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EFFECT OF SPARSOMYCIN *IN VIVO* AND *IN VITRO* ON HEPATIC POLYRIBOSOMES AND PROTEIN SYNTHESIS

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Abstract—Sparsomycin (20 μg/20 g of body wt), when administered intraperitoneally to mice, induced marked disaggregation of hepatic polyribosomes and inhibited incorporation of [^{14}C]leucine into hepatic proteins by 90 per cent. However, addition of sparsomycin *in vitro* to a cell-free amino acid incorporating system did not cause disaggregation of polyribosomes, yet inhibited the incorporation of [^{14}C]leucine into proteins by 80 per cent. In studies concerned with initiation of protein synthesis, it was observed that sparsomycin inhibited the factor-dependent initiation of new polyphenylalanine chains, as well as factor-dependent binding of either phe-*t*RNA or met-*t*RNA to 40S ribosomal subunits. These findings are interpreted as suggesting that, *in vivo*, sparsomycin disaggregates polyribosomes probably by interfering with the initiation process.

SPARSOMYCIN, a sulfur-containing antibiotic which inhibits protein synthesis in both bacterial and mammalian cells, is a useful tool for studies concerned with the mechanism of protein biosynthesis. It is one of a few agents which specifically inhibits protein synthesis in both 70S and 80S ribosomal systems.[1–4] It acts by a different mechanism than that utilized by some other known inhibitors of protein synthesis.[5–8] This antibiotic has been found to block peptide bond formation and thus prevents the transfer of amino acids from *t*RNA to the nascent polypeptide chains.[3–5]

Our present study was concerned with the effect of sparsomycin *in vivo* and *in vitro* on polyribosomes and protein synthesis in mouse liver. The results indicated that the administration of sparsomycin *in vivo* resulted in disaggregation of hepatic polyribosomes and in inhibition of hepatic protein synthesis. On the other hand, addition of this antibiotic to a cell-free amino acid incorporating system inhibited protein synthesis, but it did not cause disaggregation of hepatic polyribosomes, but rather decreased the release of ribosomes from mRNA. These paradoxical results obtained with sparsomycin on hepatic polyribosomes *in vivo* and *in vitro* and the possible sites of action of this antibiotic are discussed. A preliminary report has been presented earlier.[9]

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MATERIALS AND METHODS

Female mice (Hilltop Lab Animals, Scottdale, Pa.) were maintained on a commercial ration (Wayne Lab Blox). The animals were fasted overnight and had free access to water throughout. The following morning, mice weighing 20–22 g were divided into groups. Experimental animals were injected intraperitoneally with sparsomycin (20 μg/20 g of body wt; a gift from the National Cancer Institute) and/or cycloheximide (2 mg/20 g of body wt; Upjohn Company), while control animals received intraperitoneally equal volumes of saline. In incorporation experiments *in vivo*, L-leucine[^{14}C] (U), 5 μCi/mouse (10 mCi/m-mole; Amersham/Searle), was administered intraperitoneally. In each experiment, livers from four mice in each group were pooled. Preparation of postmitochondrial supernatant (PMS), determination of size distribution of polyribosomes of deoxycholate-treated (DOC) PMS, preparation of C-polyribosomes and amino acid incorporation *in vitro* using either PMS or polyribosomes were carried out as described earlier.[10] Determination of radioactivity incorporated into total liver proteins was carried out as described earlier.[11] Alkaline and acid ribonuclease activities in various hepatic subcellular fractions were estimated by the method of Rahman.[12]

Initiation factors and salt-washed ribosomes from crude liver ribosomes were prepared as reported previously.[13] Ribosomes were prepared by centrifugation of DOC-treated PMS for 2 hr at 40,000 rev/min at 4° using a Spinco 40 rotor in a model L2-65 ultracentrifuge. The ribosomal pellets were rinsed and resuspended in 10 ml buffer A, which contained: 20 mM Tris HCl, pH 7·4; 5 mM Mg^{2+}; 100 mM KCl; 1 mM dithioerythritol; 0·25 mM EDTA and 10% glycerol. The ribosomal suspension was treated with a high salt buffer (20 mM Tris HCl, pH 7·4; 15 mM Mg^{2+}; 2 M KCl; 1 mM dithioerythritol; 0·25 mM EDTA; 10% glycerol) to a final KCl concentration of 1 M. The suspension was maintained at 0° for 1 hr with occasional stirring and then the ribosomes were sedimented at 60,000 rev/min in the Spinco 65 rotor for 3 hr. The supernatant containing initiation factors was carefully removed, KCl was dialyzed out, and it was concentrated by $(NH_4)_2SO_4$ precipitation. The precipitate was dissolved in a buffer containing 20 mM Tris HCl (pH 7·4), 5 mM Mg^{2+}, 200 mM KCl, 1 mM dithioerythritol, 0·25 mM EDTA, 15% glycerol and dialyzed against this same buffer and stored at −20°. To prepare salt-washed ribosomes, the pellets, obtained above after centrifugation of 1 M KCl-treated ribosomes, were resuspended in a buffer containing 20 mM Tris HCl (pH 7·4), 10 mM Mg^{2+}, 500 mM KCl, 1 mM dithioerythritol, 0·25 mM EDTA and centrifuged through 5 ml of 25% sucrose containing the above buffer for 5 hr at 40,000 rev/min. The pellets were resuspended and dialyzed against buffer A.

Ribosomal subunits, 60S and 40S, were prepared as described by Leader and Wool.[14] The nature and purity of the isolated ribosomal subunits were examined by analyzing the RNA components of isolated subunits on linear sucrose gradients as described earlier.[15]

Labeled mouse hepatic phenylalanine-*t*RNA and methionyl-*t*RNA were prepared by the method of Moldave[16] with some modifications described by Pilkis and Korner.[17] pH 5 enzyme was prepared from livers of mice and was used as a source of *t*RNA and of aminoacyl-*t*RNA synthetases. *t*RNA was charged with either [^{14}C](U)-L-phenylalanine (513 mCi/m-mole) or L-methionine (methyl[^{14}C]) (59 mCi/m-mole), along with 19 unlabeled amino acids. A mixture of transferases I

and II was prepared from pH 5 supernatant of mouse liver by the method of Moldave.[16]

RESULTS

Studies in vivo. Sucrose density gradient profiles of hepatic polyribosomes of DOC-treated PMS of control animals and of animals treated with sparsomycin 2 hr before being killed are presented in Fig. 1. Hepatic polyribosomal disaggregation occurred with a low dose (2·5 μg/20 g of body wt) of sparsomycin. The degree of disaggregation of hepatic polyribosomes progressively increased after the administration of increasing amounts (2·5–10·0 μg) of sparsomycin (Fig. 1). However, polyribosome breakdown appeared to have reached a maximum at a dose of 10–20 μg/20 g of body wt.

Polyribosomal profiles were observed in one experiment after 20, 35, 50 and 65 min following the administration of sparsomycin (20 μg/20 g of body wt). They revealed a progressive disaggregation beginning after 20 min, where the change was minimal, and becoming maximal after 65 min, where the pattern revealed marked disaggregation similar to that illustrated in Fig. 2. The polyribosomal profiles presented in Fig. 2 indicate that when sparsomycin was administered at a dose of 20 μg/20 g of body wt, maximal disaggregation of hepatic polyribosomes was seen by 1 hr after the administration of sparsomycin and this effect persisted up to 4 hr. Under these conditions, incorporation of L-leucine-[^{14}C] *in vivo* into total liver proteins of sparsomycin-treated animals was inhibited by 90 per cent after 1 hr and this inhibition was observed up to 3 hr after the administration of sparsomycin.

In order to test for the possibility that the disaggregation of hepatic polyribosomes by sparsomycin was due to changes in ribonuclease activity, this was investigated in several experiments. Acid and alkaline ribonuclease activities in various subcellular fractions of livers of control and sparsomycin-treated mice were estimated. The

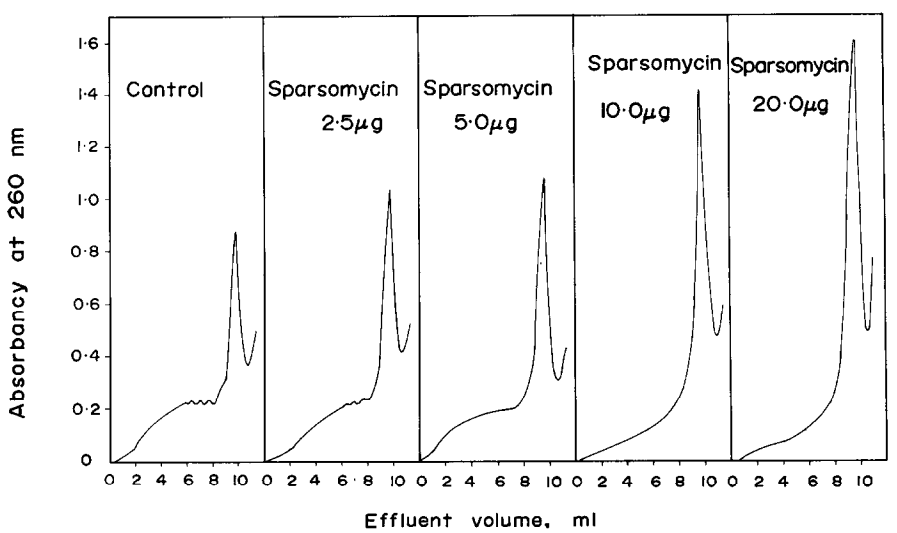

FIG. 1. Sucrose density gradient patterns of polyribosomes prepared from deoxycholate-treated postmitochondrial supernatants of livers of control and sparsomycin-treated animals. Experimental animals were injected intraperitoneally with 2·5, 5, 10 and 20 μg of sparsomycin per 20 g of body wt 2 hr before being killed. Control animals received saline. Livers from four animals were pooled in each group.

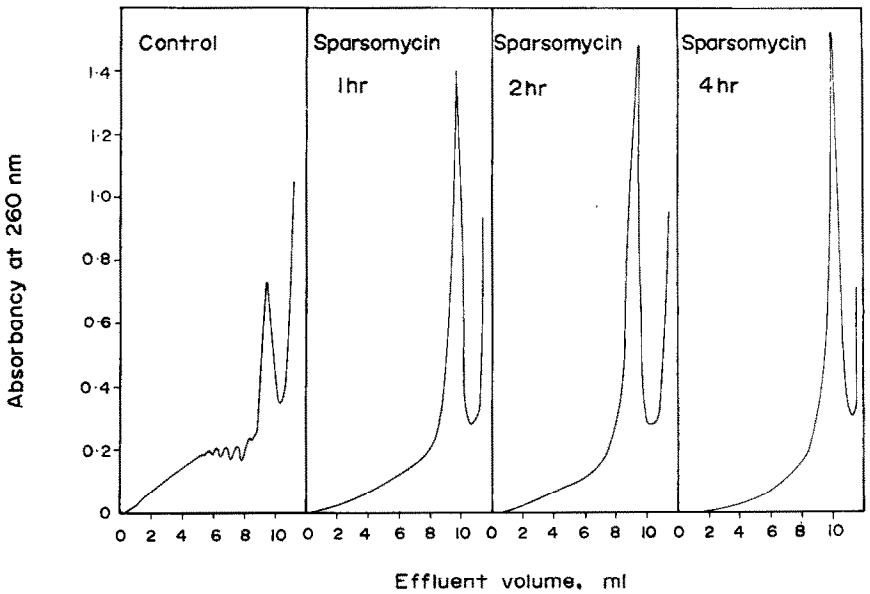

FIG. 2. Sucrose density gradient patterns of polyribosomes prepared from deoxycholate-treated postmitochondrial supernatants of livers of control and experimental animals. Experimental animals were injected intraperitoneally with 20 μg/20 g of body wt of sparsomycin and the animals were sacrificed at selected intervals (1–4 hr). Control animals received saline. Livers from four animals were pooled in each group.

results (unpublished data) indicated that the levels of both acid and alkaline ribonuclease activities in particulate, postmitochondrial supernatant and microsomal fractions of liver were not increased due to the administration of sparsomycin. In fact, we observed that the levels of ribonuclease activity in the livers of mice treated with sparsomycin were 15–20 per cent less compared to that of control mice. Thus, this finding rules out the possible interference by sparsomycin in the metabolism of the polyribosome cycle via altered ribonuclease activity.

Studies in vitro. The time course of L-leucine[^{14}C] incorporation by a cell-free system using postmitochondrial supernatant (PMS) in the presence of sparsomycin is depicted in Fig. 3. In this experiment, PMS was obtained from livers of control mice and the PMS was incubated for various times in the presence of sparsomycin (2 μg/ml of PMS). The results in Fig. 3 indicate that, using mouse liver PMS, incorporation of leucine[^{14}C] *in vitro* into proteins was inhibited approximately 83 per cent by sparsomycin after a 5-min incubation period and this inhibition (at least 68 per cent) persisted up to 60 min of incubation. In another group of experiments, the time course (2–10 min) of L-leucine[^{14}C] incorporation into proteins by a cell-free system using purified C-polyribosomes[10] with or without sparsomycin was studied. The results were similar to those described in Fig. 3 and revealed a 60–80 per cent inhibition of incorporation due to sparsomycin. After a 10-min incubation period, the ribosomes were analyzed on sucrose gradients. The results in Fig. 4 show that the degree of disaggregation of polyribosomes in the presence of sparsomycin (Fig. 4c) was much less than that of controls (Fig. 4b) even though there was significant inhibition *in vitro* of leucine[^{14}C] incorporation into proteins due to sparsomycin. Actually the antibiotic appeared to cause an inhibition of the release of ribosomes from the

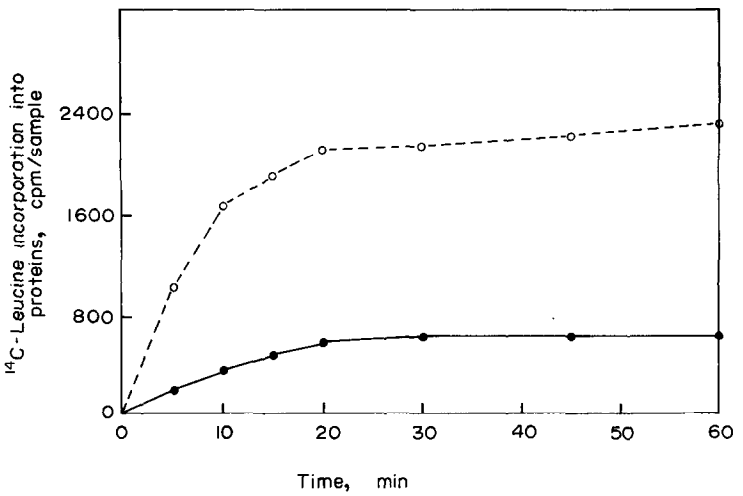

FIG. 3. Effect of sparsomycin on the kinetics of [^{14}C]leucine incorporation *in vitro* into proteins using a cell-free system containing postmitochondrial supernatant of mouse liver. Three experiments were carried out. In each experiment, post-mitochondrial supernatant was prepared from pooled livers of ten fasted mice. Sparsomycin, 2 μg, was added to 1 ml postmitochondrial supernatant and incubated at 25° with an amino acid incorporating system which contained 1·5 μmoles 2-mercaptoethanol, 1·0 μmole ATP, 0·4 μmole GTP, 10 μmoles phosphoenolpyruvate, 6·5 μg pyruvate kinase, 30 μmoles Tris HCl (pH 7·4), 150 μmoles NH_4Cl, 3·5 μmoles $MgCl_2$ and 0·5 μCi L-leucine-[^{14}C]. At indicated intervals, equal portions of the reaction mixture from control and experimental samples were removed. The reaction was stopped with 0·6 N perchloric acid containing 20 mg Celite per ml and the precipitates were washed. Hot 5% trichloroactic acid-insoluble radioactivity was measured. Control, (○); experimental, (●).

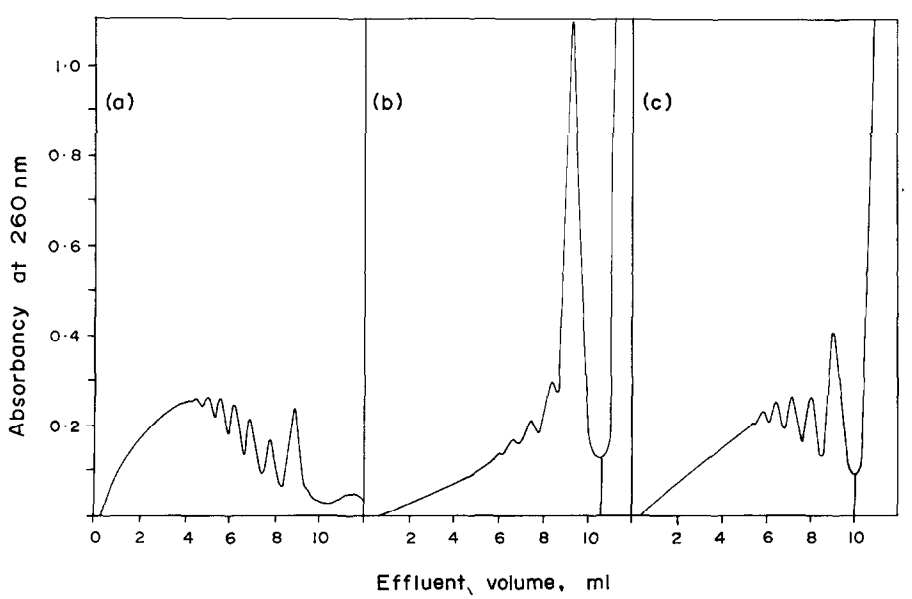

FIG. 4. Sucrose density gradient analysis of C-polyribosomes after incubation using a complete cell-free system, with or without added sparsomycin. C-polyribosomes were prepared from pooled livers of mice, as described in Materials and Methods, and aliquots containing equal amounts of O.D. 260 units were incubated with a complete cell-free system described in the legend to Fig. 3. (a) Unincubated control, incubated for 0 min at 0°; (b) control, incubated for 10 min at 37°; (c) experimental, incubated for 10 min at 37° with sparsomycin (4 μg/ml of incubation mixture).

messenger RNA (compare Fig. 4c with 4b). This observation is in agreement with data reported by Trakatellis[8] from a similar experiment.

Recently it has been frequently shown in several mammalian systems, including liver, that initiation factors obtained from ribosomal washes (KCl washes of ribosomes) stimulate initiation of new polyphenylalanine chains at low Mg^{2+} concentrations,[13,18–20] and further, these factors are specifically involved in the formation of initiation complex, 40S-aminoacyl-*t*RNA–mRNA.[21–23] It has been further shown that several agents, such as sodium fluoride[19] and aurintricarboxylic acid,[24] strongly inhibit the initiation of new polyphenylalanine chains as well as the binding of aminoacyl-*t*RNA to the 40S ribosomal unit. Therefore, we tested whether sparsomycin interferes with the initiation process of polyphenylalanine synthesis and with the binding of aminoacyl-*t*RNA to the 40S ribosomal subunit.

The results of four experiments in which the effect of sparsomycin upon initiation of polyphenylalanine synthesis was measured revealed that the addition of sparsomycin (5 or 10 μg) to the complete system caused a 94 per cent decrease in the polyphenylalanine synthesis which is dependent upon initiation factors. The addition of aurintricarboxylic acid (56 μg) caused a 91 per cent decrease in polyphenylalanine synthesis. Table 1 shows the initiation factors-dependent binding of [^{14}C]phenylalanine-*t*RNA and [^{14}C]methionyl-*t*RNA to 40S ribosomal subunits. The results show that the initiation factors specifically catalyzed the poly U-dependent binding of [^{14}C]phe-*t*RNA as well as AUG-dependent binding of [^{14}C]met-*t*RNA to 40S

TABLE 1. EFFECT OF SPARSOMYCIN ON INITIATION FACTORS-DEPENDENT BINDING OF [^{14}C]phe-*t*RNA AND [^{14}C]met-*t*RNA TO 40S RIBOSOMAL SUBUNITS*

System	Amount of [^{14}C]phe-*t*RNA bound (cpm)	Amount of [^{14}C]met-*t*RNA bound (cpm)
Complete	451	306
Complete – 40S subunits	19	14
Complete – Initiation factors	16	25
Complete – Poly-U	43	
Complete – AUG		17
Complete with 60S subunits replacing 40S subunits	82	31
Complete + sparsomycin	27	19
Complete + aurintricarboxylic acid	34	17

* Initiation factors and 60S and 40S ribosomal subunits were prepared from livers of fasted mice as described in Materials and Methods. The assay for the binding of [^{14}C]phe-*t*RNA to 40S subunits at low Mg^{2+} concentrations was carried out by the method of Shafritz *et al.*[18] Assay for the binding of [^{14}C]met-*t*RNA was carried out as described by Nirenberg and Leder.[25] The complete system in a total volume of 0·2 ml contained 40S subunits (60 μg); initiation factors (100 μg protein); poly-U (50 μg) or AUG ($0{\cdot}2^{O.D.260}$ unit); [^{14}C]phe-*t*RNA (5800 cpm) or [^{14}C]met-*t*RNA (5800 cpm); mg^{2+} (1 μmole); Tris HCl, pH 7·5 (4 μmoles); KCl (20 μmoles); dithioerythritol (0·2 μmole); and GTP (0·4 μmole). When 40S ribosomal subunits were replaced in the complete system, the reaction mixture contained 120 μg 60S ribosomal subunits. When inhibitors were added to the complete system, the reaction mixture contained 5 μg sparsomycin or 56 μg aurintricarboxylic acid. The reaction mixtures were incubated for 15 min at 30° in the case [^{14}C]phe-*t*RNA binding and for the binding of [^{14}C]met-*t*RNA, the reaction mixtures were incubated for 5 min at 23°. The reactions were stopped by the addition of 3 ml cold buffer (20 mM Tris HCl, pH 7·5) and immediately filtered through Millipore filters. The filters were washed three times with cold buffer, dried and counted after the addition of 15 ml Bray's solution, in a Packard liquid scintillation spectrometer.

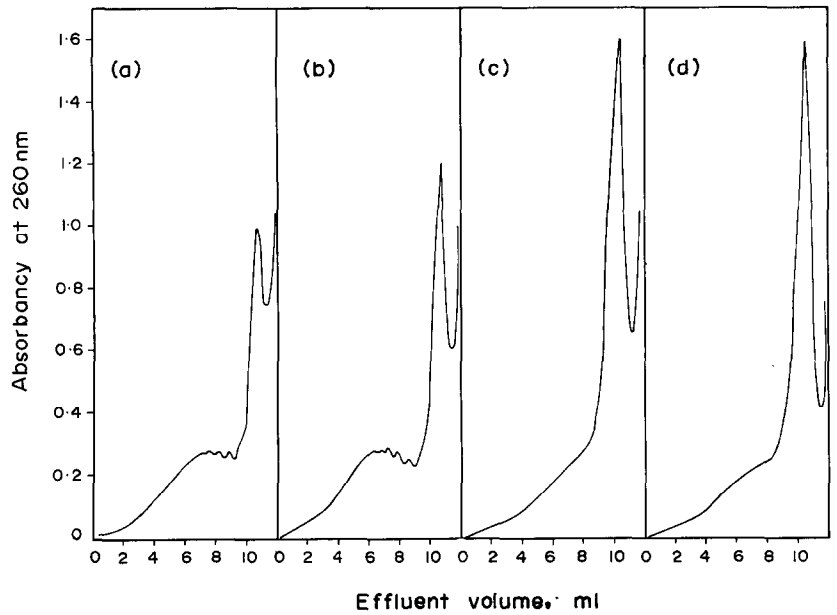

FIG. 5. Sucrose density gradient patterns of polyribosomes prepared from deoxycholate-treated postmitochondrial supernatants of livers of control and experimental animals. All mice were fasted overnight and then divided into four groups, each containing four mice. The groups received the following treatments: (a) saline; (b) cycloheximide (1 mg/10 g of body wt) 1 hr before killing; (c) sparsomycin (20 μg/20 g of body wt) 1·5 hr before killing; and (d) sparsomycin (20 μg/20 g of body wt) at zero time, cycloheximide (1 mg/10 g body wt) after 1·5 hr and the animals were killed 1 hr later. Four experiments were carried out and in each experiment livers from four mice were pooled in each group.

ribosomal subunits; the addition of sparsomycin completely reduced this factor-promoted binding of aminoacyl-*t*RNA to small ribosomal subunits.

Effect of cycloheximide in vivo. It has been shown that the administration of cycloheximide causes a reformation of polyribosomes previously disaggregated by either puromycin or ethionine in rat liver[26] or by sodium fluoride in reticulocytes.[27] In the present study we investigated whether cycloheximide could reassemble the polyribosomes disaggregated by sparsomycin. The results presented in Fig. 5 indicate that polyribosomes disaggregated by sparsomycin (Fig. 5c) could not be reformed to any significant extent after the administration of cycloheximide (Fig. 5d).

DISCUSSION

The results presented in this study demonstrate that sparsomycin inhibits protein synthesis both *in vivo* and *in vitro*; however, it causes disaggregation of polyribosomes *in vivo* but not *in vitro*. Previous studies using a cell-free system from *Escherichia coli*[3–5] and from mouse liver[8] suggested that sparsomycin binds to the larger ribosomal subunits and inhibits peptidyl transferase, an enzyme responsible for peptide bond formation. Accordingly, sparsomycin or any other agent that inhibits protein synthesis at translocation or at the peptidyl synthetase stage should freeze polyribosomes and therefore should not lead to disaggregation of polyribosomes. On the other hand, if a compound inhibits protein synthesis by primarily interfering with

the initiation process, then such a situation should lead to disaggregated polyribosomes. Our results on the effects of sparsomycin *in vivo* on hepatic polyribosomes indeed suggest that the latter mechanism may be involved. The results presented in Figs. 1 and 2 indicate that the administration of sparsomycin to mice, instead of inhibiting the release of ribosomes from polyribosomes, actually results in disaggregation of polyribosomes. Such a disaggregated polyribosomal pattern could result either from an interference in the rate of attachment of ribosomes to *m*RNA or from faster readout. In either case, the administration of cycloheximide, an agent that appears to interfere with peptide chain release,[28–30] should result in the preservation of polyribosomes. The data in Fig. 5 indicate that the administration of cycloheximide to mice did not reform hepatic polyribosomes previously disaggregated by sparsomycin. Hence, this suggests that the disaggregation of polyribosomes induced by sparsomycin may not be due to a decreased rate of attachment or to an increased rate of readout of ribosomes from polyribosomes, but rather it may be due to an effect of sparsomycin at the initiation site.

The results show that sparsomycin strongly inhibited the factor-dependent initiation of new polyphenylalanine chains, which indeed suggests that sparsomycin *in vivo* may act at the initiation process by not allowing further attachment of new ribosomal subunits at the initiation sites of *m*RNA. Further studies supporting that sparsomycin may act at the initiation level have been presented in Table 1. Sparsomycin inhibited the initiation factors-dependent binding of aminoacyl-*t*RNAs to the 40S ribosomal subunits. It is interesting to note that sparsomycin is as effective an inhibitor as aurintricarboxylic acid, which is a well known inhibitor of initiation of protein synthesis.[24] Therefore, it is possible that sparsomycin *in vivo* may stabilize the initiation complex or inhibit the formation of the initiation complex, thus preventing the attachment of ribosomal subunits onto *m*RNA.

In earlier studies dealing with the influence of dietary tryptophan on the regulation of hepatic polyribosomes, we observed that the administration of tryptophan to fasted mice[11] or to mice pretreated with toxic agents such as ethionine,[31] actinomycin D[32] or puromycin,[33] which induce polyribosomal disaggregation, caused a corrective effect on the hepatic polyribosomes and protein synthesis. The effect of tryptophan on hepatic polyribosomes in fasted mice is probably due to an increase in the levels of cytoplasmic *m*RNA, which may initiate new chains of polyribosomes.[34] Therefore, it became of interest to determine how tryptophan would act on or influence sparsomycin-treated mice. The administration of tryptophan could neither reform the polyribosomes broken down by sparsomycin nor restore the normal rate of protein synthesis.

Since initiation of protein synthesis does not occur or is minimal when using polyribosome preparations *in vitro*, the addition of sparsomycin to a cell-free amino acid incorporating system would not be expected to result in any greater disaggregation of polyribosomes than that which occurs in the control. Therefore, the partial inhibition of disaggregation of polyribosomes incubated in the presence of sparsomycin in a cell-free system (Fig. 4c) is most probably due to inhibition of peptidyl synthetase activity, as reported by other workers.[5,8] Since the administration of sparsomycin *in vivo* results in marked disaggregation of hepatic polyribosomes, it is possible that sparsomycin *in vivo* may not inhibit peptidyl synthetase or, even if it does, it is to a much lesser degree than its effect at the initiation site.

Our present studies suggest that sparsomycin, depending upon the conditions, may have a dual effect on the structural integrity of hepatic polyribosomes. Thus, as one studies in detail the action of certain antibiotics such as sparsomycin, it becomes apparent that they may act in more than one way, depending upon a variety of circumstances.

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