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Comparison of cycloheximide effect on hepatic protein synthesis at the microsomal level *in vivo* and *in vitro*

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CYCLOHEXIMIDE is an inhibitor of protein synthesis *in vivo*[1, 2] and *in vitro*.[3] However, in our previous studies[4–7] we were able to show that cycloheximide administration to rats *in vivo* stimulated the L-^{14}C-phenylalanine incorporation *in vitro* in an incubation system with hepatic microsomes isolated from treated animals. We established that this cycloheximide-induced stimulation on the system *in vitro* was localized in the microsomal fraction, was independent of changes of cofactor requirements, unrelated to the stabilization of endogenous RNA, and probably dependent on the mediation of the adrenal glands.

There were reports in the literature[2, 3, 8–10] that the aggregation of the ribosomes in the microsomal fraction might be affected by cycloheximide and that such a shift in the degree of aggregation could account for the influence of cycloheximide on protein synthesis.

We have now investigated some of the differences between cycloheximide-induced effects on protein synthesis *in vivo* and *in vitro*. We find that the degree of aggregation of the microsomal fraction is not altered by cycloheximide treatment *in vivo* or *in vitro* under our experimental conditions. We find that cycloheximide pretreatment *in vivo* inhibits amino acid incorporation into protein *in vivo* (except in adrenalectomized animals), but stimulates protein synthesis *in vitro*. The addition of cycloheximide to the incorporation system *in vitro* reverses the stimulation of protein synthesis brought about by prior injection of cycloheximide *in vivo*.

MATERIALS AND METHODS

Cycloheximide was obtained from the Cancer Chemotherapy National Service Centre (thanks to Dr. J. A. R. Mead). Animals used were female Sprague–Dawley derived rats weighing 160 g each,

supplied by Dublin Laboratory Animals (Dublin, Va.). The rats treated with cycloheximide were given 1 mg/kg i.p. in a volume of 0·8 ml water. Control animals were injected with water only. All experimental animals were starved in the 24-hr period between injection and sacrifice, but were allowed water *ad libitum*.

In studies with amino acid incorporation *in vivo*, control and cycloheximide-treated rats were injected i.p. with uniformly labeled L-^{14}C-leucine (20 μc/2 μmole/0·8 ml) 20 min before killing by stunning and decapitation. The livers were removed and homogenized under standard conditions with 5 vol. of medium, according to Henshaw *et al.*[11] After differential centrifugation at 600 *g*, 15,000 *g*, and 105,000 *g*, the microsomal pellets were redispersed in the manner described by Henshaw *et al.*[11] Aliquots of material to be studied (15 O.D.$_{260}$ units) were carefully layered over 4 ml of a linear gradient of 5–20% sucrose in the resuspension buffer and in turn layered over 1 ml of 50% buffered sucrose. Centrifugation was for 1 hr at 39,000 rpm in the SW-39 head of the Spinco model L ultracentrifuge. Ten-drop fractions were then taken from the bottom of the gradient, diluted with 1 ml water, and analyzed for optical density (O.D.) at 260 mμ. After the O.D. measurement, each sample was transferred to tubes containing 1 mg of carrier protein. Radioactivity was then determined by precipitation with 10% trichloroacetic acid and purification by a simplified Siekevitz[12] procedure, followed by counting in a scintillation counter as described by Jondorf *et al.*[7]

In studies *in vitro*, microsomal fractions were prepared from the unlabeled livers of control and cycloheximide-treated rats as described by Jondorf *et al.*[7] Protein concentrations of microsomal fractions were adjusted to the same values after analysis by the method of Lowry *et al.*[13] Incubations were then performed for 15 min at 37° for the incorporation of L-^{14}C-phenylalanine, as previously described,[7] in the presence of 105,000 *g* supernatant material from control animals. After the incubation, the materials were chilled, diluted with ice-cold medium of Henshaw *et al.*[11] to a total volume of 12·5 ml, and then centrifuged for 1 hr in the Spinco model L centrifuge at 105,000 *g*. The pellets at the end of this time were rinsed with suspending medium and redispersed as described by Henshaw *et al.*[11] Aliquots of the redispersed incubated microsomes were then layered on sucrose gradients as above and centrifuged for 1 hr at 39,000 rpm. Ten-drop fractions were collected at the end of this time and analyzed for O.D. at 260 mμ and for radioactivity as before.

RESULTS

In vivo *incorporation experiments*. Sucrose density gradient analysis of the microsomal fractions from livers of control and cycloheximide-treated rats indicates that the sedimentation profiles are essentially identical, each with a narrow heavy peak and a broader light peak (Fig. 1). The ratios of aggregated to free ribosomes (cf. Henshaw *et al.*[11]) in control and cycloheximide-treated hepatic

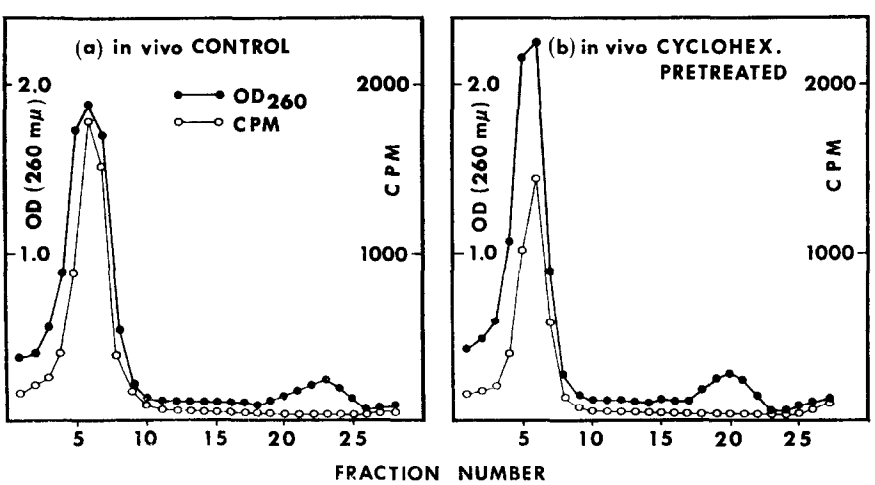

FIG. 1. Sucrose gradient analysis of hepatic microsomal fractions from (a) control and (b) cycloheximide-treated rats after labeling *in vivo* with L-^{14}C-leucine.

microsomal fractions are 6·3 and 5·6 respectively (Table 1). Whereas phenobarbital pretreatment of rats increases the ratio of aggregated to free ribosomes,[14] and thereby affords a partial explanation for the changes in the amino acid incorporation observed after phenobarbital pretreatment, cycloheximide has no such effect. The results also indicate that the incorporation of L-^{14}C-leucine into

TABLE 1. SUMMARY OF DATA FROM FIGS. 1 AND 2 SHOWING TOTAL PEAK (AGGREGATED, agg and FREE, fr) OPTICAL DENSITIES (O.D.) AND ASSOCIATED RADIOACTIVITY (cpm)

	ΣO.D.$_{agg}$	ΣO.D.$_{fr}$	Σcpm$_{agg}$	Σcpm$_{fr}$	$\frac{\Sigma O.D._{agg}}{\Sigma O.D._{fr}}$	$\frac{\Sigma cpm_{agg}}{\Sigma O.D._{agg}}$
In vivo						
Cont.	8·47	1·34	5936	274	6·3	701
Cyc.	8·47	1·50	4262	312	5·6	503
In vitro						
Cont.	5·39	2·32	539	226	2·3	100
Cyc.	5·46	2·48	1038	357	2·2	190
Cyc. + cyc.	5·39	3·07	351	261	1·8	65

The last two columns indicate ratios of aggregated to free ribosomes and specific activities of aggregated ribosomes

microsomal protein is inhibited in the cycloheximide-treated rats. The sp. act. (cpm/O.D.$_{260}$) at the peak of the heavy aggregated ribosome region are 1005 and 644 respectively for control and cycloheximide-treated microsomes. The overall incorporation into microsomal protein is inhibited in the cycloheximide-treated rats by 28 per cent (Table 1). This is in agreement with Young *et al.*[1] and Traketellis *et al.*[2] In corresponding experiments with adrenalectomized rats (5 days postoperative), cycloheximide administration did not inhibit amino acid incorporation into microsomal protein *in vivo*.

In vitro *incorporation experiments*. The distribution of O.D. and radioactivity in incubated hepatic microsomal fractions from control and cycloheximide-pretreated animals is shown in Fig. 2a and b. It is clear that there are no differences in the ratios of aggregated to free ribosomes (Table 1) in control and cycloheximide-pretreated samples. However, the radioactivity associated with the aggregated ribosomes in the sample from cycloheximide-pretreated animals is much greater than the corresponding amino acid incorporation from the controls. The aggregated ribosomes from cycloheximide-pretreated sources have a greater peak specific activity (198) than the control (104) and greater specific activity overall (Table 1).

The results of a critical experiment are illustrated in Fig. 2c. Microsomal fractions from livers of animals pretreated with cycloheximide *in vivo*, incubated with L-^{14}C-amino acid in the presence of cycloheximide *in vitro*, and then applied to a sucrose gradient as described, reveal the difference between cycloheximide action *in vivo* and *in vitro*. Amino acid incorporation, as evidenced by radioactivity associated with the aggregated ribosomes, is inhibited to a level below that of the control (Table 1). This result shows quite clearly that the addition of cycloheximide to the incubation system *in vitro* not only inhibits protein synthesis as expected,[3] but also reverses the stimulation of amino acid incorporation associated with cycloheximide pretreatment *in vivo* described by Jondorf *et al.*[4-7]

DISCUSSION

The physical and functional heterogeneity of microsomal cell fractions derived from the endoplasmic reticulum has been described by Fouts[15] and Siekevitz.[16]

It is now well established that only the aggregated ribosomes of the microsomal fraction are active in protein synthesis and that a dynamic model, in which ribosomes in the aggregated polysomes move as independent entities along molecules of messenger RNA, is consistent with the findings in various cellular and subcellular systems.[2, 11, 17-24]

We find that cycloheximide pretreatment *in vivo* has no tangible effect on the dynamic equilibrium between aggregated and free ribosomes existing during conditions of protein synthesis in the liver

either *in vivo* or *in vitro*. This is in agreement with previous work on mouse liver[2] and reticulocytes,[8] but conflicts with the findings of Wettstein *et al.*[3] and of Williamson and Schweet,[9] who detected a shift toward polyribosomes in systems under the influence of cycloheximide.

The effect of incubation on the equilibrium between aggregated and free ribosomes during amino acid incorporation *in vitro* is a shift toward free ribosomes. This shift is the same for microsomes from

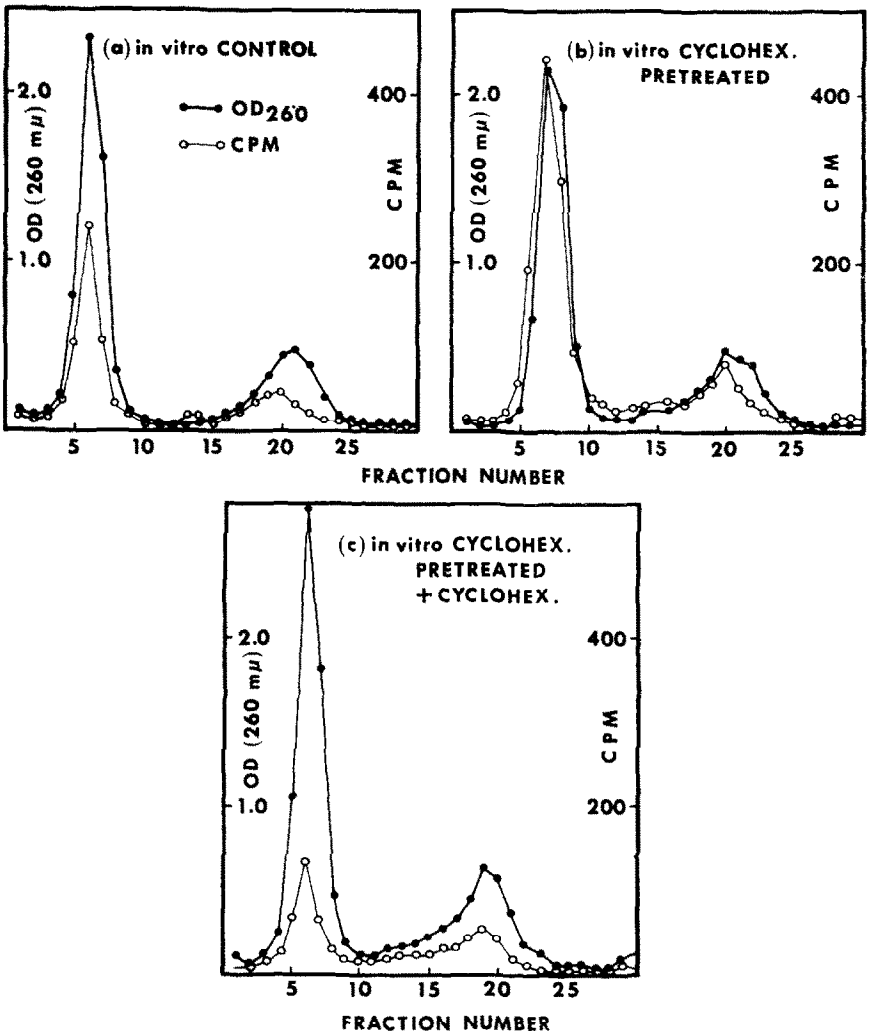

FIG. 2. Sucrose gradient analysis of hepatic microsomal fractions after labeling *in vitro* with L-^{14}C-phenylalanine. (a) Control rats, (b) cycloheximide-treated *in vivo*, and (c) cycloheximide-treated *in vivo* and incubated in the presence of cycloheximide (100 μg) *in vitro*.

control and cycloheximide-pretreated sources and is consistent with the dynamic model of protein synthesis.[21-23]

Despite the similartieis of the O.D. profiles obtained with control and cycloheximide-pretreated hepatic microsomal fractions, the radioactivity associated with the aggregated ribosomes leads to the conclusion that cycloheximide pretreatment inhibits protein synthesis *in vivo*[1, 2] and stimulates protein synthesis *in vitro*.[4-7] This stimulation of protein synthesis *in vitro* can be reversed by adding cycloheximide to the incubation mixtures.

Differences between effects *in vivo* and *in vitro* on amino acid incorporation brought about by cycloheximide pretreatment have also been observed by Traketellis *et al.*[2] These differences may be related to hormonal influences or to some factor in the supernatant fraction. We favor an interpretation involving hormonal influences on the basis also of previous work with adrenalectomized rats[5, 25] and recombination experiments with microsomes and supernatant material from control and cycloheximide-treated sources.[7]

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