Biochemical Pharmacology, Vol. 31, No. 7, pp. 1219–1225, 1982.
Printed in Great Britain.

0006-2952/82/071219–07 $03.00/0

INITIAL INHIBITION AND RECOVERY OF PROTEIN SYNTHESIS IN CYCLOHEXIMIDE-TREATED HEPATOCYTES*

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(*Received* 22 *June* 1981; *accepted* 10 *September* 1981)

Abstract—Previous studies conducted with intact rats had demonstrated that protein synthesis was reversibly inhibited by cycloheximide. Polysome aggregation occurred during inhibition with a return to normal during recovery, suggesting that the block of translational activity involved termination and release of polypeptides. This study involving freshly isolated hepatocytes was undertaken to clarify the mechanism of the biphasic response to cycloheximide. Cycloheximide at 1 μM inhibited [^{3}H]leucine incorporation into both cellular and secreted proteins by at least 86%, without having deleterious effects on membrane integrity as indicated by trypan blue uptake and lactate dehydrogenase (LDH) (EC 1.1.1.27) release. After removal of cycloheximide, incorporation of labeled amino acids into cellular protein and protein secreted into the medium returned to control levels. Kinetically, incorporation into secreted protein exhibited a lag of 30–45 min, indicating that a longer recovery period for restoration of proteosynthetic ability is required for membrane-bound polysomes. During the first 100 min of the recovery period, 30% of the cellular protein, which had been prelabeled during cycloheximide inhibition, was secreted into the medium; treated cells, however, secreted prelabeled protein at a lower initial rate. To elucidate the mechanism of action of cycloheximide, the content of the cytoplasmic ribonucleoprotein complexes (RPC), polysome size classes, and the distribution of radioactivity among the various ribosome classes were determined during inhibition and recovery. Larger size class polysomes (7+) were increased by cycloheximide treatment and remained increased during recovery. During inhibition, there was enhanced [^{3}H]leucine labeling with increasing polysome size, implicating termination as the rate-limiting step, whereas during the recovery phase the labeled nascent polypeptides were removed from the ribonucleoprotein complex at a 3- to 4-fold greater rate than control, indicating an accelerated release of completed proteins.

Cycloheximide, a potent inhibitor of protein synthesis in eucaryotic organisms, has been widely used in intact animals, primary and growing cells in culture, and cell-free systems, over a large range of doses and for short and long durations [1–3]. Recent investigations indicate that cycloheximide affects all stages of translation such as initiation [4–8], elongation [9–15], and termination [5, 16, 17], although the initial inhibition seems to be dose dependent [18]. Furthermore, in studies with animals *in vivo* [19–23] and with mammalian cells in tissue culture [24–26], cycloheximide reversibly inhibits protein synthesis. In intact animals, a dose- and time-dependent recovery, including a phase of stimulation of labeled amino acid incorporation, has been demonstrated. In tissue culture studies, enhanced initiation of translation of new polypeptides occurs after the removal of the inhibitor [25]. Because of the toxicity of the antibiotic in various experimental animals and variations in sensitivity of different experimental systems in which the inhibitor has been employed, it is difficult to draw any definitive conclusions regarding its mechanism of action during inhibition and subsequent recovery and/or stimulation of protein synthesis.

To eliminate systemic effects associated with the whole animal and to avoid cellular dedifferentiation inherent in growing cells, we have chosen to study the mode of action of cycloheximide on protein synthesis with isolated hepatocytes. In the present study, the kinetics of synthesis and secretion of liver protein, and the cytoplasmic content and distribution of polysomes and newly synthesized nascent polypeptides during inhibition and recovery phases were investigated using non-toxic doses of cycloheximide.

EXPERIMENTAL PROCEDURE

Materials

Hanks' balanced salt solution (without Ca^{2+}, Mg^{2+}), Swim's S-77 Medium, and trypan blue stain were purchased from the Grand Island Biological Co. (Grand Island, NY). Cycloheximide, soybean trypsin inhibitor (type II-S), and Triton X-100 were supplied by the Sigma Chemical Co. (St. Louis, MI). Collagenase (type II) was obtained from Worthington Biochemicals (Freehold, NJ). Flow Laboratories (Rockville, MD) was the source of fetal calf serum. Ribonuclease-free sucrose was provided by Schwarz-Mann (Orangeburg, NY). Heparin was purchased from Organon, Inc. (West Orange, NJ). Crystalline bovine serum albumin was supplied by

* This work was supported by Biomedical Research Support Grant 5-S07-RR05413 from the National Institutes of Health.

† This work was performed in partial fulfilment of the requirements of the degree of Doctor of Philosophy from Hahnemann Medical College and Hospital.

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Miles Laboratories (Kankakee, IL). L-[4,5-^{3}H]Leucine (45–76 Ci/mmole), L-[U-^{14}C]leucine (354 mCi/mmole), and NCS solubilizer were purchased from Amersham Radiochemicals (Arlington Heights, IL). Rats (272 ± 15 g, adults male Wistar) were obtained from the West Jersey Farm Division of the Parco Scientific Co. (Wenonah, NJ) and were fed water and Ziegler laboratory chow (Gardners, PA) *ad lib*.

Methods

Preparation of isolated hepatocytes. Hepatocytes were prepared according to the procedures of Berry and Friend [27], Seglen [28] and Jeejeebhoy *et al.* [29] as follows. The rats were anesthetized with Ketaset (98% ketamine, 2% acepromazine) and given 400 U.S.P. units of heparin, intraperitoneally. The liver was initially perfused *in situ* with 400 ml of Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution containing 25 mM $NaHCO_3$, pH 7.6, at 25 ml/min (15 min). After excision, the liver was placed on a nylon mesh support and perfused with Swim's S-77 Medium containing collagenase (100 units/ml)/heparin (2 units/ml)/soybean trypsin inhibitor (0.08 mg/ml)/2.5% bovine serum albumin/$CaCl_2$ (5 mM), pH 7.5, at 25 ml/min. This perfusion solution was recirculated for 15–30 min. The cells were dislodged and filtered through one layer of cheesecloth, and the hepatocytes were collected by gravity sedimentation (10 ± 1 min). After resuspension in incubation medium [Swim's S-77 Medium containing 2.5% bovine serum albumin/10% fetal calf serum/heparin (2 units/ml)], the cell number was adjusted to 4–8 × 10^6 cells/ml and cell viability was assessed by trypan blue exclusion. Routinely, 1.5–4.3 × 10^8 cells were isolated with 90–96% viable.

Incubation of hepatocytes. Hepatocyte suspensions (2 × 10^6 cells/ml) were incubated in polycarbonate flasks, at 37°, under a constant stream of 95% O_2–5% CO_2, with constant shaking (72 cycles/min), for 15 min before addition of cycloheximide and/or radioactive amino acid. At the indicated times, aliquots of the cell suspension were removed for determination of cell viability by trypan blue exclusion, lactate dehydrogenase (LDH) released into the medium, and incorporation of radioactively labeled amino acid.

Incorporation of radioactively labeled amino acid was conducted as follows: [^{3}H]leucine (10 μCi/ml) was added to the hepatocyte suspension with or without cycloheximide. For total protein synthesis the reaction was stopped at indicated times by the addition of 25–100 μl of cell suspension to cold 10% trichloroacetic acid (TCA). To determine [^{3}H]leucine incorporation into cellular and secreted proteins, 175 μl of cell suspension was removed from the incubation vessel and cells were collected by centrifugation in a Beckman airfuge set at 20 psig for 5 sec; 25 μl of supernatant fluid was removed and added to cold 10% TCA, and 150 μl of cold 10% TCA was added to the packed cells. All acid-insoluble material was dissolved in 0.5 N NaOH and precipitated with TCA twice to remove free [^{3}H]leucine before the determination of radioactivity.

To study protein synthesis after cycloheximide removal, double-labeling experiments were conducted. Two samples (10 ml of heptocyte suspension) were incubated with [^{3}H]leucine (10 μCi/ml) in the presence or absence of cycloheximide. After 1 hr of incubation the cells were washed twice with 40 ml of cold incubation medium. The cycloheximide-treated cells were resuspended in incubation medium with [^{14}C]leucine (10 μCi/ml); the untreated cells were resuspended without label or, in some experiments, in the presence of [^{14}C]leucine as indicated in the text. Both samples were then reincubated at 37° and, at indicated times, aliquots were removed to determine [^{3}H]leucine and [^{14}C]leucine radioactivity present in the total hepatocyte suspension, liver proteins and proteins in the medium.

Isolation of cytoplasmic ribunucleoprotein complexes (RPC) and analysis of polysome aggregation state. In experiments in which cytoplasmic RPC were isolated, hepatocytes (1.0 × 10^8 cells) were incubated in 50 ml of incubation medium. Cells were collected by centrifuging in a Sorvall SS-34 rotor for 5 min at 30 *g*. The RPC were isolated according to the procedures of Palmiter [30] as described previously [17]. The freshly isolated RPC were layered over 0.5 to 1.5 M sucrose gradients containing 25 mM NaCl/5 mM MgCl/25 mM Tris–HCl, pH 7.5, and centrifuged at 87,000 *g* for 245 min at 4° in a Spinco SW-27 rotor. The gradient was removed from the top and monitored continuously at 260 nm by a flow cell in a Gilford spectrophotometer. The absorbance was recorded, and fractions (0.8 to 1.2 ml) were collected with an ISCO fraction collector. The complexes consisted of 37 ± 5% monomeric units (40S, 60S, and 80S) and 63 ± 5% polysomes (disome and greater) or a polysome/monomer ratio of 1.7.

Analytical methods. Cell concentration and viability were determined in an American Optical Neubauer Hemacytometer. Approximately 200 cells were counted for each determination and the final concentration of trypan blue was 0.3%. LDH activity in the medium was measured spectrophotometrically by monitoring the disappearance of NADH at 340 nm using LDH reagent kits obtained from the CalBiochem-Behring Corp. (San Diego, CA). Radioactivity was determined in a Beckman LS-3100 liquid scintillation spectrometer as described previously [31].

RESULTS AND DISCUSSION

Inhibitory phase following cycloheximide administration

Effect of various concentrations of cycloheximide on hepatocytes. When the effect of various cycloheximide concentrations (1 × 10^{-7} to 5 × 10^{-3} M) on cellular integrity and protein synthesizing ability of the hepatocytes was examined, a dose-dependent inhibition of [^{3}H]leucine incorporation was observed with lower cycloheximide concentrations (0.1, 1.0 and 10 μM), while higher concentrations (100 μM and above) inhibited protein synthesis greater than 95% (Fig. 1). The decrease in label incorporation for all cycloheximide concentrations examined was statistically significant ($P < 0.05$) when compared to control values.

Control cell viability, as indicated by trypan blue uptake, exhibited a gradual decrease during incu-

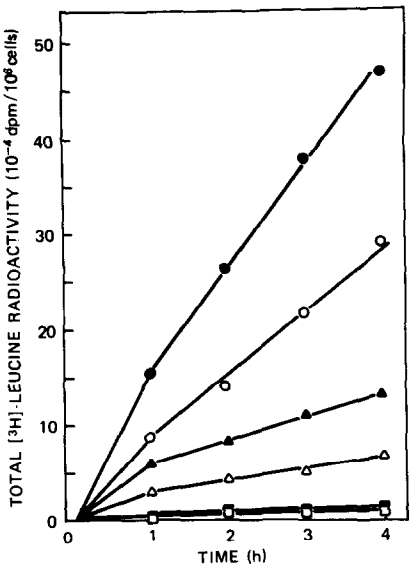

Fig. 1. Dose dependence of the inhibition of protein synthesis by cycloheximide. Incubation conditions and determinations of [^{3}H]leucine incorporation were described under Methods. Cycloheximide and 10 μCi/ml of [^{3}H]leucine (46 Ci/mmole) were added at zero time. Key: control, (●); cycloheximide concentrations: 0.1 μM (○); 1 μM (▲); 10 μM (△); 100 μM (■); and 1000 μM (□). Data are the means of four experiments.

bation whereas LDH released into the medium showed a 5–10% increase. Treatment with cycloheximide concentrations of 1.0 μM or less caused no statistically significant changes ($P < 0.05$) in either of these two variables. However, concentrations

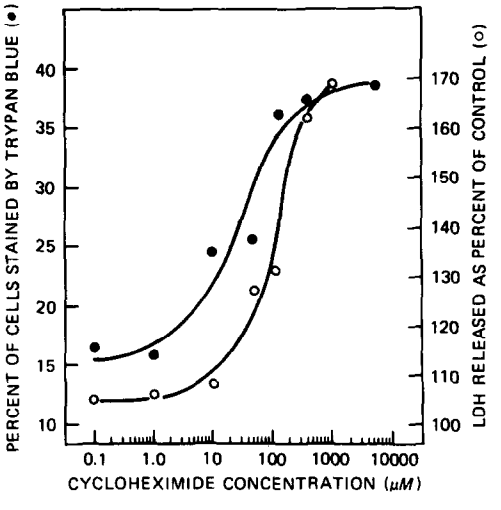

Fig. 2. Trypan blue uptake and LDH release as a function of cycloheximide concentration. Cells were incubated with various concentrations of cycloheximide, and at 2 hr and 2.5 hr samples were removed for LDH and trypan blue uptake determinations, respectively. Data represent the means of five to six experiments. Cumulative variation in LDH determinations was 7.1%. Standard deviations for trypan blue determinations were 2.5% for control cells and for cells treated with less than 10 μM cycloheximide. At higher concentrations of cycloheximide the standard deviation was less than 8.5%.

greater than 10 μM resulted in the impairment of membrane integrity as assayed by these criteria (Fig. 2).

Since 1.0 μM cycloheximide inhibited protein synthesis 85% but did not cause damage to the cell population as measured by trypan blue exclusion and LDH leakage, this concentration was employed in subsequent experiments for perturbing the protein synthetic system.

Incorporation of [^{3}H]*leucine into cellular and secreted proteins.* Since leucine is known to be incorporated into the internal sequence of a protein, incorporation of radioactively labeled leucine was used to measure the rate of protein synthesis in the present study. In 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. As shown in Table 1, the incorporation of [^{3}H]leucine into cellular protein was inhibited by 86%, whereas the label incorporated into proteins secreted into the medium during 1 hr of incubation of the isolated hepatocytes in the presence of 1 μM cycloheximide was decreased by 94%. Of interest, the extent of [^{3}H]leucine incorporation into the polypeptides associated with the cytoplasmic RPC was unaffected, suggesting that the sensitive step of cycloheximide inhibition is associated with the cytoplasmic ribosomes.

Quantitative changes and distribution of radioactivity in various polysome size classes. To further understand the protein synthetic state of the polysomes, evaluation of the RPC by sucrose gradient centrifugation was conducted. Data presented in Fig. 3 show that the content of the complexes was not altered; however, cycloheximide did cause a change in the distribution of ribosomes along the mRNA resulting in an increase of the larger size class polysomes (7+ polysomes). The ratio of larger size class polysomes (7+) to smaller polysomes (≤6) was increased more than 2-fold, from 0.33 in control cells to 0.70 in cycloheximide-treated cells. The distribution of [^{3}H]leucine radioactivity associated with the complexes is shown in Fig. 4. In the treated cells, the radioactivity associated with the top of the gradient (soluble proteins co-precipitated with the polysomes) was decreased to 32% of the control. [^{3}H]Leucine incorporation into polypeptides associated with monosomes, disomes and trisomes was inhibited 45%, whereas tetrasomes through hexasomes contained 85% of the radioactivity compared to untreated cells. However, radioactivity present in the larger size class polysomes was substantially higher in the treated cells than control. The data presented in Figs. 3 and 4 strongly suggest the possibility that cycloheximide blocks the termination and release step, while allowing initiation and elongation to proceed at a slower rate. This is in agreement with Oleinick [18] who observed that cycloheximide, at 0.1 to 10 μM, affects termination to a greater extent than elongation and initiation in exponentially growing Chinese hamster ovary cells. Furthermore, the inbibitory effect of cycloheximide on polypeptide termination and release is in accord with our previous studies employing a nonlethal dose of the antiobiotic *in vivo* [17].

Table 1. Radioactivity of [^{3}H]leucine-labeled protein of hepatocytes treated with 1 μM cycloheximide*

Condition	Leucine incorporation			
	Cellular protein (pmoles/10^6 cells)	Secreted protein (pmoles/10^6 cells)	Cytoplasmic ribonucleoprotein complexes (pmoles/10^6 cells)	(dpm $\times$ $10^{-4}/A_{260}$)
Control	2.45 ± 0.84 (3)	0.15 ± 0.07 (3)	0.016 ± 0.004 (4)	0.58 ± 0.12 (5)
Treated	0.34 ± 0.17† (3)	0.01 ± 0.004 (3)	0.015 ± 0.004 (4)	0.59 ± 0.10 (5)

* Cycloheximide (1 μM) and 10 μCi/ml of [^{3}H]leucine (76 Ci/mmole) were added at zero time and cells were incubated for 60 min. Each value is the mean ± S.D. for the number of experiments in parentheses.

† P value less than 0.05.

Recovery phase following cycloheximide removal

Utilization, turnover and resynthesis of liver proteins. Recovery of liver protein synthesis following inhibition by cycloheximide in rats has been noted [19–23, 32]; for isolated cells, especially hepatocytes, however, information concerning the kinetics of reversal of protein synthesis inhibition has not been reported. To distinguish prelabeled proteins from the newly synthesized proteins, double-labeling experiments were conducted. As depicted in Fig. 5 [^{3}H]leucine-prelabeled cells, in the presence or absence of cycloheximide, showed a constant level of radioactivity throughout the 90-min incubation period following cycloheximide removal. This suggests that there was neither a reutilization of [^{3}H]leucine nor a turnover of labeled proteins. When the cycloheximide-pretreated cells were reincubated with [^{14}C]leucine, 2.65 pmoles of leucine was incorporated into total protein during the first hour of reincubation, approaching the 2.8 pmoles of leucine incorporated by the control cells during the previous hour. This suggests that after the removal of inhibitor the rate of protein synthesis by these cells was recovered to almost the normal rate.

Synthesis of cellular protein during the first 45 min of the recovery period (Fig. 5, inset) accounted for virtually all of the protein synthesized at this time. The kinetics of the [^{14}C]leucine incorporation into total and cellular protein were similar. The incorporation of leucine into the newly synthesized protein secreted into the medium exhibited a pronounced lag period of 30–45 min. This lag period is only partially attributable to the transit time required for secretion [33, 34]. Since membrane-bound polysomes have been suggested to be more sensitive to cycloheximide inhibition than free polysomes [35, 36], it is possible that the synthesis of secretory

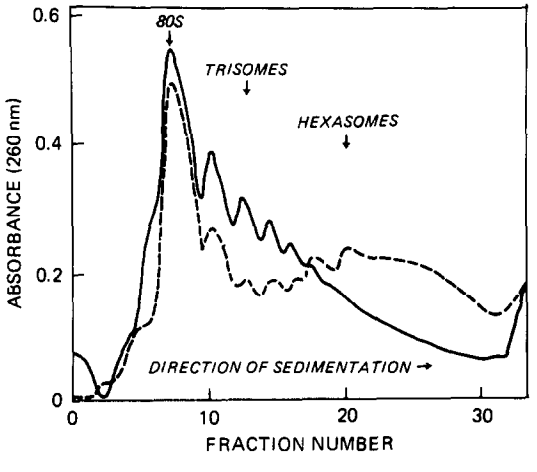

Fig. 3. Sedimentation profile of polysomes isolated from control and cycloheximide-treated hepatocytes. Cells were incubated for 1 hr in the presence (- - - -) or absence (————) of 1 μM cycloheximide. Approximately 8.0 A_{260} units of RPC were applied to each sucrose gradient and centrifuged as detailed under Methods.

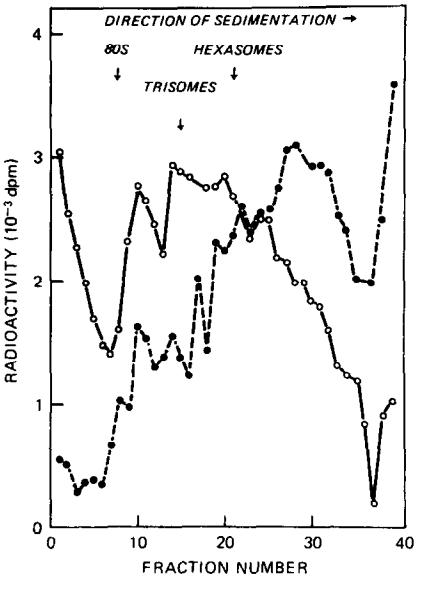

Fig. 4. Radioactivity profile of nascent polypeptides associated with polysomes from control and cycloheximide-treated hepatocytes. Cells were incubated for 1 hr with 12 μCi/ml [^{3}H]leucine (76 Ci/mmole) in the presence (- - - -) or absence (————) of 1 μM cycloheximide. Approximately 16.0 A_{260} units of RPC were applied to each gradient.

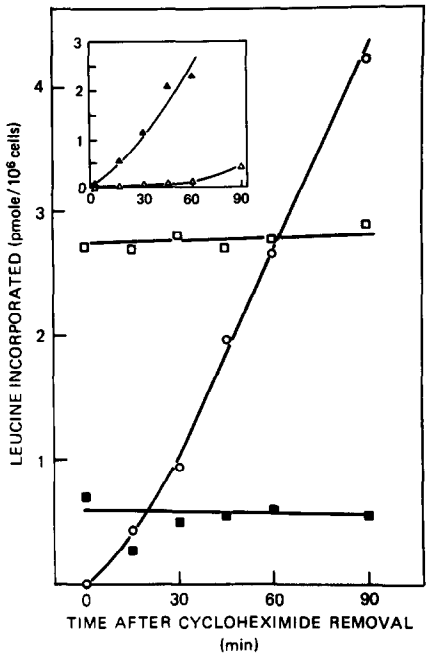

Fig. 5. Restoration of protein synthesis by hepatocytes following removal of cycloheximide. After a 1-hr incubation with 10 μCi/ml of [^{3}H]leucine (76 Ci/mmole) in the presence or absence of 1 μM cycloheximide, cells were washed twice, resuspended in incubation medium, and reincubated. Treated cells were reincubated with 10 μCi/ml of [^{14}C]leucine (354 mCi/mmole) in the absence of inhibitor. Key: [^{3}H]leucine incorporated into total protein of control (□) and cycloheximide-treated cells (■); [^{14}C]leucine incorporated into total (○), cellular (△), and secreted protein (△) was determined at various times indicated.

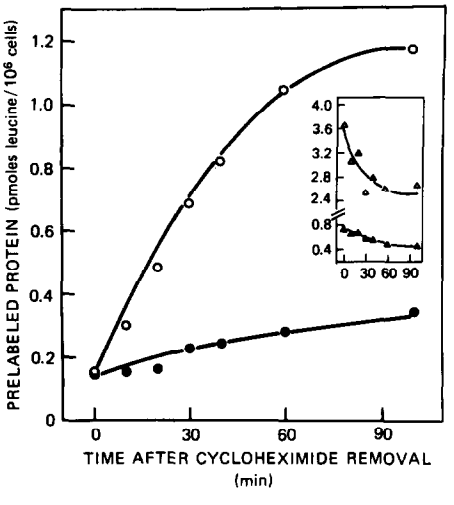

Fig. 6. Time course of protein secretion after cycloheximide removal. After a 1-hr incubation with 10 μCi/ml of [^{3}H]leucine (76 Ci/mmole) in the presence or absence of 1 μM cycloheximide, cells were washed twice, resuspended in incubation medium, and reincubated without additional label. Secreted proteins: control (○); treated cells (●). Cellular protein (inset): control (△); treated cells (▲).

proteins requires a longer recovery period. Also, Morland *et al.* [37] have reported a 10–20% inhibition of protein secretion during cycloheximide treatment. Therefore, a residual inhibition of the transport process may contribute to the observed lag as well. At 60 min, 2.3 pmoles and 0.12 pmole of leucine were incorporated into cellular and secreted protein respectively (Fig. 5, inset). The sum of these values equals 91% of the total protein synthesized during this time (Fig. 5), suggesting that during the recovery phase synthesis of both intra- and extracellular protein was restored almost to normal.

Secretion of proteins after cycloheximide removal. To differentiate between the rate of synthesis and secretion being the controlling factor, the time course for the secretion of [^{3}H]leucine-prelabeled cellular protein during the recovery period was followed as shown in Fig. 6. As the amount of [^{3}H]leucine-labeled protein in the medium increased, there was a concomitant decrease in cellular [^{3}H]leucine-labeled proteins (inset). During the 100 min in which secretion was followed, 30% of the prelabeled protein present in the cell at the beginning of recovery was transported to the medium in both control and cycloheximide-treated cells. This is in accord with the findings of Feldhoff *et al.* [38] which showed that one-third of total protein synthesized in untreated hepatocytes was transported into the medium. However, during the first 30 min after cycloheximide removal, the treated cells secreted protein at a much slower rate relative to the control. During reincubation, cells still retained a high degree of membrane integrity as indicated by dye exclusion. Also, the lag period observed in the treated cells gives indirect evidence of the intactness of the membrane. If prelabeled protein were to pass a ruptured cell membrane, the radioactivity in the supernatant fraction would increase drastically [39]. Thus, the delay of the appearance of [^{3}H]leucine-prelabeled and newly synthesized [^{14}C]leucine-labeled proteins in the medium must result from a residual effect of cycloheximide inhibition of protein transport [37].

Change and distribution of radioactivity in various size classes of polysomes. If the rate-limiting step of protein synthesis affected by cycloheximide is the termination and release, enhanced dissociation of the nascent polypeptides from polysomes would be expected during the recovery phase. To investigate this possibility, after 1 hr of reincubation with [^{14}C]leucine, the cells were collected and the cytoplasmic RPC were isolated. The content of the complexes and the polysomal profiles were similar to those presented in Fig. 3, with the cycloheximide-treated cells still showing an increase in larger size classes of polysomes. The specific radioactivity of [^{14}C]leucine was $0.18 \pm 0.01 \times 10^4$ and $0.15 \pm 0.01 \times 10^4$ dpm/A_{260} for the control and treated cells respectively. These data suggest that nascent polypeptide label incorporation had been restored at 83% of control. After cycloheximide removal, there was a significant decrease in those nascent polypeptides which had been prelabeled during the inhibitor phase. When the [^{3}H]leucine specific radioactivity was examined, $0.45 \pm 0.01 \times 10^4$ and $0.12 \pm 0.01 \times 10^4$ dpm/A_{260} remained associated with control and treated cell polysomes respectively. When

Table 2. Synthesis and release of nascent polypeptides associated with polysomes*

Time of addition		Radioactivity distribution among polysome size classes							
		[^{14}C]Leucine (dpm/fraction)				[^{3}H]Leucine (dpm/fraction)			
Cycloheximide (1 μM)	[^{14}C]Leucine (1 μCi/ml)	1–3	4–6	7+	Total	1–3	4–6	7+	Total
None	1 hr	1,648	1,187	1,740	4,575	5,710	1,416	1,846	8,972
0 hr	1 hr	1,669	823	2,002	4,494	1,071	268	553	1,892
		(101)	(70)	(115)	(98)	(19)	(19)	(30)	(21)
1 hr	None					9,344	2,090	4,008	15,440
						(164)	(148)	(217)	(172)

* Hepatocytes were incubated for 1 hr in the presence of [^{3}H]leucine (10 μCi/ml), washed, and reincubated for another hour. The additions of cycloheximide and [^{14}C]leucine (348 mCi/mmole) are indicated in the table. Polysomes were isolated at the end of the incubation and 7.3 A_{260} units were layered with a 0.5 to 1.5 M sucrose gradient. After centrifugation, various size classes were pooled and radioactivities were determined. Data presented were obtained from a typical experiment. Values given in parentheses are the percentages of control.

compared to the specific radioactivities of the cytoplasmic RPC after the initial hour of incubation (Table 1), these values indicate that, during the 1 hr reincubation period, treated cells had released 80% of the radioactivity previously incorporated while control cells had released only 20%. The greater reduction of [^{3}H]leucine in the treated cells during the recovery phase suggests that pre-existing polypeptides were terminated and released at a rapid rate. This rapid termination and release is also shown by the [^{3}H]leucine radioactivity associated with various size classes of polyribosomes (Table 2). There was a 3- to 5-fold reduction in prelabeled polypeptides throughout the entire polysomal population while newly synthesized proteins showed no major differences from the control. Since the newly synthesized polypeptides are expected to be released along with the pre-existing [^{3}H]leucine-labeled proteins during the recovery phase, it is concluded that ribosomes must move along the mRNA at a faster rate resulting in an enhanced termination and release.

To further substantiate the notion that cycloheximide affects termination and release, cells were prelabeled with [^{3}H]leucine for 1 hr before the addition of cycloheximide. As expected, during the subsequent hour of incubation there was an accumulation of pre-existing labeled nascent polypeptides associated with the various polysome size classes, with the larger size polysomes (7+) exhibiting the greatest increase in radioactivity over control (Table 2).

In conclusion, the present study has extended our *in vivo* findings to isolated hepatocytes and confirmed that a nontoxic dose of cycloheximide (1 μM) inhibited termination to a greater extent than other translational steps. These results are in accord with findings reported by several laboratories which shown that in growing Chinese hamster ovary cells [18], rabbit reticulocytes [5], and a cell-free system [11], cycloheximide concentrations between 0.1 and 50 μM appear to impair termination preferentially. Structural evidence has demonstrated the importance of ribosomal protein L29 [40], located close to the peptidyl transferase center [41], in cycloheximide affinity for the 60S subunit. Another protein required for cycloheximide sensitivity has also been described [42]. This protein was not required during the EF-2 translocation step, indicating that the cycloheximide sensitive step is subsequent to translocation. Data presented in this study demonstrate that during the recovery phase where there was enhanced disappearance of prelabeled nascent polypeptides from all polysome size classes subsequent to inhibitor removal, restoration of protein synthetic ability resides with the translational apparatus. Thus, the steps subsequent to translocation may be involved in the recovery phase as well.

High doses of cycloheximide are known to affect various aspects of chromosomal and nuclear organization [43], DNA and RNA synthesis [44–46], and the integrity of the liver cell [47]. Therefore, the use of a low, non-toxic dose of cycloheximide in our study provides reasonable assurance that protein synthetic ability could be perturbed without causing undue alterations in other biochemical functions of the cell. Since the use of a non-proliferating cell suspension eliminated any influences which could be incurred from replication or cell division, the present study allows for a direct comparison of results obtained with rat hepatocytes *in vivo* and *in vitro*. A consistent mechanism of action has been proposed for a chosen cell type. The exact mode of how cycloheximide affects the termination and the mechanism by which the cell determines whether a protein should be released or remain bound have not been clarified. Nevertheless, using hepatocyte suspensions as an *in vitro* model, perturbed with cycloheximide or other agents, it seems likely that progress will be made in uncovering control mechanisms of mammalian protein synthesis.

Acknowledgements—We acknowledge the excellent technical assistance of Ms. Louise Ames and Ms. Kathleen Doyle, and thank Ms. Leslie Sweeney for preparation of the manuscript.

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