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Production *in vivo* of falloff monosomes in mouse liver by sparsomycin

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The antibiotic and antitumor agent, sparsomycin, has been shown to be a potent inhibitor of protein synthesis in both prokaryotes and eukaryotes [1]. A system *in vitro* demonstrated that this agent inhibits the peptidyl transferase activity of 50S ribosomal subunits [2]. Furthermore, addition of sparsomycin to a cell free amino acid-incorporating system inhibited the incorporation of [^{14}C]leucine into proteins by 80 per cent but did not cause polysome disaggregation [3]. On administration *in vivo*, sparsomycin inhibited incorporation of labeled amino acid into mouse liver protein and also caused complete hepatic polysome disaggregation [3–5]. An inhibitor of peptidyl transferase activity would be expected to freeze ribosomes on mRNA and so the polysome disaggregation observed *in vivo* is an intriguing finding. In this paper, we confirm the inhibition of protein synthesis and polysome disaggregation produced by sparsomycin in mouse liver *in vivo* and provide evidence as to how this disaggregation occurs based upon a determination of the dissociability and the 4S to 5S molar RNA ratio of disaggregated 80S monosomes. We have previously utilized these parameters to characterize hepatic monosomes produced by other inhibitors of protein synthesis [6–9].

Materials and methods. To determine the acute effect of sparsomycin on the synthesis of mouse liver protein, three experimental animals, male Swiss Webster albino mice weighing 25–30 g were given a single i.p. injection of 1 μg sparsomycin (Drug Development Branch, National Cancer Institute) per g body wt, dissolved in 0.1 ml of 0.9% NaCl solution. Three control mice were injected i.p. with 0.1 ml of a 0.9% NaCl solution. One hr later, experimental and control mice were injected i.p. with 3 μCi [^{14}C]leucine/100 g body wt (New England Nuclear Corp., sp. act., 260 mCi/m-mole). Thirty min later the animals were sacrificed and trichloracetic acid (TCA) precipitable protein was prepared from 1-g samples of liver. The liver samples were homogenized in 5 vol. of 0.25 M sucrose with 20 strokes of a Potter–Elvehjem homogenizer with Teflon pestle. This homogenate was made 10% with respect to TCA, mixed for 1 min with a Vortex mixer, and centrifuged at 10,000 *g* for 10 min at 4°. The pellet was successively washed, once with 10% TCA and three times with 5% TCA. The lipids were extracted with acetone followed by single extractions with ethanol, ethanol and ether (3 : 1), and ether. The precipitate was dried and then hydrolyzed in 5% TCA at 90° for 20 min. The hot TCA insoluble residue was solubilized in 0.5 N NaOH and reprecipitated with 10% TCA. This final TCA insoluble residue was solubilized in 4 ml of 0.5 N NaOH and 1 ml of this solution was suspended in 15 ml Aquasol (New England Nuclear Corp.). Radioactivity was measured in a Mark III liquid scintillation spectrometer model 6880 (Searle Analytical, Inc.). Counting efficiency was approximately 85 per cent as determined by the channels ratio method [10]. Protein content was determined by the method of Lowry *et al.* [11].

For electron microscopic observations, small pieces of liver from control and sparsomycin-treated animals were fixed at 0–4° for 1–2 hr in 2% osmium tetroxide buffered with *S*-collidine at pH 7.4. After fixation, the tissues were dehydrated in a graded series of alcohols and embedded in Epon 812 and Araldite. Ultrathin sections cut with an LKB ultramicrotome were stained with lead hydroxide and examined in an electron microscope.

To determine the degree of polysome disaggregation produced by sparsomycin, postmitochondrial supernatant from experimental and control mouse livers was prepared, layered on linear sucrose gradients, centrifuged, and scanned as previously described [8]. For a determination of the dissociability of the sparsomycin 80S monosomes, the postmitochondrial supernatant from experimental mouse livers was made 0.3 M with respect to KCl, layered on linear sucrose gradients containing 0.3 M KCl, centrifuged for 240 min, and scanned.

To determine the tRNA content of the monosomes produced by sparsomycin, monosome pellets from the livers of mice treated with sparsomycin were resuspended in 5 mM Tris–HCl (pH 7.4) and the number of tRNA molecules per ribosome was determined as previously described [8]. This determination is based upon the method of Kabat [12] and relies upon the theory that since each ribosome contains one 5S rRNA molecule, the number and type of 4S tRNA molecules can be estimated by a calculation of the 4S to 5S molar RNA ratio, with and without prior Pronase treatment. Pronase treatment before RNA extraction removes nascent peptides from peptidyl tRNA and permits extraction of tRNA originally present as tRNA, aminoacyl tRNA, and peptidyl tRNA. Only tRNA from tRNA and aminoacyl tRNA is extracted when RNA is extracted without Pronase pretreatment.

Results. A single injection of 1 μg sparsomycin/g body wt reduced the incorporation of [^{14}C]leucine into TCA precipitable protein by 95 per cent, i.e. from 194 $\pm$ 19 dis./min/mg protein to 9 $\pm$ 7 dis./min/mg protein.

The livers of mice given a single dose of 1 μg sparsomycin/g body wt and killed at 30 min, 1 hr, 4 hr, 12 and 24 hr after the injection were examined by electron microscopy. At 1 and 4 hr, detachment of ribosomes from the endoplasmic reticulum channels was prominent. Numerous single ribosomes were seen scattered throughout the cytoplasm. Cytoplasmic accumulation of lipid droplets was noted in liver cells at 12 hr. No evidence of nucleolar segregation was noted at any of the intervals studied. By 24 hr, the cytoplasmic changes reversed almost completely in many liver cells.

A comparison of Fig. 1A and B reveals that a single i.p. injection of 1 μg sparsomycin/g body wt produces complete hepatic polysome disaggregation within 1 hr when compared with the polysome profile from a control mouse. Figure 1C demonstrates that the 80S monosomes produced by sparsomycin dissociated into 40S and 60S subunits in linear sucrose gradients containing 0.3 M KCl, implying that these monosomes are free of mRNA [6–9].

From Table 1, it can be seen that control ribosomes contain 0.9 molecule tRNA and/or aminoacyl tRNA and 0.8 molecule peptidyl tRNA/ribosome. Sparsomycin monosomes contain 0.9 molecule tRNA and/or aminoacyl tRNA and 0.7 molecule peptidyl tRNA/ribosome.

Discussion. Our results confirm those of Trakatellis [3] and Sarma *et al.* [5], demonstrating that sparsomycin administered *in vivo* in mice reduces incorporation of [^{14}C]leucine into liver proteins by 95 per cent and produces complete hepatic polysome disaggregation within 1 hr.

In the present study, electron microscopy demonstrated that administration of sparsomycin *in vivo* causes detachment of ribosomes from the endoplasmic reticulum. However, this finding may be an artifact, since Sarma *et al.* [4] previously reported that, even though sparsomycin *in vivo* causes apparent detachment of hepatic membrane-bound ribosomes by electron microscopy, these ribosomes remained attached to the membranes by a density gradient centrifugation.

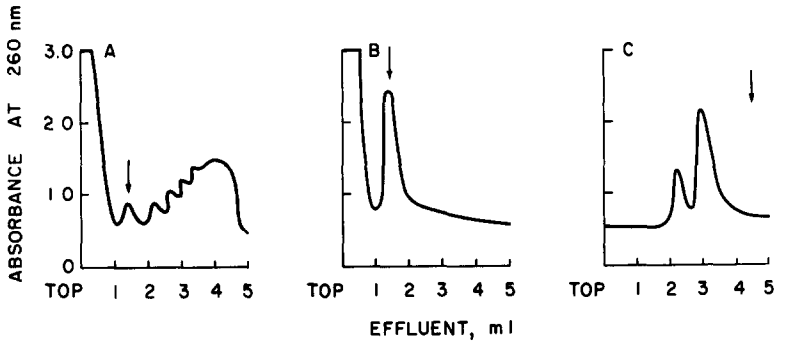

Fig. 1. Ribosome-polysome profiles from sparsomycin-treated and control mouse livers in 0.5 to 1.2 M linear sucrose gradients. The arrow indicates the position of the 80S peak. (A). Control ribosome-polysome profile (0.9% NaCl solution), postmitochondrial supernatant, centrifuged for 90 min.; and (B). and (C). 1 hr after a single i.p. injection of 1 μg sparsomycin/g body wt. In panel B, the postmitochondrial supernatant was centrifuged for 90 min.; in panel C, the postmitochondrial supernatant was made 0.3 M with respect to KCl and centrifuged for 240 min.

The 80S monosomes produced by sparsomycin are dissociable in a 0.3 M KCl solution and contain tRNA and/or aminoacyl tRNA as well as peptidyl tRNA. Thus, sparsomycin monosomes differ from both runoff ribosomes, which dissociate in 0.3 M KCl but lack peptidyl tRNA, and complexed ribosomes, which do not dissociate in 0.3 M KCl. It has been shown that ribosomes free of mRNA are dissociable into subunits, while ribosomes attached to mRNA are not dissociable even when stripped of nascent peptides [13]. Thus, the dissociability of sparsomycin monosomes suggests that they are free of mRNA, and the 4S to 5S ratio with and without Pronase treatment suggests that they may have arisen by prematurely falling off mRNA during elongation, since tRNA and/or aminoacyl tRNA and peptidyl tRNA are still attached. The production of falloff monosomes by administration of sparsomycin *in vivo* may require the presence of an enzyme(s) or other factor(s) not present in systems *in vitro*.

The uniqueness of falloff monosomes produced by administration of sparsomycin *in vivo* in mice should be emphasized. We have previously reported only one other agent which produces falloff monosomes *in vivo* in mouse liver, this being the hepatotoxic and hepatocarcinogenic agent dimethylnitrosamine [6]. Since sparsomycin and dimethylnitrosamine appear to disaggregate polysomes via a similar mechanism, it may be of interest to further compare and contrast the two.

Table 1. Transfer RNA content of ribosomes*

Type of ribosome	4S:5S Ratio with Pronase	4S:5S Ratio without Pronase
Control ribosomes (0.9% NaCl solution)	1.7	0.9
Sparsomycin monosomes	1.6	0.9

* Ribosome pellets were suspended in 5 mM Tris–HCl (pH 7.4). RNA was extracted immediately or after incubation for 1 hr at 30° with 3 mg/ml of Pronase and 4 mg/ml of sodium dodecyl sulfate prior to extraction. RNA was precipitated overnight at −18° with 2 vol. ethanol and 0.2 M NaCl. RNA was then analyzed by electrophoresis in 4% polyacrylamide gels and the gels were scanned at 260 nm, as previously described [6]. The 4S to 5S molar RNA ratio was calculated by multiplying the value of the 4S peak by 1.5 and by dividing by the value of the 5S peak [12].

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