Short Communications

Contamination of Free-Ribosome Fractions by Detached Previously Membrane-Bound Ribosomes

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A rough-membrane fraction isolated from rat liver by a procedure designed to prevent membrane denaturation was subjected to the gradient treatment normally used to isolate free ribosomes. Under these conditions, at most 20% of the ribosomes were detached from membrane with less than 5% sedimenting into the free-polyribosome pellet.

There is considerable evidence that, in rat liver, some ribosomes are not membrane-bound but are scattered freely in the cytoplasmic matrix (Palade, 1955; Bloemendal *et al.*, 1964; Redman, 1969). A commonly used procedure for the isolation of these free (membrane-unassociated) ribosomes from liver homogenates is to subject a microsomal fraction to a prolonged ultracentrifugation over a gradient containing 2M-sucrose (Bloemendal *et al.*, 1964; Blobel & Potter, 1967). The free ribosomes (i.e. subunits, monoribosomes and polyribosomes) were originally considered to sediment quantitatively under these conditions (Blobel & Potter, 1967), but it is now known that free ribosomes may also be found in the 2M-sucrose layer (Sarma *et al.*, 1972) and in the membrane bands above this layer (Vernie *et al.*, 1971). Free polyribosomes sediment preferentially (Sarma *et al.*, 1972) and are recovered in the pellet free of contaminating rough vesicles (Bloemendal *et al.*, 1967).

The possibility that at least some of the free polyribosomes isolated by this type of procedure were membrane-bound polyribosomes which had been detached from membranes during the gradient procedure was apparently eliminated by the studies by Benedetti *et al.* (1966) and Loeb *et al.* (1967). Only a small fraction of the previously membrane-bound ribosomes were recovered in the pellet when a purified rough-membrane fraction was subjected to centrifugation over 2M-sucrose.

On the other hand, Bont *et al.* (1972) found that on resubjecting isolated rough membranes to discontinuous gradient centrifugation a fraction of membrane-free polyribosomes was again recovered in the pellet, provided that the rough membranes were suspended in a medium of high sucrose concentration (>1M) or containing post-microsomal supernatant. A negligible recovery of these membrane-free ribosomes was obtained when the rough membranes were denatured, i.e. exposed to dilute sucrose solu-

tions, before gradient centrifugation as in the earlier studies by Benedetti *et al.* (1966) and Loeb *et al.* (1967). Bont *et al.* (1972) interpreted these observations to mean that the original membrane fraction was actually a mixture of membranes and free ribosomes but that the latter became membrane-bound when the membranes were denatured.

This is, however, only one of two possible explanations. In view of mixing experiments with labelled free ribosomes (see Andrews & Tata, 1971; Sarma *et al.*, 1972), it seems unlikely that isolated rough-membrane fractions are composed of or derived from free ribosomes and membranes. The alternative explanation, i.e. that detachment of ribosomes from membranes is more significant if the rough membranes are not denatured, and hence that isolated free ribosomes are seriously contaminated with detached, previously membrane-bound ribosomes, must therefore be considered. This possibility was re-examined in the present paper.

Female Wistar rats weighing 150–220 g were usually starved for 18–25 h before each experiment. The animals were killed by cervical dislocation and the liver was homogenized with 2–3 vol. of 0.25M-sucrose-TKM buffer (see Blobel & Potter, 1967). The homogenate was then centrifuged at 8000g_{av} for 10 min (SW39 rotor; Spinco) to obtain a post-mitochondrial supernatant. Post-microsomal supernatant was prepared by centrifuging post-mitochondrial supernatants at 164000g_{av} for 75–90 min (50Ti rotor). The upper 11 ml of supernatant was collected.

A membrane fraction containing little free ribosomal material was prepared from the post-mitochondrial supernatant in the presence of high sucrose concentration or post-microsomal supernatant as follows.

Post-mitochondrial supernatant (4 ml) was layered over a 'cushion' consisting of 1.3M-, 1.5M- or 2.0M-

sucrose-TKM (1 ml) and the system was centrifuged at $26000g_{av.}$ for 15 min (SW39 rotor). The membranes in and on the dense sucrose layer were carefully collected by using a bent Pasteur pipette, dispersed in post-microsomal supernatant (final volume 4 ml) and layered over 1.3–2.0 M-sucrose-TKM (1 ml) as above. The gradients were centrifuged at $32000g_{av.}$ for 20 min (SW39 rotor) and the purified membrane fraction was collected and dispersed in post-microsomal supernatant or 1.1 M-sucrose-TKM.

In some experiments, membranes were deliberately denatured (Bont *et al.*, 1972) by omitting the sucrose 'cushion' and resuspending the first and subsequent pellets in 0.25 M-sucrose-TKM. Phase-contrast microscopy confirmed that membranes prepared by this procedure were extensively aggregated (see Bont *et al.*, 1972), in contrast with those prepared in high sucrose concentration or post-microsomal supernatant, which resembled membranes in the original post-mitochondrial supernatant.

To study the detachment of membrane-bound ribosomes, the native or denatured membranes, suspended in post-microsomal supernatant or 0.25 M-sucrose-TKM respectively (2.5 ml), were layered on a discontinuous sucrose gradient consisting of 1.25 ml of 2 M-sucrose-TKM (lower layer) and 1.25 ml of 1.5 M-sucrose-TKM (middle layer). The gradients were centrifuged at $131000g_{av.}$ for 20–24 h (SW50 rotor; Spinco). The 2 M-sucrose and membrane layers were separately collected, diluted with TKM buffer (to 0.35–0.40 M with respect to sucrose) and sedimented at $133000g_{av.}$ for 120 min or $164000g_{av.}$ for 85 min (50Ti rotor; Spinco). In two experiments, the detached ribosomes contaminating the membrane bands in the 1.5 M-sucrose layer were isolated by using a sedimentation gradient (K. O'Toole & J. K. Pollak, unpublished work). Briefly, the bands in the 1.5 M-sucrose layer were divided into smooth and rough fractions (see Bloemendal *et al.*, 1967) and the rough fraction was diluted with TKM buffer (to 0.35–0.40 M with respect to sucrose) and layered on linear continuous 0.44–0.88 M-sucrose gradients. After a 60 min centrifugation at $133000g_{av.}$ (SW50 rotor), the gradient

supernatants were decanted and the free ribosomes were sedimented ($131000g_{av.}$ for 120 min, 50Ti rotor). The smooth-membrane fraction and the 2 M-sucrose-layer ribosomes were sedimented similarly.

Table 1 demonstrates that when either native or denatured membranes were subjected to the discontinuous-gradient procedure only about 10% of the RNA entered the 2 M-sucrose layer. Moreover, since less than 5% of the total RNA actually sedimented (Table 1) and since usually 50% or more of the sedimentable RNA in a post-mitochondrial supernatant is recovered in this fraction (Blobel & Potter, 1967), it follows that the free polyribosome fraction isolated by most authors for functional studies is not significantly contaminated by detached membrane-bound ribosomes. In this respect, the present observations do not agree with those of Bont *et al.* (1972), who found that, when a purified rough-membrane fraction that had not been denatured was re-centrifuged over the discontinuous gradient, at least 20% of the total ribosomes were recovered in the 2 M-sucrose pellet (compare with results in Table 1).

Since the 2 M-sucrose pellet contains only a fraction of the detached ribosomes present in the gradient (Sarma *et al.*, 1972), the observations of Bont *et al.* (1972) also suggest that very extensive (e.g. 40%) total detachment of ribosomes actually occurred. In the present experiments, free or detached ribosomes in the pellet and the 2 M-sucrose layer and any in the membrane fractions were taken into consideration. Nevertheless, total ribosomal detachment was less than 20%. In fact, it should be noted that the purified membrane fraction could be contaminated with native free ribosomes. Only about 5% contamination by the free ribosomes normally recovered from a post-mitochondrial supernatant would account for the observed ribosomal detachment. For this reason, it must be stressed that the observed recovery of free ribosomes from the purified membrane fraction represents a maximum value for detachment.

Although it could be argued that, in the present study, detached membrane-bound ribosomes above

Table 1. *Detachment of membrane-bound ribosomes on the discontinuous gradient*

See the text for details. RNA was determined by the method of Munro & Fleck (1966). Results are expressed as means $\pm$ S.E.M., with numbers of experiments in parentheses.

Condition of membranes layered on gradient	RNA layered on gradient (mg/g)	Percentage of total sedimentable RNA recovered from discontinuous gradient in:			
		2 M-sucrose pellet	2 M-sucrose layer	free ribosomes isolated from 1.5 M-sucrose-TKM layer	total detachment
Native	0.52 ± 0.12 (6)	3.0 ± 0.2 (6)	8.3 ± 0.3 (6)	5.8 ± 0.4 (2)	17.1
Denatured	0.65 ± 0.03 (2)	1.9 ± 0.1 (2)	8.8 ± 0.7 (2)	—	—

the 2M-sucrose layer re-attached to membrane when the suspension was diluted for the sedimentation gradient step (see above), this possibility was eliminated as follows. A native smooth-membrane fraction was prepared as described by Bont *et al.* (1972) and denatured by the addition of 2 vol. of a suspension of free ribosomes in TKM buffer. Free ribosomes were then separated from this suspension and from appropriate controls by using the 0.44–0.88M-sucrose gradient method (see above). The denaturation of the membranes did not influence the efficiency of the separation, since $108 \pm 5\%$ of the free ribosomes recovered in the absence of membranes were recovered in the presence of membranes (two experiments).

The cause of the discrepancy between the present results and those of Bont *et al.* (1972) is not known. It is possible, however, that since the rough membranes used by Bont *et al.* (1972) had already experienced the shear forces produced during an 18h gradient centrifugation, detachment of membrane-bound ribosomes occurred more easily on the second gradient. In the present investigation, the free-ribosome-poor membrane fraction was prepared by low-speed differential centrifugation, and the shear forces operating during isolation should have been minimal. Moreover, provided that post-microsomal supernatant had been prepared beforehand, the isolation was completed within 2h rather than 18h of liver homogenization (see Bont *et al.*, 1972). Therefore any ribosomal detachment occurring in the present study should more nearly reflect detachment occurring during the fractionation of a post-mitochondrial supernatant.

The present results suggest, therefore, that during fractionation of a post-mitochondrial supernatant

by prolonged ultracentrifugation over 2M-sucrose, the free-polyribosome pellet is not appreciably contaminated with detached membrane-bound ribosomes, and minimal contamination of the unsedimented free-ribosome fractions with detached membrane-bound ribosomes occurs. The evidence thus supports the view that nearly all of the isolated free ribosomes are present as such *in vivo*.

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