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Sparsomycin-Induced Disaggregation but not Detachment of Hepatic Membrane-Bound Polyribosomes[1] (40116)

H. SIDRANSKY,[2] E. VERNEY, C. N. MURTY AND D. S. R. SARMA[3]

Department of Pathology, University of Pittsburgh, School of Medicine, Pittsburgh, Pennsylvania 15213 and Department of Pathology, The George Washington University Medical Center, Washington, D.C. 20037

For a number of years our laboratory has been concerned with the mechanisms by which selected hepatotoxic agents act on hepatic polyribosomes and protein synthesis in experimental animals. Among the agents investigated were actinomycin D (1–3), puromycin (4–6), ethionine (5, 7–9), aflatoxin B_1 (10), CCl_4 (5, 8, 11), hypertonic NaCl (12–15), dimethylnitrosamine (16), and sparsomycin (5, 17). The studies were concerned with how these agents affect the total polyribosomes as well as the free and membrane-bound polyribosomes of the livers of rats and mice. Studies dealing with the nature of attachment of ribosomes to membranes in livers of animals exposed to hepatotoxins were also undertaken. They revealed that, even though agents such as ethionine, puromycin, CCl_4, and sparsomycin caused extensive disaggregation of membrane-bound polyribosomes, they did not cause detachment of ribosomes from the membranes (5). This led to further studies which revealed that normal membrane-bound ribosomes attached to membranes could be influenced *in vitro* by $[K^+]$ (increasing $[K^+]$ leads to detachment) (8). Similar findings have been reported by Adelman *et al.* (18) who also demonstrated that ribosomes are bound to membranes by a direct interaction between the membrane and the large ribosomal subunit which are labile at high KCl concentrations. Also, evidence has been presented that disaggregated membrane-bound ribosomes obtained after *in vivo* treatment of mice or rats with ethionine or CCl_4 could be released from membranes with high KCl concentrations (8).

Recently we investigated the effect of $[K^+]$ *in vitro* on detachment of hepatic ribosomes after sparsomycin treatment *in vivo* and observed that the ribosomal attachment to membranes was different than that found earlier after ethionine or CCl_4. Sparsomycin has been demonstrated *in vivo* to cause marked disaggregation of hepatic polyribosomes (5, 17, 19, 20). The present report deals with the findings which suggest that the hepatic ribosomes on membranes after sparsomycin treatment *in vivo* are attached via peptides as well as by ionic bridging influenced by $[K^+]$.

Materials and methods. Female mice weighing 25 g and female rats weighing 150–180 g (Hilltop Lab Animals, Inc., Scottdale, PA) were fasted overnight before using.

Labeled membrane-bound polyribosomes were obtained by administering [^{14}C]-6-orotic acid (58 mCi per mmole), 2.5 μCi per mouse or 5 μCi per rat, intraperitoneally 20 hr before killing.

Sparsomycin, obtained as a gift from the National Cancer Institute, 1 μg/g of body weight, was administered intraperitoneally 2 hr before killing. Dimethylnitrosamine, obtained from Eastman Organic Chemicals, 1.25 mg/mouse was administered intraperitoneally 1 hr before killing. Control animals received saline (0.9% NaCl).

Preparation of membrane-bound ribosomes and determinations of radioactivity in the ribosomes were essentially the same as described earlier (5, 8). Ten milliliter aliquots of 1.38 *M* sucrose layer (containing 0.05 *M* Tris, pH 7.6, and 0.005 *M* $MgCl_2 \cdot 6H_2O$ (TM), rat liver cell sap, and membrane-bound polyribosomes freed of free ribosomes) were adjusted to different concentrations of KCl (2.5 *M* KCl in 0.25 *M* sucrose containing

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[2] Address for reprints: Dr. H. Sidransky, Department of Pathology, The George Washington University Medical Center, 2300 Eye Street, N.W., Washington, D.C. 20037.

[3] Present address: Department of Pathology, University of Toronto School of Medicine, Toronto, Canada.

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TM was added to give final concentrations of 0.1, 0.2, or 0.4 *M* KCl). Total volumes were made up to 12.5 ml with TM and the tubes were incubated on ice for 20 min and 12.5 ml of incubating mixture were loaded on to a discontinuous gradient of 7 ml of 2.0 *M* sucrose in TM and 7 ml of 1.38 *M* sucrose in TM. The tubes containing 26.5 ml were centrifuged for 20 hr at 29,000 rpm at 2° in a 30 rotor in a Spinco model L3-40 centrifuge. The 1.38 *M* sucrose layer and above supernatant was aspirated, treated with 10% deoxycholate to give a final concentration of 1% deoxycholate, diluted with TKM (TM + 0.025 *M* KCl) to 30 ml and centrifuged for 4 hr at 40,000 rpm at 2° in a 42.1 rotor to pellet membrane-bound ribosomes. The 2.0 *M* sucrose layer along with the pelleted detached ribosomes was diluted three times its volume with TKM and centrifuged 2 hr at 40,000 rpm at 2° in a 42.1 rotor to pellet detached ribosomes. One aliquot (10 ml) of 1.38 *M* sucrose layer (containing TM, rat liver cell sap, and membrane-bound polyribosomes) was incubated with puromycin (6 mg per tube), 4 mg ATP, 2.25 mg GTP, 26 mg PEP, 0.015 ml pyruvate kinase (60 I.U.) and 2.5 ml dialyzed liver cell sap for 30 min at 37° before it was incubated on ice with different concentrations of KCl. In each case, the radioactive detached ribosomes (2 *M* sucrose and pellet) and retained or undetached ribosomes (1.38 *M* sucrose layer) were determined. Percentage of detachment was calculated as detached ribosomes × 100/total ribosomes (detached + undetached). Each value was corrected for blanks.

Incorporation of [^{14}C]phenylalanine into polypeptide using ribosomes obtained from postmitochondrial supernatants under the direction of poly(U) was performed as described earlier (21).

Results. Previously we reported that membrane-bound polyribosomes of normal fasted animals prepared by the method of Blobel and Potter (22) and incubated in the presence of 0.4 *M* KCl *in vitro* revealed a 30% detachment of ribosomes while membrane-bound polyribosomes of CCl_4- or ethionine-treated animals prepared and incubated similarly revealed a 53–65% detachment of ribosomes (8). Thus, even though the membrane-bound hepatic ribosomes had become disaggregated by CCl_4 or ethionine treatment *in vivo*, they were still attached to membranes and only after KCl incubation *in vitro* became detached (8). In contrast to these findings, when rats were treated *in vivo* with sparsomycin, which caused marked disaggregation of hepatic polyribosomes (5), incubation of membrane-bound ribosomes with KCl *in vitro* failed to increase the detachment of ribosomes over that in controls (Table I). This finding led us to explore why the disaggregated polyribosomes of the sparsomycin-treated rats behaved differently than did those of CCl_4- or ethionine-treated rats.

Earlier findings suggested that membrane-bound ribosomes were attached to membranes in at least two ways: when engaged in protein synthesis, they are attached to membranes by growing polypeptides as well as by binding that can be influenced by [K^+]; and when they are not engaged in protein synthesis, they are attached only by the latter (8, 18). This suggested to us that conceivably the disaggregated ribosomes of sparsomycin-treated animals may still be attached via peptides even though they are not active in protein synthesis. To test for this we conducted experiments whereby hepatic membrane-bound ribosomes of control and sparsomycin-treated mice were preincubated *in vitro* with puromycin before incubating them with increasing concentrations of KCl. Puromycin reacts with ribosomal bound peptidyl-tRNA

TABLE I. INFLUENCE OF DIFFERENT CONCENTRATIONS OF KCl ON THE DETACHMENT OF RIBOSOMES FROM MEMBRANE-BOUND POLYRIBOSOMES OF LIVERS OF CONTROL (SALINE) AND SPARSOMYCIN-TREATED RATS.[a]

KCl *M*	Percentage of detachment of ribosomes	
	Control	Sparsomycin
0.10	13.5 ± 1.9	13.0 ± 1.9
0.20	15.7 ± 3.0	19.0 ± 2.2
0.40	22.5 ± 3.2	27.0 ± 2.7

[a] Rats received intraperitoneally as follows: control, saline 4 hr before killing or sparsomycin (1 μ/g of body weight) 2 hr before killing. Each rat received 5 μCi of [^{14}C]-6-orotate intraperitoneally 18–20 hr before killing. Membrane-bound polyribosomes (1.38 *M* sucrose layer containing 0.05 *M* Tris, pH 7.6, and 0.005 *M* Mg^{2+} and rat liver sap) were prepared and incubated with different concentrations of KCl and detached ribosomes were obtained. Groups of each experiment consisted of two rats. The results are the average of seven experiments ± SE of the mean.

TABLE II. INFLUENCE OF PUROMYCIN AND DIFFERENT CONCENTRATIONS OF KCl ON THE DETACHMENT OF RIBOSOMES FROM MEMBRANE-BOUND POLYRIBOSOMES OF LIVERS OF CONTROL (SALINE), SPARSOMYCIN-, OR DIMETHYLNITROSAMINE-TREATED MICE.[a]

Group	No. of experiments	Puromycin incubation	Percentage of detachment of ribosomes		
			0.1 *M* KCl	0.2 *M* KCl	0.4 *M* KCl
Control	3	−	9.9 ± 1.7	15.4 ± 2.0	22.8 ± 3.2
		+	13.6 ± 2.6	22.3 ± 2.1	45.3 ± 4.3
Sparsomycin	3	−	13.4 ± 1.5	19.9 ± 2.1	24.6 ± 5.2
		+	15.1 ± 1.1	29.4 ± 3.6	47.5 ± 3.6
Control	2	−	5.8 ± 3.4	10.0 ± 3.0	15.4 ± 5.6
		+	14.8 ± 6.1	30.6 ± 2.2	47.6 ± 12.4
Dimethylnitrosamine	2	−	9.3 ± 0.6	18.0 ± 3.9	23.0 ± 5.1
		+	16.4 ± 1.5	33.6 ± 6.7	56.5 ± 4.1

[a] Aliquots (10 ml) of 1.38 *M* sucrose layer (containing TM, liver cell sap, and membrane-bound polyribosomes) were incubated with 6 mg puromycin, 4 mg ATP, 2.25 mg GTP, 26 mg PEP, 0.015 ml pyruvate kinase and 2.5 ml dialyzed liver cell sap for 30 min at 37° before they were incubated on ice with different concentrations of KCl. The results are the means ± SE of the means.

to form a peptidyl puromycin product which is then released from the polyribosome (23). The results, summarized in Table II, revealed that puromycin preincubation accentuated the release of ribosomes from the membranes of the sparsomycin-treated group. This suggests that the disaggregated ribosomes of the experimental animals contain peptides which are maintaining the attachment of ribosomes to the membranes.

In one experiment we studied the ability of isolated ribosomes from sparsomycin-treated rats and control rats with poly(U) to incorporate [^{14}C]phenylalanine into polyphenylalanine. The ribosomes of the experimental group incorporated 55% less than that of controls.

Discussion. Our present findings that the sparsomycin-induced disaggregation of polyribosomes into monomers that still retain peptides which may become liberated by puromycin treatment *in vitro* are consistent with results reported by Hayes *et al.* (20). These investigators presented evidence that there was an increase in the tRNA content of hepatic total ribosomes of sparsomycin-treated mice in comparison with that of run-off ribosomes. Thus, they considered the disaggregated polyribosomes due to sparsomycin to be fall-off ribosomes (ribosomes falling off from mRNA). Also, by measuring dissociability of these 80 S ribosomes in 0.3 *M* KCl, we (8), as well as they (20), found that they dissociated into two ribosomal subunits, suggesting that the ribosomes were free of mRNA.

Our present results emphasize that certain hepatotoxic agents may *in vivo* disaggregate membrane-bound polyribosomes into monomers which structurally may be different from those induced by other agents. For example, administration of hepatotoxins such as ethionine and CCl_4 lead to disaggregation of polyribosomes to monomers that do not contain peptides (run-off ribosomes) (5). On the other hand, administration of an agent such as sparsomycin leads to disaggregation of polyribosomes of monomers that still contain peptides (fall-off ribosomes) (20). Although both classes of ribosomes under these experimental conditions do not maintain protein synthesis, the attachment of the single ribosomes of the two types to membranes are different: the former type maintains attachment by a cationic type of bridging while the latter type has, in addition, peptides which influence attachment.

Sparsomycin, a sulfur-containing antibiotic, inhibits protein synthesis in both bacterial and mammalian cells (24). It has been found to block peptide bond formation and thus prevents the transfer of amino acids from tRNA to the nascent polypeptide chains based upon previous studies using a cell-free system from *Escherichia coli* (25) and from mouse liver (19). These studies suggested that sparsomycin binds to the larger ribosomal subunits and inhibits peptidyl transferase, an enzyme responsible for peptide bond formation. Also, sparsomycin has been reported to inhibit *in vivo* the factor-dependent initiation of new polyphenylalanine chains as well as factor-dependent binding of either phe-tRNA or met-tRNA to 40 S ribosomal sub-

units (17). Using cycloheximide, an agent that acts to interfere with peptide chain release (26) and has been used to determine whether it can cause a reformation of polyribosomes previously disaggregated by hepatotoxic agents (27), we observed that it could not reassemble the ribosomes disaggregated by sparsomycin (17). Our present findings suggest that sparsomycin causes fall-off ribosomes which are defective as indicated by the decreased formation of polyphenylalanine when assayed *in vitro* with poly(U). Thus, sparsomycin appears to act in a number of ways in disturbing the protein synthetic mechanisms in the liver.

Since Chiga *et al.* (20, 28) reported that dimethylnitrosamine, as well as sparsomycin, acts *in vivo* to induce fall-off ribosomes, it became of interest to test how dimethylnitrosamine would act in relation to ribosomal attachment to membranes. Our results (Table II) reveal that it was necessary to use puromycin as well as KCl to cause detachment of ribosomes *in vitro* from membranes, results identical to those found with sparsomycin (Table II). Thus, at least two hepatotoxic agents seem to behave in a similar manner in their effect on ribosomes.

There appears to be at least three ways by which polyribosomes may breakdown into ribosomes depending upon the inducing agent. They are as follows: (a) run-off ribosomes, which occur normally or after treatment with agents such as ethionine and CCl_4 (5, 29, 30); (b) mRNA containing ribosomes, 80 S ribosomes still retaining mRNA, which are found after RNase digestion (31); and 3) fall-off ribosomes, which are found after treatment with sparsomycin or dimethylnitrosamine. Our current study presents support for the existence of this third category of ribosomes.

Summary. The nature of attachment of hepatic ribosomes to membranes after sparsomycin treatment *in vivo* of rats and mice was investigated. The results based upon the influence of treatment *in vitro* with different concentrations of KCl and with puromycin on the detachment of membrane-bound ribosomes from membranes of livers of sparsomycin-treated animals revealed that these ribosomes are attached to membranes by peptides as well as by ionic bridging and are considered as fall-off ribosomes. Similar effects have been described after treatment *in vivo* with dimethylnitrosamine. In contrast, treatment *in vivo* with ethionine or CCl_4 produced run-off ribosomes which are attached only by ionic bridging since they become detached *in vitro* with KCl treatment alone.

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