the RNA/protein ratio should be 1.0 as in our preparation. Electron microscopy and the A_{260} to A_{280} ratio of 1.6 also indicates good ribosomal purity. Enzyme determinations show a minor contamination with the microsomal fraction.

Incorporation of leucine, isoleucine and valine. The amino acid incorporation was fitted by the Hill equation as in our earlier studies on brain homogenates,^{20,21} in which the maximal incorporation rate was also diminished by an excess of another branched-chain amino acid. The present

inhibition could not be accounted for by any transport effects. Neither could it be due to diminished aminoacylation of tRNA as in inhibition of incorporation by phenylalanine in excess,^{32,33} because no inhibition was detected in cell sap by an excess of another branched-chain amino acid. The mutual inhibition of incorporation into the acid insoluble fraction by the branched-chain amino acid was fully ribosome-dependent and detergent-sensitive. Phenylalanine influences the formation of brain polysomes, their stability, polypeptide chain initiation and elongation.³⁴⁻³⁷ These reactions may also be involved in the present inhibition with branched-chain amino acids.

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