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Initial inhibition by cycloheximide of translational activity of rat liver polysomes *in vivo*

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It has been variously proposed that the primary effect of cycloheximide at the translational level is on the initiation, or elongation, or termination and release of the polypeptide chains. Analysis of numerous literature data on the mechanism of cycloheximide inhibition of protein synthesis shows inconsistent and sometimes contradictory findings depending on the system used, such as yeast [1, 2], L-cells [3], hamster cells [4, 5], chick embryo cells [6], rabbit reticulocytes [7–10], and intact animals such as mouse [11], rabbit [12] and rat [13–16]. It is also difficult to compare, and, therefore, to draw conclusions from systems employing growing versus non-growing cells, cells in tissue culture versus intact animals, and mice versus rats.

In order to define further the mode of action of cycloheximide on protein synthesis in the intact rat during the initial inhibition of liver protein synthesis [17–19], approaches used in the investigation of the stimulated translational activity of rat liver polysomes in our previous paper [20] were employed. The translational state of rat liver polysomes, the proportion of various size classes of polysomes, and their protein-synthesizing activity were examined 2 hr after a single injection of a non-lethal (2 mg/kg body wt) or a lethal (20 mg/kg body wt) dose of cycloheximide.

All studies were performed on 190 ± 10 g male Wistar rats. The maintenance, treatment with cycloheximide, [^{3}H]leucine incorporation (100 μCi/100 g body wt), and removal of livers were described previously [19]. Cytoplasmic ribonucleoprotein complexes, polysomal size classes, and puromycin-released polypeptides were isolated and determined as described by Ch'ih *et al.* [20]. Protein was determined by the method of Lowry *et al.* [21]. Trichloroacetic acid-precipitable radioactivity was determined as described [22]. Data were presented as the means ± S.D. of a number of experiments (N). Each experiment consisted of at least two to three rats. The probability that an effect of the experimental condition was due to chance was measured by Students *t*-test on the mean difference between paired observations in a series of different experiments; P values less than 0.05 were considered significant.

Animals were treated with cycloheximide 1 hr prior to the administration of [^{3}H]leucine; after a 60-min labeling period, the livers were removed for processing. Since in our previous study [18] we demonstrated no changes from controls of the specific radioactivity of leucine (0.26 μCi/μmole and 0.24 μCi/μmole for the control and treated), and since leucine is known to be incorporated into the internal sequence of a polypeptide chain, [^{3}H]leucine incorporation was used to measure the rate of protein synthesis in the present study. In

Table 1. Effect of cycloheximide on polysome size classes and distribution of radioactivity*

Sucrose gradient	Normal			Treated		
	Content (%)	Radioactivity		Content (%)	Radioactivity	
		(dis./min)	(dis./min/mg ribosome)		(dis./min)	(dis./min/mg ribosome)
Top fraction		420 ± 120			83 ± 4+	
Monomers 40S	1.0 ± 0.4	75 ± 24		1.2 ± 0.1	42 ± 3	
60S	1.2 ± 0.3	61 ± 24		2.5 ± 0.4+	43 ± 3	
80S	6.0 ± 1.1	86 ± 26	887	4.5 ± 0.3	122 ± 15+	1671
Polysomes						
2	5.5 ± 0.8	75 ± 29	852	4.4 ± 1.0	153 ± 21+	2125
3	5.4 ± 0.8	62 ± 27	721	4.7 ± 0.8	93 ± 15	1224
4	5.5 ± 0.7	56 ± 15	636	4.9 ± 0.8	105 ± 18+	1313
5	5.5 ± 0.7	58 ± 17	637	5.9 ± 0.6	117 ± 29+	1232
6	5.4 ± 0.7	56 ± 21	659	5.8 ± 0.6	129 ± 29+	1372
7+	64.5 ± 3.4	583 ± 194	571	66.1 ± 3.9	1443 ± 134+	1337
Polysomes/monosomes ratios:						
7+/80S	10.8			14.7		
Polysomes (2–7+)/80S	15.3			20.4		

* Data presented are means ± S.D. from four separate experiments; in each experiment freshly isolated cytoplasmic ribonucleoprotein complexes from normal (15.4 to 16.4 A_{260} units) and treated (15.8 to 16.8 A_{260} units) rat liver were used. Specific radioactivity (dis./min/mg of ribosome) is calculated using the equivalence of A_{260} of 10 = 1 mg ribosome.

+ Indicates P value is less than 0.05.

addition, any incorporation of this label into proteins would eliminate the possibility that a complete blockage of protein synthesis at the initiation or elongation step had occurred. The amount of cytoplasmic ribonucleoprotein complexes isolated from liver exhibited no difference between the control and the 2 hr cycloheximide-treated animal (A_{260}/g of liver: 72 for the control and 69 for the treated), indicating that cycloheximide treatment did not cause either an increase of the nucleolytic or proteolytic activities, or a decrease of the proteosynthetic apparatus.

In order to further understand the protein synthetic state of the polysomes, a detailed evaluation of the complexes was conducted. Quantitative values expressed as per cent of total, presented in Table 1, indicated that the relative amounts of the various monomeric and polymeric units were similar, except that an increase of the larger ribosomal subunit (60S) and a decrease of the 80S monosomes were observed with the treated rat. The polysomes/monosomes ratios, however, showed a 30–40 per cent increase. That there was no drastic increase of monosomes and smaller polysomes during the cycloheximide treatment suggested that initiation of protein synthesis was not affected. The increase of the polysomes/monosomes ratios, however, reflected the possibility that the antibiotic blocked the termination and release step, but allowed initiation and elongation to proceed, producing polysomes of a larger size than normal as ribosomes piled up along mRNA [23].

As to the radioactivity determined from the various regions of the gradient (Table 1), the control showed 420 dis./min on the top of the gradient; in the treated only 83 dis./min were found. The decrease of 80 per cent of the radioactivity present in the released polypeptides exhibited by the cycloheximide-treated fraction was in agreement with the previously observed inhibition of [^{3}H]leucine incorporation into various intra- and extracellular proteins [17–19]. When radioactivity from the various size classes of polysomes was determined, the treated liver polysomes showed a 1.5- to 2.5-fold increase. The total radioactivity recovered from the sucrose gradients was 1532 and 2330 dis./min/15 A_{260} units for the control and treated respectively. If we express the radioactivity associated with 80S monosomes and larger polysomes (2 polysomes to 7 polysomes) as dis./min/mg of ribosomes (Table 1) and as dis./min/polysome abundance in each ribosome class (Table 2), significantly higher amounts (2- to 2.5-fold) of radioactivity were found in the cycloheximide-treated rat. The 2-fold increase of the protein-synthesizing activity of the monoribosomes exhibited by the treated rat reflected that the monomeric unit did not contain an abnormal amount of inactive single ribosomes, "run off" ribosomes [24]. The absence of both the "run off" ribosomes and the accumulation of mono-

Table 2. Comparison of the specific radioactivity associated with ribosome class between normal and cycloheximide-treated*

Ribosome class	Radioactivity (dis./min/polysome % in fraction)		Ratio (treated/untreated)
	Control (untreated)	Cycloheximide (treated)	
80S	14.3	27.1	1.9
2 Polysomes	13.6	34.8	2.6
3–6 Polysomes	10.6	20.8	2.0
7+ Polysomes	9.0	21.8	2.4

* Data are calculated from the mean values presented in Table 1.

Table 3. Polypeptide chains released *in vitro* with puromycin*

Condition of rat	Total (A) (dis./min)	(Treated/untreated)	Released polypeptide chains High mol. wt. fraction (B) (dis./min)	(Treated/untreated)	Low mol. wt. fraction (C: A–B) (dis./min)	(Treated/untreated)
Normal	1290 ± 360		961 ± 335		329	
Treated	2596 ± 46+	2.0	1974 ± 204+	2.1	632	1.9

* Data are presented as mean ± S.D. from four separate experiments; in each experiment, 30 A_{260} units of freshly isolated cytoplasmic ribonucleoprotein complexes were used.
+ P value is less than 0.05.

mers and lighter polysomes (Table 1) further suggested that neither the initiation nor the elongation step of the ribosomal cycle was the rate-limiting step [25]. The increase of the polysomes/monosomes ratio (Table 1) and the specific activity of the various polysomal classes observed (Table 2) suggested that the termination and release of polypeptides were affected.

To substantiate this notion further, nascent polypeptides released from the ribonucleoprotein complexes by incubation *in vitro* with puromycin were studied. The rationale was that, if the elongation process was partially inhibited by the antibiotic *in vivo*, lower than normal radioactivity should be found, and a disproportional increase of radioactivity present in the low mol. wt. peptidyl puromycin fraction should be seen. If the termination process was the rate-limiting step, higher than normal radioactivity should be found without a disporportional increase of the low mol. wt. peptidyl puromycin fraction. As shown in Table 3, a 2-fold accumulation of nascent peptide chains was seen with the total and high mol. wt. peptides (columns A and B) without a disproportional increase of the low mol. wt. fraction (column C). These results and the results presented in Tables 1 and 2 strongly suggest that the cycloheximide given to the intact rat at 2 mg/kg body wt, a dose which did not cause death of the animal and liver necrosis [26], inhibited the process of termination and release of polypeptides from the polysomes. The amounts of peptidyl puromycin released (Table 3) using purified ribonucleoprotein complexes are higher than those released using liver microsomes as reported previously [19]. An explanation for the difference may be that when rough microsomes were treated with puromycin *in vitro* a large part of the newly synthesized material remained associated with the membranous materials and was not released into the supernatant fluid [27]. During inhibition of termination and release by cycloheximide, the newly synthesized polypeptide chains probably directed themselves further toward the lumina of the microsomal vesicle and were released into the vesicle upon the *in vitro* incubation with puromycin.

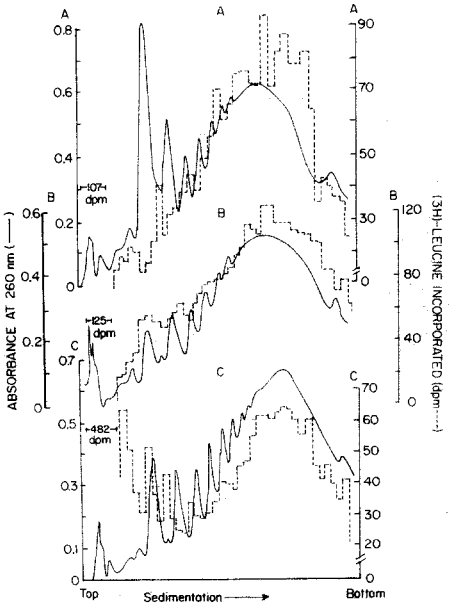

Fig. 1. Changes of polysomal patterns and radioactivity profile of cycloheximide treatment. Freshly isolated ribonucleoprotein complexes (15 A_{260} units) were used in each case. Key: (A) cycloheximide, 20 mg/kg body wt; (B) cycloheximide, 2 mg/kg body wt; (C) untreated. Absorbance at 260 mm (——); radioactivity (– – – –).

With the use of cycloheximide to unravel the mechanism controlling protein synthesis in yeast and mammalian cells in culture and in cell-free systems, it has been suggested that low levels of cycloheximide slow down the elongation process by affecting the EF-2, whereas higher levels completely inhibit elongation, and even initiation [23]. In order to test this possibility, the effect of a lethal dose (20 mg/kg body wt) on the polysomal profile and [^{3}H]leucine incorporation was compared to normal and 2.0 mg cycloheximide-treated rats (Fig. 1). In the polysomal profile from animals receiving 20 mg cycloheximide (curve A), there was a significant accumulation of 80S monosomes (13 per cent of the total) as compared to the normal (6.0 per cent) and 2 mg cycloheximide-treated (4.5 per cent), with a decrease in the polysomes/monsomes ratio, suggesting that the high dose of the antibiotic partially inhibited initiation. If this were the case, lower than normal radioactivity should have been found throughout the entire gradient. As shown in Fig. 1, the radioactivity patterns exhibited only a decrease of the top fractions (curve A) as compared to both the normal (curve C) and the 2.0 mg cycloheximide-treated (curve B). In the polysomal region, however, higher than normal radioactivity was observed. The accumulation of monosomes (absorbance profile) without an elevated level of radioactivity suggested the presence of "run-off" monomeric units incapable of synthesizing proteins due to a partial inhibition at the initiation step. Furthermore, radioactivity in the largest size polysome abundance classes is decreased relative to that observed from the low-dose (2.0 mg) animals, suggesting that elongation may be affected as well. At lethal doses, it is probably quite likely that all aspects of the translation process are affected to varying degrees.

The data presented suggest that, in intact rats, the rate-limiting step of the ribosomal cycle of protein synthesis during the initial inhibition by cycloheximide is the termination and release process. Our results are in agreement with earlier reports [5, 10, 15] that cycloheximide inhibits the termination step. Furthermore, we feel that the isolation of intact polysomes, analysis of polysomal profiles and radioactivity, and *in vitro* release of the peptidyl-puromycin molecules are necessary for proper interpretation of the mode of action with the protein synthesis inhibitor.

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Stereospecific hydrolysis of the optimal isomers of *O*-ethyl *O*-*p*-nitrophenyl phenylphosphonate by liver microsomes*

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The stereospecificity of an enzymatic reaction involving a substrate or inhibitor is generally attributed to the asymmetrical nature of the enzyme surface. Phosphonates may have four different substituents arranged around the phosphorus atom, resulting in two optically active forms. Studies with tabun, ethyl-*N*-*N*-dimethylphosphoroamidocyanidate [1, 2], showed a high degree of specificity with regard to the rate of hydrolysis of the isomers. The *l*-isomer of *O*-ethyl-*S*-(2-ethyl mercaptoethyl) ethylphosphonothiolate reacted ten to twenty times faster than the *d*-isomer in the inhibition of cholinesterase from four different sources [3], while stereoselectivity in the metabolism of cyanofenphos, *O*-ethyl *O*-*p*-cyanophenyl phenylphosphonothionate, in the rat appeared to be mainly due to selective hydrolysis of the (−)-oxon analog by an arylhydrolase [4].

The present study was undertaken to compare the rates of hydrolysis of the optical isomers of EPNO, *O*-ethyl *O*-*p*-nitrophenyl phenylphosphonate, by rat, mouse and rabbit liver microsomes. Studies were also conducted on the activation and inhibition of such hydrolysis reactions by various factors.

Chemicals. The optical isomers of EPNO were prepared by oxidation of the optical isomers of EPN, (O-ethyl O-*p*-nitrophenyl phenylphosphonothionate) as described previously [5]. $CaCl_2 \cdot 2H_2O$, $CoCl_2 \cdot 6H_2O$ and $MnCl_2 \cdot 4H_2O$ were purchased from the Fisher Scientific Co., Pittsburgh, PA, $HgCl_2$ from Reagent, Inc., Middlesex, NB, $BaCl_2 \cdot 2H_2O$ from the J. T. Baker Chemical Co., Phillipsburg, NJ, and EDTA from Matheson, Coleman & Bell, Cincinnati, OH.

Enzyme preparation. Male mice (20–25 g) (Dublin ICR strain), male rats (250–300 g) (Sprague–Dawley strain), and a male rabbit (3700 g) (New Zealand White strain) were decapitated and their livers removed and homogenized as 20% homogenates in 0.05 M Tris–HCl buffer, pH 7.4, at 0–4%. Differential centrifugation was carried out as described previously [6]. The microsomal fractions were washed, suspended in the same buffer and centrifuged at 100,000 *g* for 1 hr at 4°. The pellets were suspended in the same buffer and used as a source of the hydrolase.

Reaction. The reaction mixture consisted of a microsomal preparation equivalent to 300 mg liver in a total of 3 ml of 0.05 M Tris–HCl buffer, pH 7.4. The reaction and control cuvettes were equilibrated at 37° for 3 min before adding the appropriate EPNO isomer to the reaction cuvette in 5 μl acetone. The final concentration of the isomer was 0.05 mM.

The hydrolytic reaction was followed by measuring at 405 nm the *p*-nitrophenol formed as a result of the hydrolysis of EPNO, using an AMINCO DW-2 UV-Visible Spectrophotometer. The control cuvettes were prepared in the same manner except that EPNO was not added.

The effects of a number of compounds on inhibition and activation were studied by the addition of the corresponding

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