Regulation of Mammalian Protein Synthesis *in vivo*

STIMULATED PROTEIN SYNTHESIS IN LIVER *IN VIVO* AFTER CYCLOHEXIMIDE TREATMENT

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Protein synthesis in rat liver *in vivo* was measured between 0 and 72 h after administration of a non-lethal dose of cycloheximide. There was a period of inhibition of [^3H]leucine incorporation into both intra- and extra-cellular proteins at 2 h after administration of the drug, which was followed by a recovery phase in which amino acid incorporation varied significantly among the various proteins evaluated. At 12 h there was a marked stimulation of incorporation into nascent polypeptides released from polyribosomes and plasma fibrinogen, but incorporation into ribosomal proteins as well as albumin was still inhibited. Between 12 and 48 h, nascent-polypeptide synthesis remained elevated, but ribosomal-protein synthesis recovered slowly from the inhibition to normal rates only, and plasma-albumin synthesis increased slowly to above control values up to 48 h before returning to normal. A differential pattern of incorporation was also observed for incorporation into free and membrane-bound polyribosomes.

It was reported previously (Ch'ih & Devlin, 1974; Rothblum *et al.*, 1976) that the recovery from the inhibition of protein synthesis by sub-lethal doses of cycloheximide in rat kidney was dose- and time-dependent. The inhibition was followed by a period when the rate of amino acid incorporation into proteins of various cell fractions was significantly above control values. At 24 h after cycloheximide treatment, stimulation of liver-protein synthesis was not due to change in either the precursor pool or the specific radioactivity of the labelled amino acid. There were significant differences in the degree of stimulation of incorporation into various cellular proteins; the incorporation into the microsomal fraction was only 110% of control, whereas for that into nuclear histones and non-histone proteins the increase was to 260 and 360% of control values respectively, suggesting possible qualitative and quantitative alterations in the regulatory mechanism of gene expression.

The purpose of the present study was to measure possible changes in the synthesis of specific classes of extracellular and intracellular proteins by the liver during the recovery period after cycloheximide administration. Demonstration of a selective stimulation of liver-protein synthesis may offer useful information for further study of differential gene activation at the transcriptional level.

We report here that a stimulation of extracellular protein (plasma fibrinogen) synthesis was observed

as early as 12 h after administration of a non-lethal dose of cycloheximide (2 mg/kg body wt.), at a time when there was no apparent stimulation of ribosomal-protein synthesis. The pattern of recovery for synthesis of albumin was different from that for the other two proteins. Examination of the [^3H]leucine incorporation into free and membrane-bound polyribosomes of the liver indicated significant differences. Some of the observations described in the present paper have already appeared in preliminary form (Procyk *et al.*, 1976).

Experimental

Treatment of animals

Male Wistar rats (210 ± 10 g) were fed on Purina chow and water *ad libitum* until killed. Cycloheximide (Sigma Chemical Co., St. Louis, MO, U.S.A.) in 0.9% NaCl was injected intraperitoneally at a dose of 2.0 mg/kg body wt. Rats treated with turpentine received injection of a sterile mixture of 50% commercial gum turpentine (Loos S. Dilworth, Bristol, PA, U.S.A.) in corn oil at a dose of 2.5 ml/kg body wt.; half of each dose was given subcutaneously in the scapular area and one-quarter was injected intramuscularly into each thigh as described by Weimer & Humelbaugh (1967). Control animals received equivalent amounts of 0.9% NaCl. Collection of plasma and removal of livers were carried out as described previously (Rothblum *et al.*, 1976).

Isolation of subcellular components

Livers were homogenized in 6vol. of 0.25M-sucrose/2mM-Tricine*/1mM-EDTA, pH7.6, with six strokes of a motor-driven (600rev./min) Teflon-glass Potter-Elvehjem homogenizer (clearance 0.15–0.23mm). The homogenate was filtered through two layers of cheesecloth and the filtrate centrifuged at 600g_{max}. and 9000g_{max}. for 10min each to remove nuclei and mitochondria respectively. The microsomal fraction was isolated as the pellet from centrifugation at 105000g_{max}. for 60min from the post-mitochondrial supernatant. Free and membrane-bound polyribosomes were isolated from the post-mitochondrial supernatant by the method of Bloemendaal *et al.* (1974), except that centrifugation was carried out at 22000rev./min for 22h in a Spinco SW-27 rotor. Membrane-bound polyribosomes were collected from the 1.5M/2.0M-sucrose interface. The pellet fraction was taken as the free-polyribosomal fraction. Ribosomal subunits and the peptidylpuromycin fraction were prepared by using washed microsomal fractions treated with puromycin *in vitro* to release nascent polypeptides, and 0.5M-KCl to release ribosomes, as described by Borgese *et al.* (1974). Ribosomes prepared in this manner exhibited an A_{260}/A_{280} ratio of 1.83–1.97:1, and existed in the form of subunits as separated by centrifugation on a continuous 10–25% (w/v) sucrose gradient. Newly synthesized polypeptides released by puromycin exhibited an A_{280}/A_{260} ratio of 1.09–1.24:1.

* Abbreviation: Tricine, N-tris(hydroxymethyl)-methylglycine.

Fractionation of plasma proteins

Fractionation was done by a modification of the methods of Jeejeebhoy *et al.* (1972). Albumin was isolated from the 2.15M-(NH₄)₂SO₄ supernatant by isoelectric precipitation at pH4.6. Fibrinogen was converted into fibrin by adding various amounts (0.25, 0.5, 0.75, 1.0, 5.0 and 10.0NIH units) of thrombin to 0.1 ml of the 1.23M-(NH₄)₂SO₄ fraction. The fibrin clots were left for 12h and washed with 0.9% NaCl by using a mechanical rotator for 24h. A flow diagram and electrophoretic patterns of plasma fractions are given in Scheme 1 and Fig. 1. respectively.

Amino acid incorporation in vivo

Groups of two to three animals were injected intraperitoneally with [³H]leucine (40–60Ci/mmol; Schwarz/Mann, Orangeburg, NY, U.S.A.) at 20μCi/100g, body wt., 1h before being killed.

Analytical methods

Protein was determined by the method of Lowry *et al.* (1951) by using crystalline bovine serum albumin (Miles Laboratories, Kankakee, IL, U.S.A.) as the standard. RNA concentration was determined by the orcinol reaction (Schneider, 1957) after the alkaline hydrolysis (0.3M-KOH for 18h at 37°C) of the HClO₄ (0.6M) precipitate, by using yeast RNA (Sigma Chemical Co.) as the standard. Plasma proteins were analysed by electrophoresis on 5% (w/v) polyacrylamide gel as described by Ho *et al.* (1974). Gels were scanned with a Gilford 2520 gel scanner at 280nm.

Scheme 1. Flow diagram for separation of rat plasma proteins
Italicized letters in parentheses refer to parts (a)–(d) of Fig. 1.

Fig. 1. Tracings of gel-electrophoresis patterns of plasma fractions

Electrophoresis and scanning were performed as described in the Experimental section. Migration was from left to right (cathodal direction). The separation of proteins was as described in Scheme 1 and in the text. (a) Whole plasma; (b) supernatant [1.23M-(NH₄)₂SO₄]; (c) supernatant [2.15M-(NH₄)₂SO₄]; (d) albumin after isoelectric precipitation.

Fig. 2. Effect of cycloheximide on the incorporation of [³H]leucine into rat kidney, liver and plasma proteins. Rats were injected at zero time with cycloheximide (2mg/kg) and then 60min before death with [³H]leucine (200μCi/kg). Incorporation of [³H]leucine into proteins was determined as described in the Experimental section. □, Liver proteins; ○, kidney proteins; △, plasma proteins.

Determination of radioactivity

Samples containing acid-insoluble radioactivity were determined as described by Devlin & Ch'ih (1972). Radioactivity was determined with a Packard Tri-Carb liquid-scintillation spectrometer. The background was 16–20 c.p.m., and the counting efficiency was 30–40%; quenching was corrected by using an external standard. All determinations of radioactivity were carried out to an accuracy equivalent to less than 1% s.d., and expressed as d.p.m./mg of protein.

Results

Characteristics of treated animals

To document the significance of changes in the protein-synthesis activity of treated animals, records on the general state of all animals were maintained. There was a 6% loss of total body weight 2h after cycloheximide treatment; liver weight relative to body weight was similar to control values. By 24h, treated rats weighed 12% less than the controls and there was an approximate 10% decrease in relative liver weight. At this time there were no significant differences in liver RNA content between the control

and treated rats, but treated animals showed a 7% decrease in protein content. Losses of both body and liver weight were due presumably to less food intake and dehydration during the 24h of experimentation. The changes in protein and RNA contents were small and do not suggest a major degeneration of the tissues.

Effect of cycloheximide on the incorporation of [³H]leucine into rat liver and plasma proteins as compared with kidney

In our previous paper (Rothblum *et al.*, 1976) the incorporation of [³H]leucine into liver proteins was carried out only at 2 and 24h after various doses of cycloheximide. The results presented in Fig. 2 were from experiments that measured the entire time-course of the effect of a single dose (2mg/kg body wt.) of cycloheximide on [³H]leucine incorporation into proteins of plasma and liver. Incorporation of the labelled precursor into plasma proteins after an initial inhibition returned to control values by 12h and continued increasing to reach a maximum (172%) by 48h. Within the first 24h after cycloheximide, the pattern of radioisotope incorporation into liver proteins resembled that of plasma proteins. However, the maximum radioisotope incorporations in

Fig. 3. Incorporation *in vivo* of [^3H]leucine into microsomal fraction, puromycin-released polypeptides and ribosomal subunits as a function of time after the administration of cycloheximide

Experimental conditions were the same as described in Fig. 2; puromycin-released polypeptides and ribosomal subunits were isolated as described in the Experimental section. Each value is the mean of three experiments with the livers of two rats in each group. $\circ$, Ribosomal subunits; $\square$, peptidylpuromycin; $\triangle$, microsomal fraction.

liver were obtained 24h earlier than in plasma, and reached only 137% of control values. After the initial inhibition of protein-synthetic activity, recovery to normal activity for the liver occurred at 12h, whereas for the kidney there was still a 50% inhibition at this time. By 18h, both tissues showed a significant stimulation of protein-synthesis ability.

Effect of cycloheximide on the synthesis of intracellular proteins

Fig. 3 presents the results of [^3H]leucine incorporation into microsomal fraction, puromycin-released nascent polypeptides and ribosomal proteins at various times after cycloheximide treatment. At 2h there was a significant inhibition of [^3H]leucine incorporation into all protein fractions, but by 12h the specific radioactivity of the microsomal fraction was the same as the untreated control animals,

whereas ribosomal protein synthesis was still inhibited. A pronounced stimulation of incorporation into puromycin-released polypeptides was observed. After 12h the radioisotope incorporation into the microsomal fraction increased (130% of the control value at 48h). [^3H]Leucine incorporation into newly synthesized polypeptides at 18, 24 and 48h after cycloheximide was 180%, 182% and 185% of control values respectively, whereas the radioisotope incorporation into ribosomal protein returned to control values without any subsequent stimulation. The inhibition of ribosomal-protein synthesis observed between 2 and 12h after administration of cycloheximide may reflect a decreased rate of ribosomal-protein synthesis and/or exchange of these proteins with cytoplasmic proteins.

These results demonstrate that during the recovery period after inhibition, the liver rapidly regains its capacity for protein synthesis and before returning to total normality catalyses a period of stimulated protein-synthesis, which occurs as early as 12h after treatment. The pattern of cellular-protein synthesis during the period of stimulated protein synthesis differs significantly for individual classes of proteins, and thus extends our original observation of differential stimulation with histone and non-histone proteins (Rothblum *et al.*, 1976)

Leucine incorporation into free and membrane-bound polyribosomes

To clarify further the events occurring during the stimulatory phase of protein synthesis after cycloheximide treatment, incorporations *in vivo* of [^3H]leucine into proteins of free and membrane-bound polyribosomes were measured. As shown in Table 1, 2h after cycloheximide administration the specific radioactivity of membrane-bound and free polyribosomes was inhibited by 74% and 22% respectively. The differential sensitivity to cycloheximide exhibited by the two polyribosomal populations is in agreement with the current proposal that free polyribosomes are less sensitive to inhibition by cycloheximide than are membrane-bound polyribosomes (Glazer & Sartorelli, 1972; Koffer, 1974). However, 12h and 24h after cycloheximide treatment, incorporation into membrane-bound protein was 78% and 165% of controls respectively and incorporation into free polyribosomes was 120% and 165% of controls.

Since membrane-bound polyribosomes of rat liver are engaged in the synthesis of secretory proteins, e.g. plasma proteins, whereas free polyribosomes are active in the synthesis of proteins retained in the cell (Rolleston, 1974), the threefold stimulation of [^3H]leucine incorporation into proteins associated with free polyribosomes at 24h after cycloheximide administration is in full agreement with the enhanced

Table 1. Incorporation *in vivo* of [^3H]leucine into free and membrane-bound polyribosomes after the administration of cycloheximide

Animals were given cycloheximide, (2 mg/kg), 2, 12 and 24 h before they were killed; 60 min before death, [^3H]leucine (200 $\mu\text{Ci/kg}$) was injected intramuscularly. Animals in groups of two were used in the experiments. Values are means $\pm$ S.E.M. for three experiments. For further details, see the text.

Time after cycloheximide administration (h)	Membrane-bound polyribosomes		Free polyribosomes	
	$10^{-3} \times$ Specific radioactivity (d.p.m./mg of protein)	% of control	$10^{-3} \times$ Specific radioactivity (d.p.m./mg of protein)	% of control
0 (control)	2.3 $\pm$ 0.5	100	0.65 $\pm$ 0.13	100
Cycloheximide-treated				
2	0.61 $\pm$ 0.06	26	0.51 $\pm$ 0.03	78
12	1.8 $\pm$ 0.07	78	0.78 $\pm$ 0.02	120
24	3.8 $\pm$ 0.7	165	1.9 $\pm$ 0.4	292

Fig. 4. Effect of cycloheximide on the incorporation of [^3H]leucine into plasma albumin and fibrinogen

Experimental conditions were the same as described in Fig. 2. Each point represents the mean $\pm$ S.E.M. for at least three experiments. $\square$, Albumin (d.p.m./mg of protein); $\circ$, fibrinogen, determined with 5 NIH units of thrombin/0.1 ml of fibrinogen fraction and expressed per ml of plasma (d.p.m./ml of plasma).

rate of histone and non-histone protein synthesis observed previously (Rothblum *et al.*, 1976). The data for membrane-bound polyribosomes, however, indicate a gradual recovery at 12h, followed by a modest stimulation. Such a condition may reflect a general rate of synthesis compatible with the recovery of all extracellular proteins to their normal rates of synthesis. Nevertheless, this does not

exclude the possibility of a selective change occurring in the synthesis of a specific extracellular protein.

Effect of cycloheximide on synthesis of extracellular protein in liver

Fig. 4 shows that the specific radioactivity of albumin at various times after the administration of cycloheximide was similar in most respects to the pattern for the total plasma proteins (Fig. 2). Between 12 and 24 h there was a marked difference between the specific radioactivity of the total plasma protein and albumin. At 12 h the specific radioactivity of total plasma protein returned to normal values (107%), whereas albumin radioactivity was still well below normal values (68% of control). Not until 24 h did the specific radioactivity of albumin return to the control value, suggesting that synthesis of other plasma proteins must be stimulated significantly between 12 and 18 h after cycloheximide treatment. Fibrinogen became highly labelled during this period (Fig. 4). After an initial inhibition of [^3H]leucine incorporation into fibrinogen, the recovery was very rapid, with maximal incorporation of label (over 300% of control) occurring between 12 and 18 h. There also occurred a second increase in fibrinogen labelling at 48 h, coinciding with the maximum incorporation of label into the total plasma protein and albumin. The enhanced rate of fibrinogen synthesis at 12 and 18 h was not associated with any significant increase in the plasma fibrinogen content as occurs, for example, in an 'acute-phase' response induced by injections of turpentine (Weimer & Humelbaugh, 1967; Bouma *et al.*, 1975) shown in Table 2. The rate of fibrinogen synthesis in animals treated with turpentine was increased by 882% (column B) compared with normal animals, and the net accumulation of fibrinogen in the plasma was 3.2-fold (column E) which is in agreement with published results (Bouma *et al.*, 1975). There was no significant accumulation of fibrinogen in cycloheximide-treated

Table 2. *Rates of fibrinogen synthesis and accumulation*

Animals were treated with cycloheximide or turpentine as described in the Experimental section. Experimental conditions were the same as those described in Fig. 4, except that 0.5 NIH unit of thrombin per 0.1 ml of fibrinogen fraction was used in column C to determine, on the basis of the isotope-dilution principle, the fibrinogen accumulation. Values are the means $\pm$ S.E.M. for seven experiments, except at 18 h after cycloheximide and 24 h after turpentine administration, where the values of a typical experiment are given.

Time after treatment (h)	Fibrinogen synthesis		Fibrinogen accumulation		
	Specific radioactivity (d.p.m./ml of plasma) (A)	Relative rate of synthesis (B)	Accumulation of radioactivity (d.p.m./ml of plasma) (C)	% of control (D)	Accumulation due to treatment [(E) = (B)/(D)]
0 (control)	4810 $\pm$ 134	100	4384 $\pm$ 300	100	—
Cycloheximide					
12	16372 $\pm$ 1270	340	12606 $\pm$ 1560	275	1.18
18	14483	301	11300	258	1.17
Turpentine					
24	42443	882	12077	275	3.2

animals. Fibrinogen concentration determined by electrophoresis also supports this fact (Ch'ih *et al.*, 1976).

Discussion

The inhibitory effect of cycloheximide on protein-synthesis *in vitro* and *in vivo* has been extensively investigated (Pestka, 1971), but there has been little concern about compensatory changes that must occur in the protein-synthesis activity as a consequence of the period of inhibition. Obviously, an acute period of inhibition of all protein synthesis will disrupt the balance between synthesis and degradation of the diverse proteins required by the tissue. There have been some studies of cellular changes during the recovery period after inhibition by cycloheximide (Jondorf, 1968; Glaumann, 1970; Christopher *et al.*, 1971; Ch'ih & Devlin, 1974; Hwang *et al.*, 1974; Rothblum *et al.*, 1976). Of particular interest is the marked stimulation over control of amino acid incorporation after inhibition reported by this laboratory (Ch'ih & Devlin, 1974; Rothblum *et al.*, 1976). The enhanced rate of protein synthesis can involve the indiscriminate synthesis of all cellular proteins, owing to an increase in the cellular content of the protein-synthesis apparatus. A comparison of the rate of radioisotope incorporation into the microsomal fraction with that into the ribosomal subunits (Fig. 3) suggested that the response was not an increase in the protein-synthesis apparatus. Further, as expected on the basis of the differences in the sites of synthesis (Table 1) and turnover rates of various proteins of liver origin, the recovery period after inhibition by cycloheximide must involve differential rates of synthesis of specific proteins. The stimulated synthesis of plasma

fibrinogen at 12h and of albumin at 48h after cycloheximide (Fig. 4), as well as of nuclear proteins at 24h (Rothblum *et al.*, 1976), further substantiates this notion. The threefold increase in the plasma content of fibrinogen observed in turpentine-treated rats has been attributed mainly to the enhanced synthesis of liver fibrinogen (Bouma *et al.*, 1975). The rate of incorporation *in vivo* of trace amounts of [3 H]leucine into plasma fibrinogen was significantly increased (eight- to nine-fold) in the turpentine-treated animals. Cycloheximide-treated animals, however, showed no net accumulation of fibrinogen in the plasma but a threefold increase in the rate of synthesis (Table 2). Presumably the lack of an increase in plasma fibrinogen during the period of increased biosynthesis after cycloheximide treatment could be the result of a re-adjustment of the relative rates at which individual classes of proteins turn over or a change in the intra-/extra-vascular distribution of the protein.

The results presented in Table 2 also indicate that the stimulation of plasma fibrinogen synthesis after cycloheximide treatment is not just an 'acute-phase' response such as is observed with inflammatory agents such as turpentine.

In conclusion, all these results demonstrate the value of using cycloheximide as a tool. After a brief inhibition of protein synthesis, the animal invokes many complex control circuits that co-ordinate the animal's metabolism in response to the condition present. That fibrinogen was rapidly synthesized early in the adaptive phase suggested that the liver first synthesized an extracellular protein of physiological importance to maintain homeostasis.

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