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THE ATTACHMENT OF POLYRIBOSOMES TO MEMBRANES OF THE HYPOCOTYL OF *PHASEOLUS VULGARIS*

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Summary

Microsomal membranes were isolated from the rapid growing part of hypocotyls of *Phaseolus vulgaris* var. Saxa. The type of ribosome—membrane interaction was determined using EDTA, puromycin and ribonuclease. EDTA treatment (3—5 mM) effected the preferential release of the small over the large ribosomal subunits from the membranes. Incubation of microsomes in puromycin at low and high KCl concentration revealed that ribosomes are attached to the membranes by the growing peptide chain and/or electrostatic linkage. Mild ribonuclease digestion released only about 10% of the membrane-bound ribosomes. Ribosome—membrane interaction in the hypocotyl of *Phaseolus vulgaris* resembles more that found in rat liver cells than that in cultured cells.

Introduction

In almost all animal and plant cells a certain number of ribosomes, varying greatly from one type of cell to another, is attached to cellular membranes [1,2]. It has been noted, that mammalian cells, active in the synthesis of secretory proteins, contain a large portion of membrane-bound ribosomes [3—5], whereas cells, synthesizing proteins primarily for intracellular use, have most of their ribosomes in the free state [6,7].

Aleurone cells producing and secreting amylase show an increased synthesis of ribosomes [8] and endoplasmic reticulum membranes [9]. This suggests that also in plant cells membrane-bound ribosomes might be engaged in the synthesis of secretory proteins. The ubiquity of ribosome—membrane interaction in cells without obvious secretory function point to a more general significance [1]. Recently a number of reports have provided evidences that bound ribosomes synthesize membrane proteins [10,11]. Time-course analysis

Abbreviations: Tris—KCl—5 mM $MgCl_2$, 50 mM Tris—HCl, pH 7.8 at 2°C, 50 mM KCl and 5 mM $MgCl_2$; Tris—KCl—2 mM $MgCl_2$, 50 mM Tris—HCl, pH 7.8 at 2°C,, 50 mM KCl and 2 mM $MgCl_2$.

of some growth and developmental systems showed that the enhancement of the rate of membrane phospholipid synthesis coincides with rather abrupt increase in ribosomes, especially in the rough endoplasmic reticulum [12,13,1].

The upper part of the hypocotyl of *Phaseolus vulgaris* is rapidly growing, depending on the action of growth hormone. Because the cell wall lies outside the plasma membrane one might expect enzymes involved in cell wall metabolism to be associated with membranes [14]. Cellulase activity could be shown to be associated with the microsomal fraction [15] and the synthesis of cell wall polysaccharides appears to be a membrane-related activity [16]. The experiments described in this report characterize the membrane—ribosome interaction in bean stem tissue.

Materials and Methods

Seeds of *P. vulgaris* var. Saxa were surface-sterilized for 10 min in 1% NaClO. They were then rinsed several times in sterile water, imbibed for 8 h, implanted in moist sterile vermiculite/sand 1 : 1 and grown in darkness at 27°C for 90 h. After this time, when the hypocyl was 5—6 cm long, 20-mm sections just below the cotyledons were excised and put into ice-cold water. All subsequent operations were performed at 2°C. For the preparation of free and membrane-bound ribosomes a modified method of Blobel and Potter [3] and Redman [6] was employed. All results in this paper refer to 7 g tissue equivalent of ribosomes and microsomes.

Preparation of free ribosomes and microsomes

7 g of hypocotyl sections were homogenized in a mortar in 20 ml of grinding buffer (0.25 M sucrose, 0.1 M Tris—HCl, pH 8.0 at 2°C 50 mM KCl and 5 mM $MgCl_2$). The resulting homogenate was filtered through three layers of cheesecloth and centrifuged at 1000 × *g* for 10 min. The supernatant was centrifuged for 15 min at 10 000 × *g* and the resulting postmitochondrial supernatant layered on a discontinuous sucrose gradient with 4 ml 1.4 M over 4 ml 2 M sucrose in Tris—KCl—5 mM $MgCl_2$ buffer (50 mM Tris—HCl, pH 7.8 at 2°C, 50 mM KCl and 5 mM $MgCl_2$) and centrifuged at 105 000 × *g* for 17 h. The pellet which sedimented through the 2 M sucrose was designated as free polyribosomes. They were resuspended in Tris—KCl—2 mM $MgCl_2$ buffer (50 mM Tris—HCl, pH 7.8 at 2°C, 50 mM KCl, 2 mM $MgCl_2$) and characterized in an isokinetic sucrose gradient [17,18].

For the preparation of microsomes the material which sedimented at the interphase of 2 M—1.4 M sucrose was removed with a Pasteur pipette. It was diluted with Tris—KCl—2 mM $MgCl_2$ buffer to give a final sucrose concentration of approx. 0.25 M and centrifuged for 30 min at 105 000 × *g*. The resulting microsomal pellet was resuspended in Tris—KCl—2 mM $MgCl_2$ buffer using a motor-driven Teflon pestle fitting the plastic tube of the centrifuge. After centrifugation for 30 min at 10 000 × *g* the resulting pellet, denoted as washed microsomes, was resuspended in Tris—KCl—2 mM $MgCl_2$ buffer.

Preparation of membrane-bound ribosomes

10% Triton X-100 was added to washed microsomes to give a final con-

centration of 2%. After 1 h standing in an ice bath, the mixture was centrifuged for 15 min at 10 000 × *g* and the supernatant characterized in an isokinetic sucrose gradient. Purified membrane-bound ribosomes were prepared by layering the microsome—Triton X-100 mixture onto a discontinuous 2 M—1.4 M sucrose gradient, centrifuging for 17 h at 105 000 × *g* characterizing the resulting pellet in an isokinetic sucrose gradient.

Treatment of microsomes with EDTA [21]

To washed microsomes, resuspended in 0.5 ml of Tris—KCl—2mM $MgCl_2$ buffer, 100 mM EDTA (pH 7.8 with KOH) in Tris—KCl—2 mM $MgCl_2$ buffer was added to give the final concentrations of 3 (5 or 40) mM EDTA. The mixture was brought to 1 ml with Tris—KCl—2 mM $MgCl_2$ buffer and after 1 h centrifuged for 30 min at 10 000 × *g*. The supernatant was layered onto a 5—20% linear sucrose gradient made in 50 mM Tris—HCl, pH 7.8 and 50 mM KCl and centrifuged for 12 h at 25 000 rev./min in an SW 25 rotor of a Spinco L 50 centrifuge.

High salt—puromycin treatment [19]

The reaction mixture contained washed microsomes in 0.5 ml Tris—KCl—2 mM $MgCl_2$ buffer, 0.1 ml of 5 mM puromycin pH 7.5 and 0.4 ml compensating buffer to give a final concentration of 50 mM Tris—HCl, pH 7.8, 50 (500) mM KCl and 2 mM $MgCl_2$. It was incubated at 37°C for 10 min, cooled and centrifuged for 30 min at 10 000 × *g*. The supernatant was layered onto an isokinetic sucrose gradient made in 50 mM Tris—HCl, pH 7.8, 500 mM KCl and 2 mM $MgCl_2$.

Ribonuclease treatment

To washed microsomes, resuspended in 0.5 ml of Tris—KCl—2 mM $MgCl_2$ buffer 5 μl (2 mg/ml) of pancreatic ribonuclease (40 units/mg) and Tris—KCl—2 mM $MgCl_2$ buffer was added to give a final volume of 1 ml. After 0.5, 1 and 5 min of incubation at 37°C the mixture was cooled, centrifuged for 30 min at 10 000 × *g* and the supernatant layered onto an isokinetic gradient made in Tris—KCl—2 mM $MgCl_2$ $MgCl_2$ buffer.

Sucrose density gradient analysis

Isokinetic sucrose gradients (25 ml) were calculated for a D_p = 1.4 and a sucrose concentration at the top of the gradient of 15% [17,18]. Linear and isokinetic gradients were centrifuged at 25 000 rev./min in the SW 25 rotor of a Spinco L 50 centrifuge. Temperature and duration of the centrifugation are indicated in the legends of the figures. The gradients were analysed by monitoring the absorbance at 254 nm in an LKB-Uvicord.

Electron microscopy

Mitochondrial fractions and microsomes were pelleted and fixed for 1 h in equal parts of ice-cold solutions of glutaraldehyde (2%) and OsO_4 (2%) buffered with 0.1 M cacodylate [20]. After washing in cold 0.1 M cacodylate buffer, the samples were postfixed in OsO_4 (2%) buffered with 0.1 M cacodylate for 1 h at 5°C. After a washing step in 0.1 M cacodylate at 5°C for

approx. 1 h, dehydration followed in increasing aceton concentrations (15—100%). During this procedure temperature was allowed to rise slowly up to 25° C. Embedding followed in Spurr's ERL. Thin sections cut on Reichert microtome were poststained with lead citrate.

Results

For the preparation of free and membrane-bound polyribosomes mitochondria had to be separated from the ribosomes and membranes of the endoplasmic reticulum. Centrifugation at 10 000 × *g* for 15 min was found to be suitable for separating the mitochondria from very large polyribosomes and membranes. When the mitochondrial fraction was sedimented at larger centrifugal forces, it contained large portions of polyribosomes and rough membranes (Fig. 1). Fig. 2 is a representative electron micrograph of rough membranes collected onto the 2 M sucrose layer after centrifugation of the post-mitochondrial supernatant at 105 000 × *g* for 17 h. No mitochondria were present in this fraction.

About 75—80% of the cytoplasmic ribosomes in the examined bean stem tissue were found to be in a free state. The rest was attached to membranes. This was calculated from the 260 nm absorbing material in the gradients of free and membrane-bound ribosomes (Figs 3a and 3b).

The $A_{260\ nm}/A_{280\ nm}$ ratio of free and purified membrane-bound ribosomes was 1.75—1.8, that of membrane bound ribosomes 1.65—1.7. A value of 1.7 is generally considered to be indicative of a highly purified polyribosome

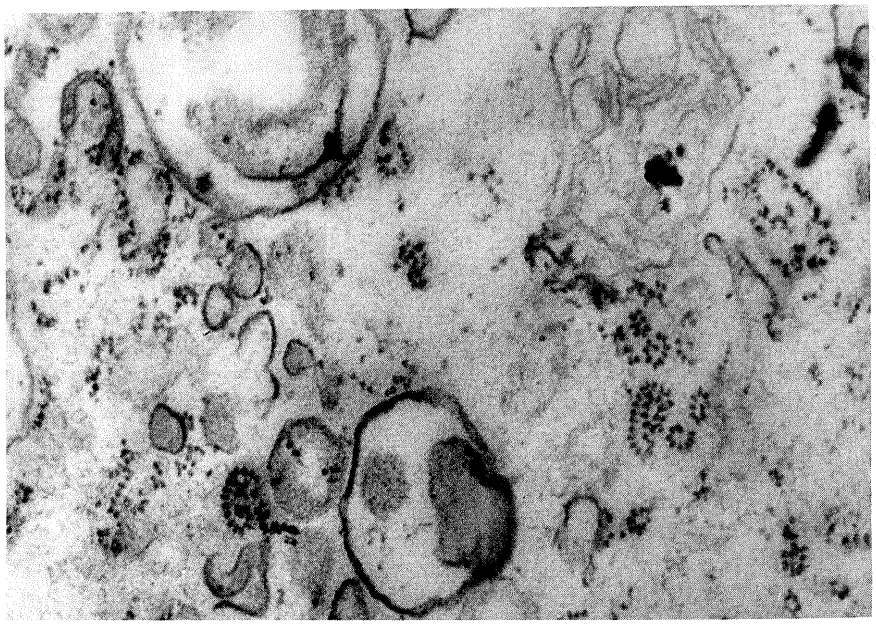

Fig. 1. Contaminated mitochondrial fraction × 60 000. The postnuclear supernatant was centrifuged for 15 min at 15 000 × *g* and the resulting pellet prepared for electron microscopy. Note the occurrence of large polyribsomes.

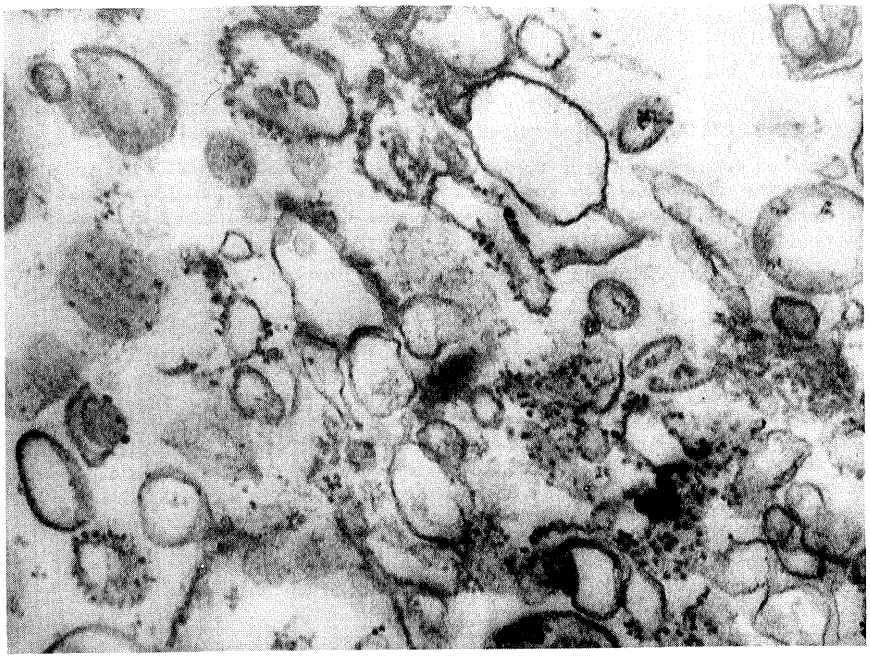

Fig. 2. Washed microsomes (fragmented endoplasmatic reticulum) × 60 000. The postmitochondrial supernatant, after centrifugation for 15 min at 10 000 × *g*, was layered onto a discontinuous sucrose gradient 4 ml 1.4 M over 4 ml 2 M sucrose in Tris—KCl—5 mM $MgCl_2$ buffer and centrifuged for 17 h at 105 000 × *g*. The membranous material (microsomes) at the interphase of 2 M—1.4 M sucrose was sedimented, washed and prepared for electron microscopy.

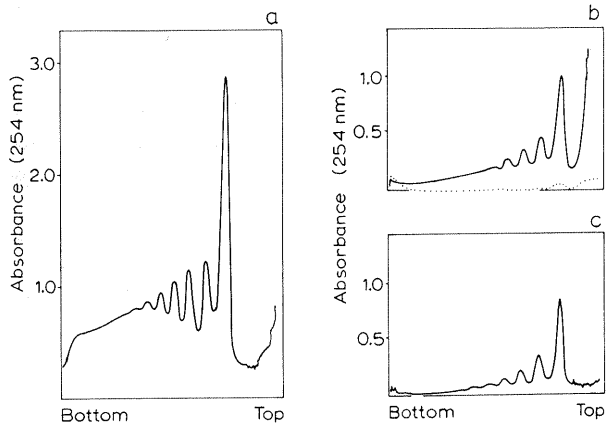

Fig. 3. Sedimentation patterns of free and membrane-bound polyribosomes. (a) Free ribosomes. (b) ———, Membrane-bound ribosomes; . . . . . ., control (contaminating free ribosomes). (c) Purified membrane-bound ribosomes. 7 g of hypocotyl segments were ground in 20 ml of buffer (0.25 M sucrose, 0.1 M Tris—HCl, pH 8.0, 50 mM KCl, and 5 mM $MgCl_2$). The postmitochondrial supernatant after centrifugation at 10 000 × *g* for 15 min was layered onto a discontinuous sucrose gradient with 4 ml 1.4 M over 4 ml 2 M sucrose in Tris—KCl—5 mM $MgCl_2$ buffer and centrifuged at 105 000 × *g* for 17 h. The resulting pellet, free ribosomes, were resuspended in 1 ml Tris—KCl—2 mM $MgCl_2$ buffer. Membrane-bound ribosomes were prepared from the microsomes collected above the 2 M sucrose layer. Microsomes were washed and treated with Triton X-100 (in the control without Triton X-100). After pelleting the membranous material at 10 000 × *g* for 15 min the supernatants contained the membrane-bound ribosomes (with Triton X-100) or contaminating free ribosomes (without Triton X-100). Purified membrane-bound ribosomes were prepared by layering the microsome—Triton X-100 mixture onto a discontinuous sucrose gradient as mentioned above, and it was centrifuged for 17 h at 105 000 × *g*. The pellet was resuspended in Tris—KCl—2 mM $MgCl_2$ buffer and denoted as purified membrane-bound ribosomes. Free and membrane-bound ribosomes were characterized in isokinetic sucrose gradients centrifuged for 3 h, 0°C, at 25 000 rev./min in the SW 25 rotor of a Spinco L 50 centrifuge.

TABLE I

ULTRAVIOLET-ABSORBANCE AND RELATIVE AMOUNTS OF RIBOSOMES OR RIBOSOMAL SUBUNITS RELEASED FROM MICROSOMES EQUIVALENT TO 7 g OF BEAN HYPOCOTYLS

Experiments were carried out as described in the legends to Figs 3—6. The amounts of ribosomes and subunits released from the microsomes were measured by the absorbance at 260 nm on representative sucrose gradients (regions of polyribosomes, ribosomes or subunits). The relative amounts were calculated from at least two experiments.

Conditions	$A_{260\ nm}$	%
Triton X-100 (membrane-bound ribosomes)	5.2	100
Triton X-100 and discontinuous gradient (purified membrane-bound ribosomes)	3.8	76
Control (without Triton X-100)	0.1	4
3 mM EDTA	0.8	20
5 mM EDTA	3.5	70
500 mM KCl	0.2	5
Puromycin at 50 mM KCl	1.5	32
Puromycin at 500 mM KCl	3.6	74
Ribonuclease incubation for 0.5 min	0.4	10
Ribonuclease incubation for 1 min	0.5	12
Ribonuclease incubation for 5 min	0.1	4

preparation [18]. Purification of membrane-bound ribosomes through a discontinuous 2 M—1.4 M sucrose gradient gives a reduction of absorbing material in the ribosome and polyribosome region of the gradient of about 20% (Figs 3b, and 3c, Table I). Part of this reduction may be due to material which was adsorbed to the ribosomes.

Size distribution of free and membrane-bound polyribosomes were very similar as seen in the gradients (Figs 3a—3c). We find about twice as many monomers as dimers. Free and membrane-bound polyribosomes too were not significantly attacked by ribonucleases at 0°C. No visible change in the polyribosome profile was observed for a preparation standing 24 h in an ice bath.

To investigate the ribosome—membrane interaction, washed microsomes were treated with EDTA [21] in a wide range of concentrations (1—40 mM), puromycin [19,25,26] at different ionic strength and ribonuclease [22,23]. For these experiments, the microsome preparation had to be without contamination of free ribosomes. Therefore microsomes as control were resuspended (see Material and Methods), centrifuged for 30 min at 10 000 × *g* and the supernatant layered onto an isokinetic sucrose gradient. As shown in Fig. 3b the preparation was nearly free of contaminating or easily detachable ribosomes. To thus washed microsomes EDTA was added and the detached ribosomal subunits were characterized in a linear sucrose gradient. Up to 2 mM EDTA no ultraviolet-absorbing material was released from the microsomes. Moreover at 2 mM EDTA and less the small amount of free ribosomes present as contaminants sedimented with the membrane fraction. A similar aggregating effect has been observed with pancreas [24] and hepatic [21] ribosomes. At 3 mM EDTA large and small ribosomal subunits are released from the membranes. Up to 5 mM EDTA the gradient shows about 50% more ultraviolet-absorbing material sedimenting in the region of the small ribosomal subunit than in that of the large one (Figs 4a and 4b). Free ribosomes, dissociated by 5

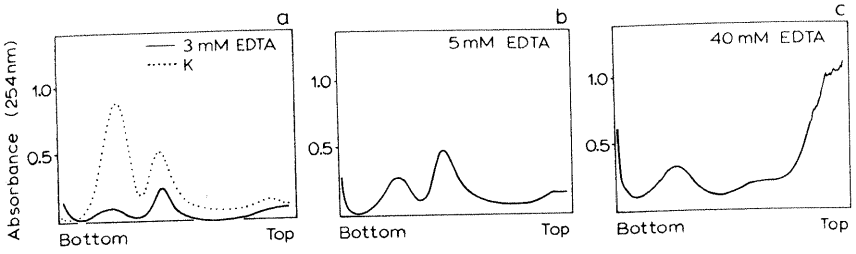

Fig. 4. Sedimentation patterns of subunits from free and membrane-bound ribosomes treated with EDTA. (a) . . . . . ., Free ribosomes (5 mM EDTA); ———, microsomes (3 mM EDTA). (b) Microsomes (5 mM EDTA). (c) Microsomes (40 mM EDTA). Free ribosomes (6.0 A_{260} nm units) resuspended in 1 ml Tris—KCl—2 mM $MgCl_2$ buffer were treated with 5 mM EDTA. After incubation for 1 h at 0°C the mixture was layered onto a 5—20% linear sucrose gradient in 50 mM Tris—HCl, pH 7.8, 40 mM KCl and centrifuged for 12 h, 2°C, at 25 000 rev./min in the SW 25 rotor of a Spinco L 50 centrifuge. Microsomes prepared as described under Fig. 2 were resuspended in Tris—KCl—2 mM $MgCl_2$ buffer with 3, 5, or 40 mM EDTA in a final volume of 1 ml and incubated for 1 h at 0°C. After centrifugation for 30 min at 10 000 X *g* the supernatants were characterized on linear sucrose gradients as EDTA-treated free ribosomes.

mM EDTA give the expected ratio of 2 : 1 for the large to small ribosomal subunit (Fig. 4a).

At EDTA concentrations above 5 mM the peak of the large ribosomal subunit is enlarging while that corresponding to the small one is more and more reduced. At 40 mM EDTA unfolding and disassembly of the subunits especially of the small ones become visible (Fig. 4c).

It is known, that ribosomes without nascent polypeptide chain dissociate into their subunits in the presence of $MgCl_2$ and 500 mM KCl whereas ribosomes with nascent chains do not dissociate under these conditions except the chains have been released by puromycin [25,26].

Therefore it should be possible to estimate the proportion of membrane-bound ribosomes with nascent chains by inducing dissociation and to show whether ionic interaction and/or nascent chains are involved in the ribosome membrane attachment [11,19].

Washed microsomes were treated with KCl at low (50 mM) or high (500

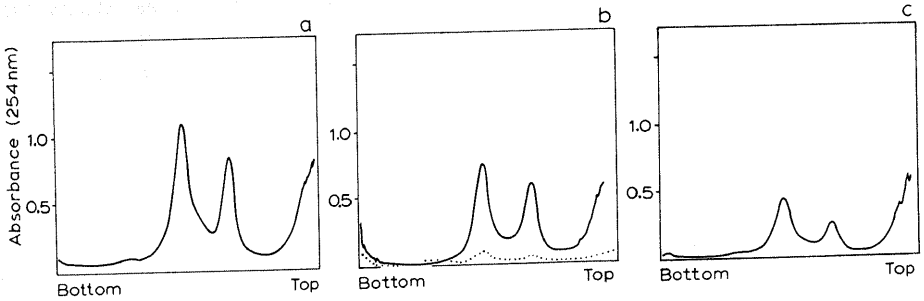

Fig. 5. Sedimentation patterns of subunits from free polyribosomes and membrane-bound ribosomes treated with puromycin at 50 and 500 mM KCl. (a) Free polyribosomes (5.0 A_{260} nm units, 0.5 mM puromycin, 500 mM KCL). (b) ———, Microsomes (0.5 mM puromycin, 500 mM KCl); . . . . . ., microsomes (500 mM KCl). (c) Microsomes (0.5 mM puromycin, 50 mM KCl). The incubations were performed in 50 mM Tris—HCl, pH 7.8, 2 mM $MgCl_2$ in a final volume of 1 ml for 10 min at 37°C. The sample without puromycin was treated at 0°C. After 10 min standing in an ice bath the mixtures were centrifuged for 30 min at 10 000 X *g* and the supernatants layered onto isokinetic sucrose gradients in Tris—KCl—2 mM $MgCl_2$ buffer. The gradients were centrifuged for 12 h, 2°C at 25 000 rev./min in the SW 25 rotor of a Spinco L 50 centrifuge.

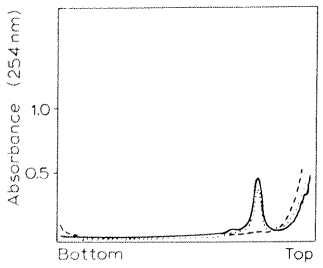

Fig. 6. Sedimentation patterns of ribosomes derived from ribonuclease-treated microsomes. Microsomes, resuspended in Tris—KCl—2 mM $MgCl_2$ buffer were incubated with 10 μg of pancreatic ribonuclease in a final volume of 1 ml for: ....., 0.5 min; ———, 1 min; - - - - - -, 5 min; at 37° C. After cooling the mixtures were layered on isokinetic sucrose gradients in Tris—KCl—2 mM $MgCl_2$ buffer and centrifuged for 3 h, 0° C, at 25 000 rev./min in the SW 25 rotor of a Spinco L 50 centrifuge.

mM) concentrations in the presence or absence of puromycin (Figs 5b and 5c). Maximal release of ribosomal subunits from membranes required both, puromycin and high KCl concentration (Fig. 5b). Low and high salt concentrations alone remove less than 10% (Table I), whereas low salt concentration with puromycin releases about 30—40% of the membrane-bound ribosomes (Fig. 5c, Table I). Mild digestion of washed microsomes for 0.5 and 1 min with 10 μg ribonuclease/ml at 37° C effects the release of about 10% of the membrane-bound ribosomes (Fig. 6, Table I). Incubations for 0.5 and 1 min show similar profiles, whereas a prolonged incubation resulted in degradation of the monosomes.

Discussion

The association of ribosomes and membranes has been characterized for mammalian cells, but very little is known as yet about the attachment of plant ribosomes to membranes [2,3]. This communication reports the isolation and characterization of membrane-bound ribosomes from bean stem tissue.

Our microsome preparation shows about 20% of the ribosomes bound to membranes. Reports on microsomes from mammalian cells demonstrated that a considerable amount of polyribosomes and cytoplasmic membranes contaminates the nuclear and mitochondrial fraction. By chosing low centrifugation forces for sedimentation of mitochondria we tried to reduce the loss of free and membrane-bound ribosomes. Nevertheless the portion of membrane-bound ribosomes in vivo might be considerably larger [3]. We have to consider that very large fragments of the endoplasmic reticulum of remnants which have not been stripped off from the nuclear membranes may contaminate the nuclear and mitochondrial fraction [3].

An absorbance ratio of 2 : 1 of the large to the small ribosomal subunit would be expected if the whole ribosomes were to become detached upon the addition of EDTA and then dissociated into the subunits or if the process of dissociation and detachment was simultaneous [21] (Fig. 4a). Treating microsomes with EDTA concentrations between 3 and 5 mM the small ribosomal subunit could be recovered from the supernatant (see Material and Methods) to a much higher extent than the large one (Figs 4a and 4b).

This result indicates that a certain portion of ribosomes is bound to membranes via their large subunit and that this ribosome membrane interaction is independent of Mg^{2+}. Similar results have been reported for mammalian cells [21].

About 10% of the membrane-bound ribosomes were released by high KCl concentration alone. It has been suggested, that the KCl-sensitive link is a direct electrostatic bond between ribosome and membrane [19]. Thus in our preparation only few ribosomes seem to be linked by electrostatic binding alone (released by high KCl) but about 30% are linked to the membrane by the nascent peptide chain alone (released by puromycin at low KCl concentration). There are about 70—80% of the ribosomes which are detached from the membranes by puromycin together with high salt concentration. Puromycin at 500 mM KCl released about twice as much ribosomes as 50 mM KCl and puromycin applied separately. This means that a considerable part of the ribosomes may be linked to membranes by both the nascent peptide chain and electrostatic bond [19]. We conclude therefore that proteins synthesized on membrane-bound ribosomes become incorporated into the membrane or are secreted into the intracisternal spaces.

Mild ribonuclease digestion of microsomes detaches only a very small portion of ribosomes from the membrane. This result makes it very unlikely that mRNA is responsible for a specific attachment of ribosomes to membranes. In tissue culture cells ribosomes may be bound to the membranes by virtue of their attachment to mRNA [22,23,28]. In cells that mainly synthesize proteins for secretion, for example rat liver cells, ribosomes bind to the membrane by the growing peptide chain [19]. Membrane attachment in growing plant tissue resembles more the type of attachment found in rat liver cells than that of tissue culture cells. For growing plant cells we have to consider, that proteins have to be excreted via the Golgi complex where most of the cell wall substances are performed [29]. This leads us to speculate that in growing plant stem tissue proteins synthesized on membrane-bound polyribosomes are preferentially engaged in cell wall metabolism. These proteins might be transported from the rough endoplasmic reticulum via the agranular endoplasmic reticulum to the Golgi complex and for some proteins at least further into the cell wall. This functional aspect is currently under study.

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