Biochemical Pharmacology, Vol. 28, pp. 691–692
Pergamon Press Ltd. 1979. Printed in Great Britain.

0006-2952/79/0301-0691 $02.00/0

Stimulation by cycloheximide of translational activity of rat liver polysomes *in vivo*

(*Received* 20 *April* 1978; *accepted* 10 *August* 1978)

Antibiotics which inhibit discrete steps of protein synthesis have been used to unravel the underlying mechanisms controlling protein synthesis [1–3] and degradation [4] in mammalian cells and intact animals. One of the frequently employed antibiotics, cycloheximide, which is a potent inhibitor of protein synthesis of eukaryotic cells, has been used at lethal and non-lethal doses for various durations (short- and long-term) in experiments *in vivo*. With lethal doses, many biochemical [5, 6] and structural [7] parameters are altered in the rat prior to the demise of the animal [5, 8]. In contrast, non-lethal doses cause a transient inhibition of protein synthesis followed by a recovery period [9–12], including a phase in which there is an above rate of amino acid incorporation into cellular proteins [11, 13, 14]. In order to define further the biochemical events occurring during this stimulatory period, the polysome pattern of liver has been examined.

All studies were performed on 190 ± 10 g male Wistar rats. The maintenance, treatment with cycloheximide (2 mg/kg body wt), [^{3}H]leucine incorporation (100μ Ci/100 g body wt), and removal of livers have been described previously [14]. Cytoplasmic ribonucleoprotein complexes were isolated according to the procedure of Palmiter [15], as described previously [16]. The separation of the various polysomal size classes from the freshly isolated ribonucleoprotein complexes was achieved by using 0.5–1.5 M sucrose gradients containing 25 mM NaCl, 5 mM $MgCl_2$ and 25 mM Tris–HCl (pH 7.5), centrifuged at 96,300 *g* for 195 min at 4° in a Spinco SW-27 rotor. The gradient was removed from the top and monitored continuously at 260 nM in a Gilford spectrophotometer. To measure the amount of each of the various size classes of polysomes, the recorded absorbance was divided into regions encompassing subunits (40S and 60S), monosomes, disomes, trisomes, tetrasomes, pentasomes, hexasomes and large polysomes (7 ribosomes and greater), and analyzed by planimetry. Peptidyl-puromycin fractions were prepared by incubating 30 A_{260} units of the ribonucleoprotein complexes with puromycin (0.5 mM) at 37° for 30 min. After incubation, the ribosomes were removed as the pellet sedimented through a 1.0 M sucrose cushion in Spinco No. 50 rotor at 42,000 rev/min. The supernatant fraction containing peptidyl puromycin was dialyzed to remove sucrose and low molecular weight (< 8000) peptidyl puromycin. The large peptidyl puromycin (mol. wt > 8000) was purified further by a 15–30% sucrose gradient containing 0.5 M KCl and centrifuged for 16 hr at 22,000 rev/min in a Spinco No. 27-SW rotor. Protein was determined by the method of Lowry *et al.* [17]. Trichloroacetic acid precipitable radioactivity was determined as described previously [18]. Data were presented as the mean ± 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 Student's *t*-test on the mean difference between paired observations in a series of different experiments; P values of less than 0.05 were considered significant.

Animals were treated with cycloheximide 23 hr prior to the administration of [^{3}H]leucine; after a 60-min labeling period, livers were removed for processing. Since in a previous study [13] we demonstrated no changes from controls of the specific radioactivity of the leucine pool, [^{3}H]leucine incorporation was used to measure the rate of protein synthesis in the present study. Before determining radioactivity of the isolated liver ribonucleoprotein complexes, the amount of the complexes, expressed as A_{260}/g of liver, was compared between the normal and the treated animals (Table 1). An 18 per cent decrease of the complexes was observed 24 hr after cycloheximide treatment, but the difference was not statistically significant. This decrease parallels the changes in body weight and relative liver weight reported previously [14] and was probably due to the hydration state of the animal. In contrast to the content of the ribonucleoprotein complexes, the radioactivity associated with the complexes was about 3.7-fold higher in the treated animal than the control. This significantly higher level of radioactivity suggested that activation of polysomal activity had occurred.

In order to understand further the protein synthetic state of the polysomes, a detailed evaluation of the complexes was conducted. The polysomal absorbance profiles showed neither a disaggregation nor an aggregation of the polysomes. Quantitative values expressed as per cent of total, presented in Table 2, also indicate no major differences. With the normal polysome profile (Fig. 1 and Table 2), the relative amount of polysomes (disomes to 7 ribosomes and greater) was 91.8 per cent of the total, indicating that intact, undegraded, and biologically active polysomes had been isolated. When the cycloheximide-treated polysomes were isolated under identical conditions, that is side by side with the untreated, the relative amount of polysomes was 91.2 per cent of the total.

Table 1. Comparison of the content and radioactivity of the cytoplasmic ribonucleoprotein complexes isolated from normal and cycloheximide-treated rat liver

Condition of rat	Ribonucleoprotein Content (A_{260}/g liver	Ribonucleoprotein Radioactivity (dis./min/g × 10^4)
Normal	72 ± 19 (26)*	1.6 ± 0.08 (7)
Treated	59 ± 19 (12)	6.0 ± 0.63† (4)

* Number of experiments is given in parentheses.
† P value is less than 0.05.

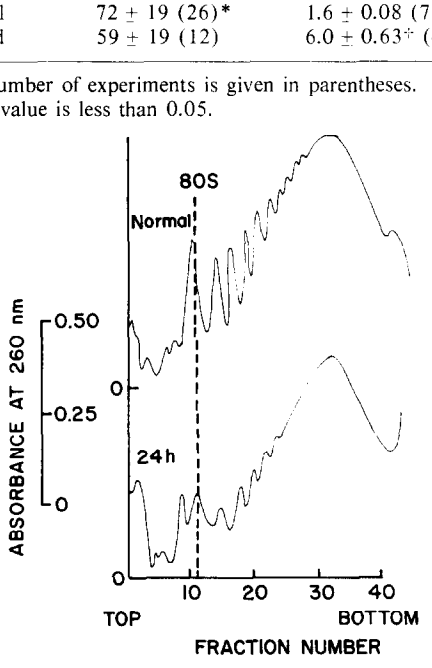

Fig. 1. Absorbance profile of normal and 24 hr cycloheximide-treated polysomes. Freshly isolated ribonucleoprotein complexes (15 A_{260} units) were used in each case. The sedimentation coefficient of monosomes (80S) is indicated by a dotted line.

Table 2. Effect of cycloheximide on polysome size classes and distribution of radioactivity*

Sucrose gradient	Normal Content (% of total)	Normal Radioactivity (dis./min)	Treated Content (% of total)	Treated Radioactivity (dis./min)
Top fractions		420 ± 120		651 ± 60†
Monomers				
40S	1.0 ± 0.4	75 ± 24	0.6 ± 0.2	287 ± 25†
60S	1.2 ± 0.3	61 ± 24	2.9 ± 0.4	314 ± 25†
80S	6.0 ± 1.1	86 ± 26	5.3 ± 1.5	382 ± 30†
Polysomes				
2	5.5 ± 0.8	75 ± 29	4.0 ± 1.3	240 ± 36†
3	5.4 ± 0.8	62 ± 27	4.7 ± 1.1	135 ± 16†
4	5.5 ± 0.7	56 ± 15	5.0 ± 0.9	143 ± 13†
5	5.5 ± 0.7	58 ± 17	5.1 ± 0.6	168 ± 15†
6	5.4 ± 0.7	56 ± 21	5.3 ± 0.8	198 ± 14†
7+	64.5 ± 3.4	583 ± 194	67.1 ± 6.2	2322 ± 198†
In vitro released peptidyl-puromycin fraction		640 ± 26 (100%)		1106 ± 128† (173%)

* Data presented are means ± S. D. from four separate experiments.
† P value is less than 0.05.

The absence of any significant accumulation of subunits and monosomes or any aggregation of polysomes suggested that cycloheximide was no longer inhibiting protein synthesis at either initiation or translocation, or even at the termination step of protein synthesis [2].

Since polysome profiles alone do not demonstrate activation of the protein synthetic system of a non-dividing cell, radioactivity distribution on the sucrose gradient was determined (Table 2). On the top of the gradient, some soluble proteins were co-precipitated with the polysomes. An increase of 55 per cent of the radioactivity exhibited by the cycloheximide treated fraction was in agreement with the previously observed per cent stimulation of [^{3}H]leucine incorporation into various cellular fractions [11, 13, 14]. This radioactivity represented only a small percentage of the total increase. As to the radioactivity associated with the various size classes of polysomes, the treated liver polysomes showed 1.5- to 4.5-fold increases (Table 2). The smaller per cent increases observed with cellular protein fractions [14] and the soluble proteins at the top of the gradient suggest a dilution of the newly synthesized proteins by the pre-existing protein pool. The larger increase observed with the various polysomes might represent the newly synthesized polypeptides not yet released from the site of synthesis. In experiments carried out to release nascent polypeptides from ribonucleoprotein complexes by incubation *in vitro* with puromycin, higher radioactivity (173 per cent of the control) was present in the peptidyl-puromycin fraction obtained from the treated complexes (Table 2), similar to results obtained with microsomes incubated with puromycin [14]. The higher rate of [^{3}H]leucine incorporation into the nascent polypeptides, without increases of polysomal size classes, observed 24 hr after a single injection of the antibiotic, reflects a faster movement of ribosomes along the mRNA. Another possible explanation for the enhanced protein synthesis observed may be a prolonged period of accumulation and decreased rate of liver mRNA inactivation [16], similar to the induction and decay of human fibroblast interferon mRNA by cycloheximide observed by Cavalieri *et al.* [19].

The data presented in this report suggest an additional limitation in the use of inhibitors of protein synthesis *in vivo* to study protein levels in animal tissues [4]. In most long-term studies with cycloheximide in the intact rat, the assumption has been that cycloheximide inhibits protein synthesis during the entire course of the experimentation and that observed decreases in protein levels or enzyme activities are attributable to the rate of degradation. In light of the findings discussed [4, 6, 9–14, 16], re-examination of some of the above mentioned experimental situations seems to be necessary for proper interpretation.

Department of Biological Chemistry,
Hahnemann Medical College and Hospital,
Philadelphia, PA 19102, U.S.A.

JOHN J. CH'IH
LINDA S. FAULKNER
THOMAS M. DEVLIN

REFERENCES

1. S. Pestka, *A. Rev. Microbiol.* **25**, 245 (1971).
2. S. Pestka, *A. Rev. Biochem.* **40**, 697 (1971).
3. S. Pestka, *Prog. nucleic Acid Res. molec. Biol.* **17**, 217 (1976).
4. A. L. Goldberg and J.F. Dice, *A. Rev. Biochem.* **43**, 835 (1974).
5. C. W. Young, P. F. Robinson and B. Sacktor, *Biochem. Pharmac.* **12**, 855 (1963).
6. J. J. Ch'ih, D. M. Olszyna and T. M. Devlin, *Biochem. Pharmac.* **25**, 2407 (1976).
7. I. Daskal, J. A. Merski, J. B. Hughes and H. Busch, *Expl. Cell. Res.* **93**, 395 (1975).
8. M. Muramatsu, N. Shimada and T. Higashinakagawa, *J. molec. Biol.* **53**, 91 (1970).
9. W. R. Jondorf, *Biochem. Pharmac.* **17**, 839 (1968).
10. H. Glauman, *Biochem. biophys. Acta* **224**, 206 (1970).
11. J. J. Ch'ih and T. M. Devlin, *J. Cell Biol.* **63**, 59 (1974).
12. K. M. Hwang, L. C. Yang, C. K. Carrico, R. A. Schulz, J. B. Schenkman and A. C. Sartorelli, *J. Cell Biol.* **62**, 20 (1974).
13. L. I. Rothblum, T. M. Devlin and J. J. Ch'ih, *Biochem. J.* **156**, 151 (1976).
14. J. J. Ch'ih, R. Procyk and T. M. Devlin, *Biochem. J.* **162**, 501 (1977).
15. R. D. Palmiter, *Biochemistry* **13**, 3606 (1974).
16. J. J. Ch'ih, L. M. Pike and T. M. Devlin, *Biochem. J.* **168**, 57 (1977).
17. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
18. T. M. Devlin and J. J. Ch'ih, *Archs. Biochem. Biophys.* **152**, 521 (1972).
19. R. L. Cavalieri, E. A. Havell, J. Vilček and S. Pestka, *Proc. natn. Acad. Sci. U.S.A.* **74**, 4415 (1977).