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ISOLATION AND CHARACTERIZATION OF MEMBRANE-BOUND RIBOSOMES FROM NERVE CELL BODIES OF IMMATURE RAT BRAIN-CORTEX

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SUMMARY: Free and membrane-bound ribosomes were isolated from neuronal perikarya of the immature rat brain-cortex. The two topographic forms of ribosomes were essentially free of contaminating organelles as shown by RNA, protein and marker enzyme analysis. Membrane-bound ribosomes amount to about a quarter of the total ribosomal population in neuronal perikarya. Both forms of ribosomes efficiently carried out cell-free protein synthesis but the membrane-bound fraction was more active than the free ribosomes.

INTRODUCTION

In the nerve cell body the existence of cytoplasmic basophilia of Nissl substance, which consists of the cisternae of the rough endoplasmic reticulum, is now well established (1,2). Although there are numerous studies on free and membrane-bound ribosomes of whole brain tissue (3) and of cerebral cortex (4,5) there is as yet no successful attempt to quantitatively isolate the two topographic forms of ribosomes from neuronal perikarya.

In the present study we have established the conditions for the preparation of essentially pure and highly active free and membrane-bound ribosomes from bulk isolated nerve cell bodies of the immature rat cerebral cortex. The method reported here could prove useful in studies aimed at the elucidation of the role of endoplasmic reticulum in neuronal protein synthesis.

MATERIALS AND METHODS

Eight-day-old rats of the Wistar strain were used throughout the study. Pactamycin was a generous gift from Upjohn Co. (U.S.A.). The source of the rest of the materials, including those used for the isolation of nerve cell bodies, has been described elsewhere (6,7).

Neuronal cell bodies from cerebral cortex were prepared as described earlier (7) except that polyvinylsulphate (4 µg/ml) was included in the isolation medium to inhibit ribonuclease activity. Neuronal perikarya from 15 cortices were pooled and homogenized in a tight fitting Potter-Elvehjelm type homogenizer in medium A (3 ml/g wet cortex, 10 up and down strokes) which

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consisted of 0.25 M sucrose, 20 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 4 µg/ml of polyvinylsulphate and 0.5 mM EGTA. The neuronal homogenate was then centrifuged at 10 000 x g for 10 min to obtain a nuclear-mitochondrial pellet and a post mitochondrial supernatant fraction.

A close examination of the distribution of RNA in subcellular fractions from rat liver (8-10) and mouse brain (11) showed that even when a relatively low gravitational force is applied to the tissue homogenate, a considerable amount of membrane-bound RNA sediments with the nuclei and mitochondria. It was therefore decided to establish conditions for a maximum recovery of ribosomal RNA from the nuclear-mitochondrial fraction without an extensive breakage of other subcellular structures. This was accomplished by repeated washing of the nuclear-mitochondrial pellet with buffered sucrose (cf. 10) containing 4 µg/ml of polyvinylsulphate. The supernatant fraction obtained from each wash was combined with the initial 10 000 x g supernatant fraction and the ionic conditions of the pooled fractions were adjusted to that of medium B (0.25 M sucrose, 20 mM Tris-HCl (pH 7.5), 12 mM $MgCl_2$ and 100 mM KCl). Following centrifugation of the combined supernatant fractions, the neuronal microsomes were obtained as a pellet. The high-speed supernatant was used for the preparation of pH 5 enzyme and the partially purified transferase fraction as described earlier (6). Free and membrane-bound ribosomes were separated on a discontinuous sucrose gradient (made in buffer B) by the method described in detail elsewhere (12).

The complete cell-free amino acid incorporation system was the same as described (6) except that 5 µCi of ^3H-labelled mixture of 15 amino acids (sp. activity 1 mCi/ml, Radiochemical Centre, Amersham) and 3-5 µg of ribosomes (given as RNA) were used in a final volume of 0.25 ml. The incubation was carried out at 37 ^oC for 30 min and the hot trichloroacetic acid-insoluble radioactivity was determined as described previously (6).

Cytochrome c oxidase was assayed spectrophotometrically (13). The activity of NADH cytochrome c reductase was determined exactly as described (14) except for incubation of the fractions in 50 mM Tris-HCl in a final volume of 1 ml. DNA was determined by a modification of the original Burton's method (15).

RESULTS AND DISCUSSION

The neuronal perikarya prepared by the present method are devoid of axons and essentially free of glial cell contamination (7), and have been shown to be highly active in incorporating amino acids into neuronal proteins *in vitro* (16,17). In our hands the yield of neuronal perikarya from cerebral cortex of 8-day-old rats was 630 µg DNA/g wet cortex with a RNA/DNA ratio of 1.26.

The RNA/protein ratios of neuronal free and membrane-bound ribosomes are shown in Table 1 which are very similar to those reported earlier for ribosomes prepared from the whole cerebral cortex (4,18). The results presented in Table 1 also show that free ribosomes are essentially devoid of membranous

TABLE 1. Purity of various subcellular fractions isolated from neuronal perikarya

Fraction	RNA/protein	NADH cytochrome c reductase nmoles/min/mg protein	Cytochrome c oxidase nmoles/min/mg protein
Homogenate	-	32.4 ± 1.6	1.6 ± 0.1
Nuclear-mitochondrial pellet (washed)	-	40.5 ± 3.0	5.5 ± 0.3
Free ribosomes	0.48 ± 0.02	none	none
Membrane-bound ribosomes	0.09 ± 0.01	69.2 ± 7.7	1.6 ± 0.2

The subcellular fractions were prepared from neuronal perikarya isolated in bulkform from the brain-cortex of 8-day-old rats (See Methods). The figures represent the mean ± SD of three determinations on four different subcellular preparations.

enzyme activities. The highest specific activity of NADH cytochrome c reductase was in the membrane-bound ribosomal fraction (Table 1); however, the presence of cytochrome c oxidase activity in this fraction suggests that the membrane--bound ribosomes were to some extent contaminated with the mitochondria. Electron microscopy depicted that the two topographic forms of neuronal ribosomes were essentially pure free and membrane-bound ribosomes except for the presence of the few mitochondria-like structures in the bound fraction (results not shown). However, inhibitor studies discussed below indicated that any contaminating mitochondria do not significantly contribute to the cell-free protein synthesis by membrane-bound ribosomes.

The initial nuclear-mitochondrial pellet contained about half of the total RNA and half of the NADH cytochrome c reductase activity found in the neuronal homogenate (Table 2). Repeated gentle washing of the nuclear-mitochodnrial fraction with ion-free medium recovered about 40 % of RNA and the same proportion of NADH cytochrome c reductase from this fraction (Table 2). However, after three washes the nuclear-mitochondrial pellet still contained approximately one-third of the total reductase activity of the homogenate and the same proportion of the total RNA. Increasing the number of ion-free washes did not result in any appreciable increase in the

TABLE 2. The effect of repeated ion-free washes on the content of RNA and NADH cytochrome c reductase in the nuclear--mitochondrial pellet.

Number of washes	% Recovered in nuclear-mitochondrial pellet compared with homogenate	
	RNA	NADH cytochrome c reductase
none	54.4 ± 2.9	50.3 ± 3.5
1	45.6 ± 1.9	36.3 ± 1.5
2	41.6 ± 1.0	32.3 ± 1.5
3	34.0 ± 0.4	29.6 ± 1.0

The initial nuclear-mitochondrial pellet obtained from neuronal homogenate by differential centrifugation was repeatedly washed with ion-free sucrose medium and analysed for RNA and the reductase. The values are the mean ± SD of three determinations on four different subcellular preparations.

recovery of either RNA or NADH cytochrome c reductase, (data not included). Membrane-bound ribosomes averaged 27 % ± 1.8 (mean ± S.D., n = 4) of the total ribosomes recovered from the neuronal perikarya.

The requirements for the incorporation of amino acids into proteins *in vitro* were very similar for the two classes of neuronal ribosomes (Table 3). The omission of ribosomes from the assay system reduced the amino acid incorporation to 20 % of the control value thus suggesting a true ribosome--dependent incorporation. The ribosome dependency of the system was further confirmed by employing ribonuclease (Table 3). Pactamycin, a selective inhibitor of initiation (19), inhibited the activities of both classes of neuronal ribosomes to the same extent (20 %, Table 3). On the other hand fusidic acid, an inhibitor of the translocation step of protein synthesis, (20) had somewhat greater inhibitory effect on the activity of membrane-free ribosomes. The relatively greater resistance of membrane-bound ribosomes to the inhibitors of translation has been demonstrated earlier with membrane-bound ribosomes from liver (6). Chloramphenicol did not inhibit the activity of either class of ribosomes (Table 3) suggesting that any contaminating mitochondria in the membrane-bound ribosomal fraction (see Table 1) do not contribute to the overall *in vitro* protein synthesis by neuronal membrane-bound ribosomes.

TABLE 3. Requirements for cell-free incorporating system and the effect of various inhibitors of translation on the protein synthesis *in vitro* by neuronal free and membrane-bound ribosomes

Conditions of incubation	Specific radioactivity (cpm/mg RNA x 10^{-3})			
	Free ribosomes	% of Control	Bound ribosomes	% of Control
Complete system	148	100	263	100
minus ribosomes	30	20	30	12
minus pH 5 enzymes	25	17	34	13
minus ATP, GTP	37	25	38	14
minus GTP	126	85	204	78
plus ribonuclease (0.1 µg/ml)	30	20	39	15
plus chloramphenicol (50 µg/ml)	-	100	-	100
plus pactamycin (5 µM)	-	79 ± 2.0	-	77 ± 0.4
plus fusidic acid (1 mM)	-	43 ± 4.8	-	56 ± 3.2
plus cycloheximide (4 mM)	-	67 ± 0.8	-	67 ± 3.8

Neuronal free and membrane-bound ribosomes were incubated *in vitro* at 37 °C for 30 min under the indicated experimental conditions. Inhibitor studies were carried out on a different set of preparation. The SD values, where given, represent the mean of at least five determinations on four different preparations. All other figures are the mean values of duplicate or triplicate determinations with a variation of less than 8 %.

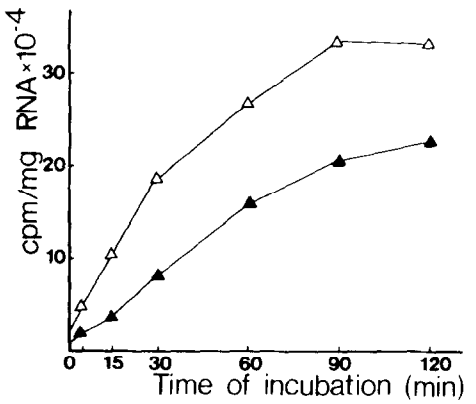

Fig. 1. Time course of amino acid incorporation into protein on neuronal free and membrane-bound ribosomes.
▲▲, free ribosomes; △△, bound ribosomes

The rate of amino acid incorporation into neuronal protein *in vitro* on both classes of ribosomes was linear up to 30 min as shown in Fig. 1. Membrane-bound ribosomes from neuronal

TABLE 4. Specific radioactivity of proteins in various subcellular fractions after labelling the neuronal perikarya with tritiated amino acids

Fraction	Specific radioactivity (cpm/mg RNA)
Homogenate	8220
Post-mitochondrial supernatant fraction	24730
Free ribosomes	6150
Membrane-bound ribosomes	9170

Neuronal perikarya were incubated with a mixture of ^{3}H-labelled amino acids for 15 min as described earlier (17) and fractionated into various subcellular fractions (see Methods). The values represent the average of duplicate determinations on two subcellular preparations.

perikarya were found to be more active than free ribosomes at all the time points studied. Thus after 15 min incubation the membrane-bound fraction incorporated 210 % more amino acids than free ribosomes. In some experiments neuronal homogenate, prior to isolation of ribosomes, was incubated with ^{3}H-labelled amino acids for 15 min as described previously (17). The hot trichloroacetic acid-insoluble radioactivity associated with the various subcellular fractions was then determined. In agreement with the results obtained with the cell-free incorporation system a more intense (50 % greater) labelling of the nascent chains was observed on the membrane-bound ribosomes as compared with the radioactivity associated with the free ribosomes (Table 4). Analysis on linear sucrose gradients revealed that the protein radioactivity sedimented in the polysomal region of the gradient (results not shown).

The functional specialization of membrane-bound ribosomes in secretory tissue is well documented (21); however, less is known about the functional significance of ribosome--membrane interactions in non-secretory tissues such as brain (4,18). There are reports that certain brain proteins may be preferentially synthesized on either class of ribosomes (22,23, but cf. 24). Membrane-bound ribosomes are more responsive to growth and development stimuli than are free ribosomes (25,26). Neuronal membrane-bound ribosomes prepared by the present method are highly active in protein syn-

thesis *in vitro* and are essentially free of contaminating organelles. Thus they may successfully be employed in studies aimed at the elucidation of the possible role of endoplasmic reticulum in the synthesis and secretion of protein in the nerve cell.

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