Biochem. J. (1976) **156**, 151–157
Printed in Great Britain

Regulation of Mammalian Protein Synthesis *in vivo*

PROTEIN SYNTHESIS IN RAT LIVER AND KIDNEY AFTER THE ADMINISTRATION OF SUBLETHAL DOSES OF CYCLOHEXIMIDE

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(*Received* 19 *December* 1975)

Protein synthesis *in vivo* was studied at various times after the administration of sublethal doses of cycloheximide to rats. Cycloheximide caused an inhibition, followed by a dose- and time-dependent stimulation, of incorporation of labelled precursor into proteins of the liver and kidney. The stimulation of protein synthesis at 24h was not due to a change of precursor pool or the specific radioactivity of the precursor used. During the stimulatory period, leucine incorporation into various cellular protein fractions varied; incorporation into total nuclear protein was the most affected.

An understanding of some of the quantitative and qualitative aspects of the regulation of protein synthesis has resulted from the judicious inhibition of the proteosynthetic system. In other areas of cellular metabolism, inhibitors have been used to perturb the system in order to investigate regulatory mechanisms during the recovery phase; in this manner, sites of control and regulatory intermediates have been determined. This approach, however, has not been used generally in a system as complex as protein synthesis. Cycloheximide is an inhibitor of protein synthesis, whose mode of action is understood (Pestka, 1971) and thus might be useful at sublethal doses for perturbing the system, permitting an evaluation of the recovery phase. In studies with animals *in vivo* (Jondorf, 1968; Glauman, 1970; Hwang *et al.*, 1974) and with mammalian cells in tissue culture (Ennis & Lubin, 1964; Christopher *et al.*, 1971) it has been observed that cycloheximide and puromycin reversibly inhibit protein synthesis. With chick-embryo cells in tissue culture, Christopher *et al.* (1971) have reported that reversal of cycloheximide inhibition is associated with an anomalously rapid rate of protein synthesis.

We report here that, after administration of sublethal doses of cycloheximide to rats, the recovery of protein synthesis in liver and kidney was dose- and time-dependent and there was a period of above-normal rate of protein synthesis. The increased rate of protein synthesis was apparently not due to a change in the amino acid pool. Examination of the rate of [^{3}H]leucine incorporation into proteins of subcellular fractions of liver indicated significant differences, suggesting alterations in the control of synthesis of individual proteins. The increased rate of synthesis of nuclear proteins during the stimulatory phase may implicate the involvement of differential gene activation during the recovery phase. Some of the observations described in the present paper have been presented in preliminary communications (Ch'ih & Devlin, 1974; Rothblum *et al.*, 1975).

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Experimental

Animals

Male Wistar rats (200±10g), maintained at 23°C under artificial illumination with a light/dark cycle of 12h, were fed on Purina Chow and water *ad libitum* until being killed. Cycloheximide (Sigma Chemical Co., St. Louis, MO, U.S.A.) in 0.9% NaCl was injected intraperitoneally. Animals were always killed between 09:00 and 11:00h to minimize diurnal variations.

Amino acid incorporation in vivo

Groups of two animals were injected intraperitoneally with [^{3}H]leucine (40–60Ci/mmol) or ^{3}H-labelled protein hydrolysate (mixture 3130-08, Schwarz/Mann, Orangeburg, NY, U.S.A.) 1h before being killed.

Isolation of subcellular components

Livers and kidneys were rinsed in ice-cold buffer, blotted dry, weighed and minced; organs of two animals in a group were pooled. All subsequent operations were performed at 0–4°C unless stated otherwise. Tissues 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 $600g_{max.}$ for 10min; nuclei were isolated from the pellet by the method of Blobel & Potter (1966) with the use of 0.1% (w/v) Triton X-100. The supernatant was re-centrifuged at $9000g_{max.}$ for 10min. Mitochondria were isolated by the procedure of Devlin & Ch'ih (1972), and microsomal fractions were purified by the method of Kamath & Rubin (1972). The postmicrosomal supernatant was taken as the cytosol fraction.

Chromatin was prepared by a modification of the method of Bonner *et al.* (1968) with Triton X-100 (0.5%) in the initial homogenization steps (Rothblum, 1975). Non-histone and histone chromosomal proteins were fractionated by the methods of Levy *et al.* (1972). Alternatively, histones were extracted from chromatin with ice-cold 0.2M-H_2SO_4.

Protein was determined by the method of Lowry *et al.* (1951), with crystalline bovine serum albumin (Miles laboratories, Kankakee, IL, U.S.A.) as the standard.

Amino acid analysis

Animals were anaesthetized with diethyl ether, and 5–7ml of blood was collected by cardiac puncture into a heparinized plastic syringe. Cells were removed by centrifugation, and plasma was processed for amino acid analysis by the methods of Stein & Moore (1954). After exsanguination, the livers were excised and prepared for amino acid analysis by the procedure of Tallan *et al.* (1954), with norleucine as an internal standard. Amino acid analysis was performed with a Beckman model 121 automatic amino acid analyser by using a standard programme for neutral and acidic amino acids.

Determination of radioactivity

Samples containing trichloroacetic acid (10%)-insoluble radioactivity were recovered by collection on Millipore cellulose acetate discs as described by Devlin & Ch'ih (1972). Samples containing acid-soluble radioactivity were extracted three times with diethyl ether to remove trichloroacetic acid. Radioactive materials were solubilized with Nuclear–Chicago solubilizer and mixed with 10ml of scintillation fluid [4.0g of 2,5-diphenyloxazole and 0.1g of 1,4-bis-(5-phenyloxazol-2-yl)benzene per litre of toluene].

Radioactivity was determined with a Packard Tri-Carb liquid-scintillation spectrophotometer; background was 16–20c.p.m., counting efficiency for ^3H was 35–45%, and 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.

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

Results

Protein synthesis in tissues after the administration of cycloheximide

The incorporation of [^3H]leucine into rat kidney proteins after the administration of doses of cycloheximide of 0.5, 2.0 and 3.0mg/kg was inhibited by 60%, 80% and 85% respectively (Fig. 1), with the inhibition lasting approx. 6, 14 and 16h. Higher than normal rates of protein synthesis, 125–170% relative to control, were observed after the period of inhibition. With 3.0mg of cycloheximide/kg approx. 70% of the animals survived for at least 24h. A stimulated rate of protein synthesis was observed in 60% of the survivors at 24h, whereas the remaining animals continued to manifest an inhibition; as this dose is greater than the LD_{50} (Ford & Leach, 1948) it is possible that the other 40% were dying, possibly because of the effects of the inhibition of protein synthesis (Young *et al.*, 1963).

A similar pattern of inhibition and stimulation of incorporation was observed in livers of treated animals. As presented in Fig. 2, at two different times

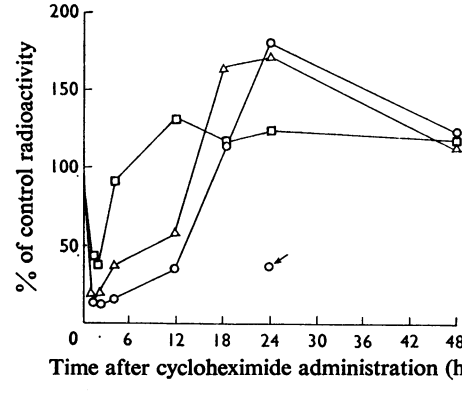

Fig. 1. *Incorporation of [^3H]leucine in vivo into total cellular protein as a function of time after the administration of cycloheximide*

Rats were injected at zero time with various doses of cycloheximide (□, 0.5; △, 2.0; ○, 3.0mg/kg). At 1h before death, [^3H]leucine (200μCi/kg) was injected intramuscularly. Animals were killed at the times indicated. Incorporation of [^3H]leucine into total cellular protein was determined as described in the text. Each value is the mean of at least three experiments with the kidneys of two rats in each experiment.

after the administration of three different doses of cycloheximide, the incorporation of [^{3}H]leucine into total protein by the cells of the liver was essentially identical with that of the kidney. In all subsequent experiments, rat liver was utilized because of ease of handling, yield of tissue and the role of the liver in the synthesis of specific extracellular proteins.

Amino acid pools and the specific radioactivity of leucine

Plasma and tissue amino acid concentrations were measured to determine possible influences of cycloheximide on pool sizes, which in turn could account for changes in the rate of incorporation. As indicated in Table 1, plasma concentrations of all measured amino acids except glycine rose within 1h of the administration of cycloheximide (2.0mg/kg). At 24h after cycloheximide administration, the concentrations of aspartate, glutamate and valine were still elevated, whereas tyrosine and proline concentrations were decreased, and the other amino acids, including leucine, had returned to control values. The concentrations of the amino acids in the liver varied in a similar pattern (Table 2). Leucine rose from a control value of 0.15 μmol/g to 0.40 μmol/g after 1h, and returned to 0.16 μmol/g after 24h. Return of most amino acid concentrations in liver to near-normal values 24h after administration of cycloheximide suggests that the accelerated rate of [^{3}H]leucine incorporation was not a reflexion of changes in the precursor pools.

The specific radioactivity of liver and plasma leucine was determined at various times after addition of cycloheximide. As shown in Table 3, after a 1h labelling period, cycloheximide (2.0mg/kg) did not inhibit the uptake of radioactivity into the acid-soluble pool of the liver. At 24h after the administration of cycloheximide, the amounts of acid-soluble radioactivity in the liver and plasma were 117% of control. The specific radioactivity of the leucine pool in liver, calculated by dividing the amount of acid-soluble radioactivity by the concentration of leucine in the liver, was 0.24 and 0.29 μCi/μmol at 2 and 24h respectively, compared with a control value of 0.26 μCi/μmol. The specific radioactivities of leucine in the plasma were 0.31 and 0.30 μCi/μmol respectively for controls and animals treated 24h previously with cycloheximide. These results support the conclusion that changes in the leucine pools could not account for the effects on incorporation.

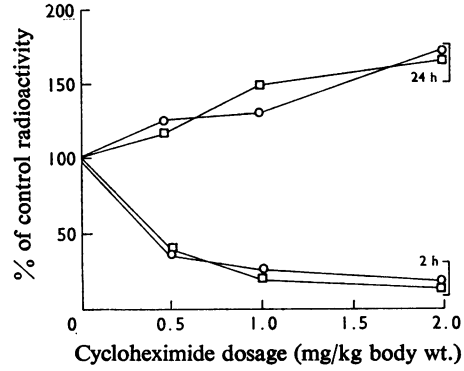

Fig. 2. *Incorporation of [^{3}H]leucine in vivo into total cellular protein of rat livers and kidney at two times after the administration of cycloheximide*

The incorporation of [^{3}H]leucine into total cellular protein of liver (○) and kidney (□) was determined at 2 and 24h after the administration of cycloheximide. For further details, see the legend to Fig. 1.

Table 1. *Concentrations of amino acids in the plasma after the administration of cycloheximide*

Animals were injected with 2mg of cycloheximide/kg 1, 2 and 24h before being killed. Values presented are means±S.D. of three experiments. For further details, see the text.

Amino acid \ Time after cycloheximide administration (h) ...	Amino acid concentration (μmol/100ml)			
	0 (Control)	1	2	24
Aspartate	4.8±2.3	9.2± 6.0	5.7± 1.6	9.3± 1.6
Glutamate	17.3±2.3	29.0±13.0	21.2± 3.0	30.5±13.0
Methionine	4.4±1.2	8.7± 0.8	8.1± 0.7	6.7± 2.4
Tyrosine	6.5±1.3	17.8± 2.3	20.1± 2.3	3.6± 0.2
Phenylalanine	4.9±1.4	11.7± 3.6	9.7± 2.4	5.6± 0.2
Proline	26.1±2.9	40.4± 5.9	38.0± 6.1	18.4± 6.0
Glycine	40.0±2.6	48.8± 8.2	50.9±12.0	53.0±10.0
Alanine	47.4±6.5	91.0±15.0	85.1±30.0	47.8± 8.0
Valine	18.1±5.5	61.3± 9.8	68.0±11.0	36.0±12.0
Isoleucine	7.3±1.9	23.6± 4.1	24.7± 5.3	10.9± 2.4
Leucine	14.9±1.8	46.2± 6.5	46.0± 8.3	18.4± 3.1

Table 2. *Concentrations of amino acids in the liver after the administration of cycloheximide*

Animals received injections of cycloheximide (2mg/kg) at zero time. Values presented are the results of a typical experiment. For further details, see the text.

Amino acid	Time after cycloheximide administration (h) ...	Amino acid concentration (μmol/g of liver)				
		0 (Control)	1	2	24	48
Aspartate		0.79	2.00	1.60	1.60	0.85
Glutamate		0.54	1.10	1.60	1.20	0.61
Methionine		0.04	0.08	0.04	0.05	0.06
Tyrosine		0.04	0.16	0.14	0.05	0.06
Phenylalanine		0.06	0.12	0.08	0.06	0.07
Proline		0.13	0.54	0.28	0.16	0.14
Glycine		1.30	2.00	2.00	1.30	1.70
Alanine		2.00	6.70	4.60	2.90	1.80
Valine		0.20	0.92	0.66	0.22	0.22
Isoleucine		0.08	0.22	0.18	0.09	0.10
Leucine		0.15	0.40	0.33	0.16	0.18

Table 3. *Specific radioactivity of leucine in the liver and plasma during the inhibitory and recovery phase of liver protein synthesis*

Values are corrected for 0.1 ml of plasma/g of liver (Caster *et al.*, 1955). Each animal received 20 μCi of [^{3}H]leucine 1 h before being killed. For further details, see the text.

Time after cycloheximide administration (h)	Liver			Plasma		
	μmol/g	10^{-3} × d.p.m.	μCi/μmol	μmol/ml	10^{-3} × d.p.m.	μCi/μmol
0 (Control)	0.14	76.9	0.26	0.15	102.3	0.31
1	0.35	134.8	0.17	0.46	185.1	0.18
2	0.29	154.7	0.24	0.46	192.7	0.19
24	0.14	90.2	0.29	0.18	119.3	0.30
48	0.17	60.2	0.17	—	85.3	—

Synthesis of the proteins of the subcellular fractions after the inhibition of protein synthesis

Measurement of an increased rate of synthesis of protein of a tissue may be a reflexion of either the synthesis of all the cellular proteins or a specific group of proteins; whereas the first case might reflect a generalized effect on the protein-synthetic apparatus, the second case might involve specific effects on translation and possibly transcription. The rates of synthesis of the proteins of the subcellular fractions of the liver were measured at zero time, and 2 and 24h after the administration of various doses of cycloheximide.

At 2h after administration of cycloheximide, (Fig. 3) synthesis of proteins of various subcellular fractions was found to be dose-dependent; there were some minor differences in the degree of inhibition. At 24h the rates of synthesis of the proteins of the various subcellular fractions had all returned to values that were near to or higher than the control values. At 0.5, 1.0 and 2.0mg of cycloheximide/kg, the relative rates of synthesis of microsomal proteins were 90%, 100% and 110% of control respectively, whereas synthesis of mitochondrial proteins was 105%, 120% and 135% of control. The highest stimulation was observed in the nuclear proteins; at 2.0mg/kg the rate of synthesis was 230% of control.

Table 4 presents the results of experiments carried out to identify further those nuclear proteins whose synthesis was most affected. At 24h after the administration of cycloheximide (2.0mg/kg) the specific radioactivity of the total chromatin proteins was 270%. When chromatin proteins were fractionated by the method of Levy *et al.* (1972) the average radioactivity of the non-histone chromosomal proteins was 360% and that of the histones was 260% (Table 4). The specific radioactivity of H_2SO_4-extracted histones was also about 260%.

Discussion

The observed inhibition by cycloheximide of protein synthesis by liver and kidney (Figs. 1 and 2) is as expected (Young *et al.*, 1963; Toinheim *et al.*, 1969; Farber & Farmer, 1973), and the recovery from the inhibition has also been reported (Jondorf, 1968; Glauman, 1970; Hwang *et al.*, 1974;

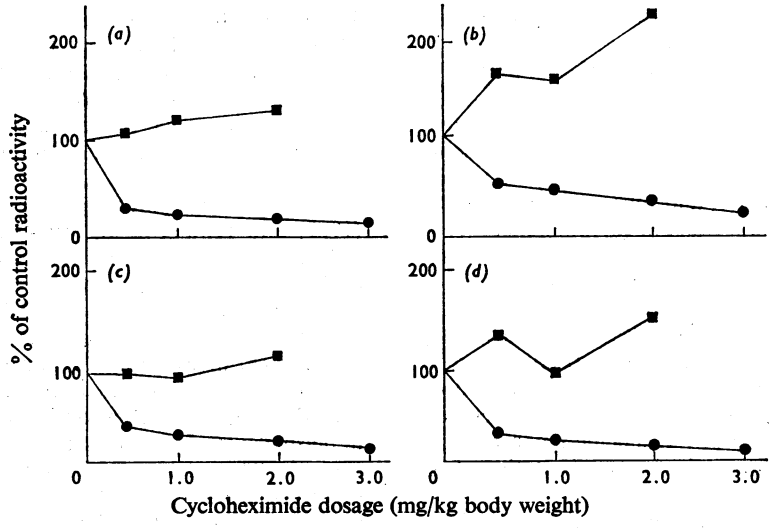

Fig. 3. *Incorporation of [^{3}H]leucine into the proteins of subcellular fractions of rat liver at two different times after the administration of various doses of cycloheximide*

Experimental conditions, the fractionation procedure and the determination of the incorporation of [^{3}H]leucine (200μCi/kg) were carried out as described in the text at 2 (●) and 24h (■) after cycloheximide administration. Each value is the mean of three experiments with two rats in each experiment. (*a*) Mitochondrial fraction; (*b*) nuclear fraction; (*c*) microsomal fraction; (*d*) cytosol.

Table 4. *Rates of synthesis of nuclear proteins*

All treated animals received single injections of cycloheximide (2mg/kg) 2 and 24h before being killed. Each group of rats consisted of five animals. For further information see the text. Histones were prepared by extracting chromatin with ice-cold 0.2M-H_2SO_4.

Experiment	Time after cycloheximide administration (h)	^{3}H-labelled protein hydrolysate (μCi/kg)	Specific radioactivity (d.p.m. of protein with % of control in parentheses)			
			Whole homogenate	Chromatin	Histone	Non-histone
1	0 (Control)	400	2314 (100)	970 (100)	535 (100)	—
	2	400	662 (28)	320 (33)	207 (39)	—
2	0 (Control)	400	3130 (100)	760 (100)	670 (100)	—
	24	400	4170 (133)	2490 (328)	1746 (260)	
3	0 (Control)	1250	8970 (100)	3780 (100)	2430 (100)	2550 (100)
	24	1250	11757 (133)	10800 (286)	7750 (312)	8190 (320)
	24	1250	10468 (117)	7590 (198)	4930 (203)	10640 (415)

Levine *et al.*, 1975). After the inhibition of protein synthesis, HeLa cells in culture show an increased rate of initiation (Penman *et al.*, 1973). Christopher *et al.* (1971) have reported an anomalously rapid rate of protein synthesis, as measured by radioactive amino acid incorporation by chick-embryo cells in culture after removal of cycloheximide and puromycin from the medium. The increased rates of

protein synthesis *in vivo* by the liver and kidney after the administration of cycloheximide (Figs. 1 and 2) extend the observations of Christopher *et al.* (1971) to whole animals.

Conclusions on alterations in the rate of protein synthesis based on changes in the rate of incorporation of an amino acid are always suspect, in that changes in the pool size, and thus specific radioactivity of the pool, could account for differences from controls. The results of this study indicate that changes of the leucine pool or the specific radioactivity of the precursor pool are not sufficiently large to account for the increased rate observed. In addition, the stimulation has been observed utilizing a radioactively labelled protein hydrolysate in place of leucine (Table 4). It is concluded that the increased rate of [^{3}H]leucine incorporation into different cellular proteins is a manifestation of an increase in protein synthesis.

The synthesis of proteins of subcellular fractions was examined to explore possible qualitative and quantitative aspects of the changing rate of total synthesis. At 24h after the administration of cycloheximide the rates of incorporation of [^{3}H]leucine into the proteins of the various subcellular fractions were not equal (Fig. 3). The pattern of incorporation suggests a possible priority in the synthesis of some proteins. If so, the result could be due to differential gene transcription (MacGilvery *et al.*, 1972), involving selective gene activation and repression. One would first expect to observe an enhanced rate of synthesis of nuclear protein, which was, in fact, observed (Fig. 3); at 24h the rate was 230% of control. Further, when deoxyribonuclear proteins were examined, the rate of total chromatin protein synthesis was 270% of control, and the rates of synthesis of non-histone and histone chromosomal proteins were 360% and 260% of control respectively (Table 4). This increased rate of non-histone protein synthesis is consistent with current models of the regulation of transcription (MacGilvery *et al.*, 1972). Current proposals include histone synthesis coupled with DNA synthesis (Borun *et al.*, 1967); however, the increased rate of histone synthesis shown in Table 4 is not associated with an increased rate of DNA synthesis (L. I. Rothblum, T. M. Devlin & J. J. Ch'ih, unpublished results). The increased rate of histone synthesis might be due to changes in histone metabolism (e.g. phosphorylation) associated with the inhibition of protein synthesis (Tanphaichitr *et al.*, 1974). Whatever the reason, it seems that when a tissue is striving to regain its normal homoeostatic balance, the synthesis of histones as well as of non-histone proteins, is co-ordinated with other metabolic events.

The results presented here do not shed light on possible hormonal changes, which might be invoked after cycloheximide administration (Levine *et al.*, 1975) and which might influence the interpretation of this study. However, the investigations by Christopher *et al.* (1971) using cells in tissue culture seem to eliminate a direct correlation between changes in hormone concentrations and the rate of protein synthesis after the administration of cycloheximide.

The cytological investigations by Verbin *et al.* (1969) and Hwang *et al.* (1974) have shown that, although the inhibition of protein synthesis by cycloheximide and puromycin is associated with ultrastructural changes in the cells of the liver, no necrotic lesions could be identified. Preliminary biochemical investigations reported by Ch'ih *et al.*, (1975) indicate that after the administration of sublethal doses of cycloheximide to rats, the changes in plasma enzyme activities are not indicative of liver necrosis. Thus it appears that the perturbation of macromolecular metabolism of the rat with sublethal doses of cycloheximide presents the investigator with a system in which to study both the qualitative and quantitative aspects of the regulation of protein synthesis as well as cellular repair mechanisms. It may be possible that investigations of the synthesis of the proteins of the various subcellular fractions will shed more light on the mechanisms involved.

We thank Mr. Michael Schwartz, St. Christopher's Hospital, Philadelphia, PA, U.S.A., for the amino acid analysis, Mrs. Linda Faulkner for technical assistance and Mrs. Frances Taylor for animal maintenance and care. Part of this paper is taken from the dissertation submitted by L. I. R. to the Graduate School of the Hahnemann Medical College and Hospital in partial fulfilment of the requirements for a Ph.D. degree. This investigation was supported by Research Grant number AM 14548 from the National Institute of Arthritis and Metabolic Diseases, NIH, USPHS.

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