Further Studies on the Stimulation of L-(^{14}C)-Amino Acid Incorporation with Cycloheximide

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SUMMARY

The administration *in vivo* of cycloheximide increases the L-(^{14}C)-amino acid incorporation *in vitro* in a rat liver microsomal system. A single injection of cycloheximide stimulates the *in vitro* system within the first hour. Two separate microsomal responses are recognized, which involve endogenous RNA and microsomal sensitivity to added messenger RNA, respectively. The former effect is more pronounced and prolonged; it persists for 5–6 days after a single injection of the drug, whereas the exogenous RNA effect returns to the level of the control in 2–3 days. The increased activity of the hepatic microsomal fraction isolated from cycloheximide-treated animals is not related to (a) changes in $MgCl_2$ requirement of the system, (b) the stabilization of the endogenous messenger RNA, (c) factors present in the supernatant fraction of the cell.

Injections of cycloheximide selectively inhibit the microsomal drug-metabolizing enzymes and prolong the hexobarbital sleeping time.

Repeated daily injections of cycloheximide slow the overall growth rate of the treated rats relative to the controls.

INTRODUCTION

Cycloheximide [Actidione, NSC-185, 3-(2-(3, 5-dimethyl-2-oxocyclohexyl)-2-hydroxyethyl)glutarimide], an antibiotic first isolated by Whiffen *et al.* (1) from *Streptomyces griseus*, has been previously shown to inhibit the growth of yeast (2), cells in culture (3), and a variety of experimental tumors (4).

In studies with intact animals, Young *et al.* (5) and Gorski and Axman (6) showed that injections of cycloheximide depressed the uptake of subsequently injected labeled amino acids into protein of selected tissues.

Studies on the influence of cycloheximide on the incorporation of ^{14}C-labeled amino acids into protein in subcellular systems (7–11) have been primarily concerned with the effects of adding cycloheximide to the incubation mixtures in various systems *in vitro*. Experiments of this type seemed to confirm that cycloheximide was a powerful inhibitor of protein synthesis.

We have previously found, however (12–14), that intraperitoneal injections of cycloheximide into rats yielded liver microsomal fractions that had greatly enhanced amino acid incorporation compared with controls. Investigation of the relationship between the administration of cycloheximide *in vivo* and the enhanced ^{14}C-labeled amino acid uptake *in vitro* in the liver microsomal system of Gelboin and Sokoloff (15), as modified by Jondorf *et al.* (16), are described in this paper.

MATERIALS

Cofactors were obtained from various suppliers as indicated: ATP,[1] GTP, NADP

[1] Abbreviations used in the text are: ATP, adenosine triphosphate; DNA, deoxyribonucleic acid; GTP, guanosine triphosphate; NADP, nicotinamide adenine dinucleotide phosphate;

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(P. L. Biochemicals, Milwaukee, Wisconsin); reduced glutathione, phosphocreatine, nicotinamide, and glucose 6-phosphate (Sigma Chemical Co., St. Louis, Missouri); creatine phosphokinase (Boehringer Mannheim Corporation, New York, New York); uniformly labeled L-(^{14}C)-phenylalanine (New England Nuclear Corporation, Boston, Massachusetts); polyuridylic acid (Miles Chemical Co., Elkhart, Indiana).

Cycloheximide was obtained from the Cancer Chemotherapy National Service Center through the courtesy of Dr. J. A. R. Mead. β-Diethylaminoethyl diphenylpropylacetate (SKF-525A) was a gift from Smith, Kline and French Laboratories, Philadelphia, Pennsylvania, through the courtesy of Dr. J. J. Kamm.

METHODS

Animal Treatment

Animals used in our experiments were female Sprague-Dawley rats of the same age, weighing 160 g each and maintained on Purina Chow rat cube diet. Groups of two or more animals were injected with cycloheximide intraperitoneally at a dose level of 1 mg/kg unless otherwise stated. The injection volume for experimental and control rats was 0.5 ml per 100 g body weight. Rats were given water ad libitum, but were starved for 24 hr to lower liver glycogen levels, and were then killed in the cold room by stunning and decapitation.

Cell Fractionation Procedures

The livers were removed, rinsed with ice-cold 0.25 M sucrose, and homogenized 4 g at a time with 5 volumes of 0.25 M sucrose in a loose-fitting Tri-R Instrument Teflon-glass homogenizer at 4000 rpm for 10 passes. The homogenates from two or more livers were pooled and centrifuged for 10 min at 600 *g* and 15 min at 15,000 *g* (at 0–4°). The microsomal pellets were obtained by subsequent centrifugation at 105,000 *g*. Each pellet was resuspended in 1.5 ml ice-cold 0.25 M sucrose. Resuspended microsomal fractions were adjusted to the same protein concentrations by the method of Lowry *et al.* (17) with bovine serum albumin V (Armour) as the standard.

In our studies on the effect of cycloheximide administration to rats on the NADPH-dependent drug metabolizing enzymes in the liver microsomes a different procedure was adopted. Groups of control and pretreated animals that had been starved for 24 hr were stunned and killed by decapitation. Livers were removed and homogenized in 3 volumes of ice-cold 1.15% potassium chloride solution. The homogenates were centrifuged in the cold at 600 *g* for 10 min and 15,000 *g* for 15 min. The 15,000 *g* supernatant material which contained the microsomes was used for the enzyme assays. For experiments with neoprontosil, microsomal fractions were prepared by centrifugation at 105,000 *g* for 1 hr and redispersal of the pellets so obtained in 0.1 M sodium-potassium phosphate buffer pH 7.6.

Incorporation Studies

Incubations for endogenous RNA-dependent (nonpreincubated) and polyU-dependent (preincubated) L-(^{14}C)-phenylalanine incorporation were performed in triplicate by a method modified from Gelboin and Sokoloff (15) as already described by Jondorf *et al.* (16) and in greater detail below.

Every nonpreincubated incubation tube contained the following components in a total volume of 0.85 ml. Potassium phosphate (pH 7.4), 10 μmoles; magnesium chloride, 5 μmoles; sucrose, 75 μmoles; ATP, 1.25 μmoles; GTP, 0.25 μmole; phosphocreatine, 20 μmoles; creatine phosphokinase, 0.125 mg; reduced glutathione (neutralized with 10 N potassium hydroxide), 50 μmoles; uniformly labeled L-(^{14}C)-phenylalanine of specific activity 10 μC/μmole, 0.025 μmole; microsomal protein, 3.6 mg; 105,000 *g* supernatant protein from control rats except where otherwise specified, 1.2 mg. The mixtures were in-

NADPH, the reduced form of nicotinamide adenine dinucleotide phosphate; PolyU, polyuridylic acid; POPOP, 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene; PPO, 2,5-diphenyloxazole; RNA, ribonucleic acid.

cubated at 37° with air as the gas phase for 15 min.

Tubes that were preincubated for 12 (or more where indicated) minutes at 37° had the same components in the mixture except for the presence of greater quantities of magnesium chloride (8 μmoles), and the absence of labeled phenylalanine. After preincubation, additional phosphocreatine (20 μmoles) and creatine phosphokinase (0.125 mg), polyuridylic acid (0.15 mg), and labeled phenylalanine (0.025 μmole) were added to the tubes which were then incubated as above.

The reactions were stopped with 3 ml of ice-cold 10% w/v trichloroacetic acid containing 1 mg/ml unlabeled L-phenylalanine. The precipitated proteins were purified according to Siekevitz (18) with the use of chloroform in the procedure omitted, and were then redissolved in hyamine (19), transferred to vials, and counted in a toluene-PPO-POPOP scintillation solution (20, 21) in a liquid scintillation counter (Nuclear Chicago, Model 724/5). Zero time counts were subtracted from the experimental counts in calculations of the net counts incorporated. All values quoted for specific activities under Results are the average values of three simultaneously incubated samples in representative experiments.

Enzyme Assays

Measurement of N-demethylation of monomethyl-4-amino-antipyrine and aminopyrine. Incubations were performed with slight modification from the method of Gillette *et al.* (22) and Booth and Gillette (23) for varying periods of time from 10 min to 1 hr (24). A typical mixture contained tissue preparation equivalent to 0.375 g liver and the following components (in amounts within the optimal range) in a total volume of 4.5 ml. Substrate, 5 μmoles; NADP, 0.5 μmole; glucose 6-phosphate, 30 μmoles; nicotinamide, 50 μmoles; magnesium chloride, 75 μmoles; 0.5 M phosphate buffer pH 7.4, 1.7 ml; neutralized semicarbazide, 5 mg. The reaction was terminated after incubation in a shaking water bath at 37° with exposure to the atmosphere, by addition of 4.5 ml of saturated lead subacetate. After centrifugation, aliquots of the protein-free supernatant material were assayed for formaldehyde by the Cochin and Axelrod (25) modification of the Nash (26) method.

Measurement of ring hydroxylation, using aniline as substrate. Incubations were performed as above, but with phosphate buffer pH 8.2, cf. Mitoma *et al.* (27), and with the omission of semicarbazide from the mixture. The reaction was terminated with 4.5 ml 10% trichloroacetic acid, and *p*-aminophenol was determined in the deproteinized supernatant by the method of Brodie and Axelrod (28). Standard amounts of *p*-aminophenol were carried through the incubation and assay procedures concurrently with the unknowns.

Measurement of azo-reduction, using neoprontosil as substrate. Incubations were performed in an atmosphere of nitrogen with microsomes from 0.5 g liver and 10 μmoles of substrate by the method of Hernandez (29) modified from Fouts *et al.* (30).

RESULTS

L-(^{14}C)-*Phenylalanine Incorporation in Vitro at Varying Levels of Cycloheximide in Vivo*

Figure 1 shows the effect of increasing the dose of cycloheximide in the range 0.05–2.0 mg/kg injected into rats, on the L-(^{14}C)-phenylalanine incorporation *in vitro*, in the hepatic microsomal system. It is clear that there is a distinct stimulating effect on both the endogenous RNA-dependent (nonpreincubated) and the polyU-dependent (preincubated) incorporation even at a dose level of 0.05 mg/kg. All the cycloheximide-treated animals yield microsomes with a greater uptake of amino acids in the *in vitro* system. It was noted that throughout the concentration range investigated the percentage increase in the endogenous RNA-dependent incorporation system was greater than the corresponding increase in the preincubated, polyU-dependent system.

Animals injected with cycloheximide at

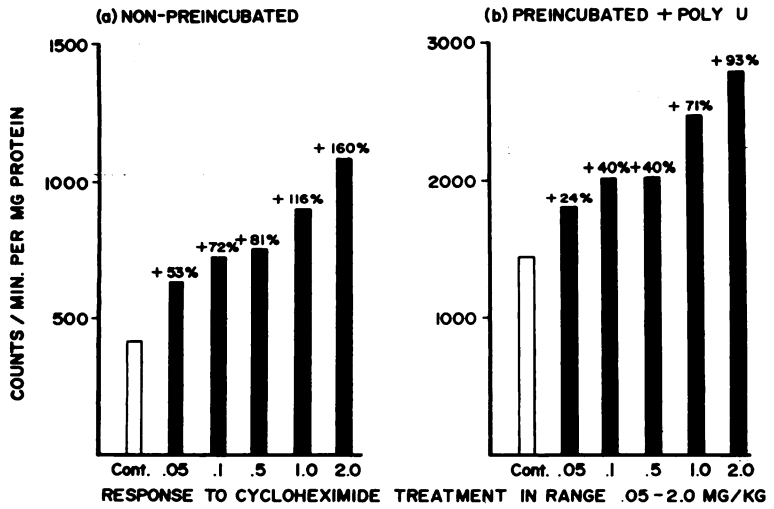

FIG. 1. *Stimulating effect of treating rats with cycloheximide at varying dose levels ranging from 0.05 to 2.0 mg/kg on the (a) endogenous RNA-dependent (b) polyU-dependent, hepatic microsomal* L-(^{14}C)*-phenylalanine incorporation in vitro*

Experimental details as indicated in Methods.

dose levels greater than 2 mg/kg rarely survived for twenty-four hr.

Effect of Magnesium Chloride on the Incorporation of L-(^{14}C)*-Phenylalanine in the Microsomal System*

Figure 2 demonstrates that the enhancing effect on the L-phenylalanine uptake

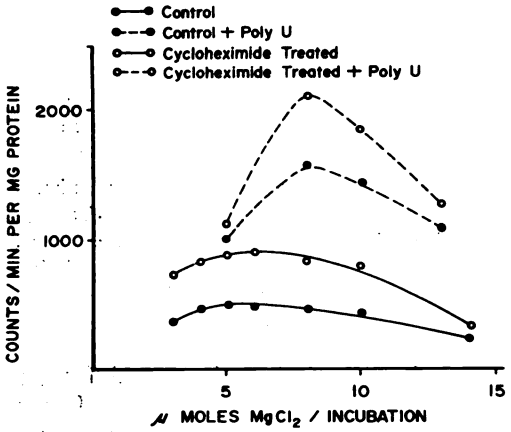

FIG. 2. *Effect of magnesium chloride on the (a) endogenous RNA-dependent (b) polyU-dependent,* L-(^{14}C)*-phenylalanine incorporation by microsomal fractions isolated from control and cycloheximide-treated rats.*

Experimental details are given in Methods section.

in vitro is not due to a shift in the optimal magnesium chloride requirement of the endogenous RNA-dependent (5 μmoles) and polyU-dependent system (8 μmoles), respectively. The optimal requirement for magnesium chloride is the same in the microsomal system isolated from cycloheximide-treated and control rats. It is known that mammalian *in vitro* incorporation systems are very sensitive to $MgCl_2$ concentrations (16, 31, 32), but the enhanced amino acid incorporation *in vitro* resulting from cycloheximide administration *in vivo* is not dependent on alterations in the requirement for Mg^{++} after cycloheximide treatment. The increase in amino acid incorporation in the microsomal system isolated from cycloheximide-treated rats is observed at the optimal and at various suboptimal $MgCl_2$ concentrations in the system *in vitro*.

Effect of Cell Sap (105,000 g Supernatant Fraction) on the Amino Acid Incorporation in the Microsomal System

Table 1 shows that the increased amino acid incorporation in the *in vitro* system brought about by administration of cycloheximide to experimental animals is predominantly localized in the microsomal

TABLE 1

The amino acid incorporation into protein in vitro, with various recombinations of hepatic microsomal and 105,000 g supernatant fractions from control and cycloheximide-treated rats

Supernatant as well as microsomal fractions from control and cycloheximide-treated (Cyclohex.) sources were adjusted to matching protein concentrations. In all other respects, conditions for incubations were as indicated under Methods. Values are stated as counts per minute per milligram of protein.

Microsomes	Supernatant	Nonpreincubated	Preincubated + PolyU
Control	Control	445	2122
Control	Cyclohex.	461 (+ 3%)[a]	2383 (+ 12%)
Cyclohex.	Control	931 (+ 109%)	3271 (+ 54%)
Cyclohex.	Cyclohex.	1032 (+ 131%)	3651 (+ 72%)

[a] Figures in parentheses represent percentage changes from control values.

fraction. The contribution to the enhanced activity, which is localized in the supernatant fraction, is relatively minor and of the order of 10% rather than 100%. This leads to the conclusion that cycloheximide treatment of rats does not affect the transfer RNA content or activity of the cell sap in the liver.

Effect of Preincubation of the Microsomal System on Endogenous and Exogenous Messenger RNA Directed L-(^{14}C)-*Phenylalanine Incorporation*

In Fig. 3 the rate of loss of endogenous messenger RNA-dependent amino acid incorporation in microsomes from control and cycloheximide-treated rats is compared. Microsomal preparations are preincubated for varying periods of time (from 3 to 24 min) before extra energy source and labeled amino acid are added to the system [cf. Kato *et al.* (32)], which is then incubated for 15 min. There is a complete loss of endogenous RNA-dependent amino acid-incorporating ability in the microsomal systems from both control and cycloheximide-treated rats after preincubations of 12 min. Cycloheximide treatment *in vivo* does not therefore enhance the stability of microsomal RNA in the *in vitro* system under investigation. When artificial messenger RNA is added to the system after varying periods of preincubation, maximum sensitivity to the added messenger is observed after the time when preincubation has effected the complete loss of endogenous RNA-dependent amino acid incorporation. In this respect the cycloheximide effect resembles that observed with 3-methylcholanthrene (33) or with phenobarbital (32).

In the preincubated system the addition of various greater amounts of polyU (200–400 μg) for the incubation, in order to test

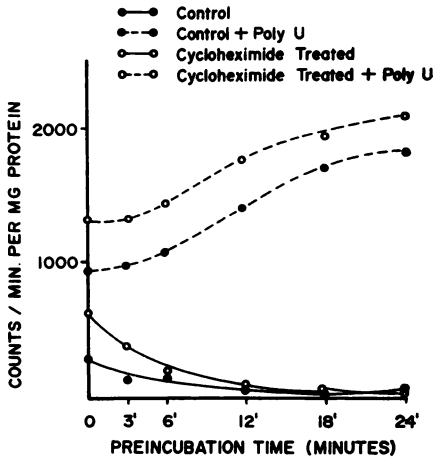

FIG. 3. *Effect of variations in the preincubation time on (a) endogenous RNA-dependent (b) polyU-dependent,* L-(^{14}C)-*phenylalanine incorporation by microsomal fractions isolated from control and cycloheximide-treated rats*

See Methods for experimental details.

the sensitivity of the microsomal fraction to exogenous RNA, produces no further increase in the amino acid incorporation of the control and cycloheximide-treated microsomes. Thus it can be concluded that the hepatic microsomal fractions from control and cycloheximide treated animals become saturated with exogenous RNA at the same concentration. Furthermore, the

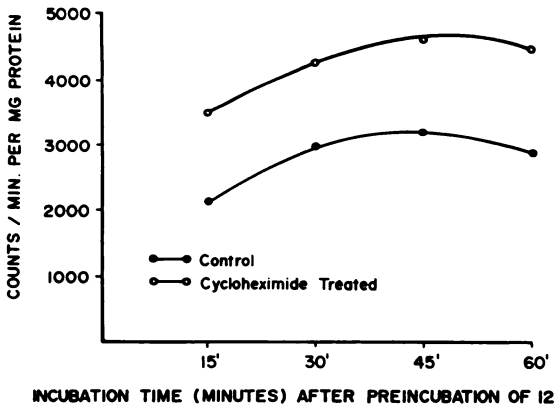

FIG. 4. *Effect of extending the incubation time on the preincubated polyU-dependent* L-(^{14}C)-*phenylalanine incorporation of hepatic microsomal fractions isolated from control and cycloheximide-treated rats*

differential activities appear to be independent of some factor related to a decrease in ribonuclease activity in the cycloheximide-treated animals, since the increased sensitivity to polyU is maintained over a range of polyU concentration in excess of saturation (cf. 32).

The effect of prolonging the incubation time with saturating amounts of polyU (150 μg) in the preincubated system is shown in Fig. 4. We found that the increased polyU-dependent phenylalanine incorporation in the microsomal system from cycloheximide-treated rats is maintained over the whole time range investigated. The activity does not level off before that of the control, and consequently the cycloheximide effect is independent not only of any possible prolongation of stability of endogenous RNA, but also of any preferential degradation of the sensitivity to added polyU.

Effect of Time after Injection on the Appearance of Cycloheximide Stimulation of Amino Acid Incorporation in the in Vitro System

Figure 5 demonstrates that cycloheximide administration *in vivo* elicits a response in the *in vitro* amino acid incor-

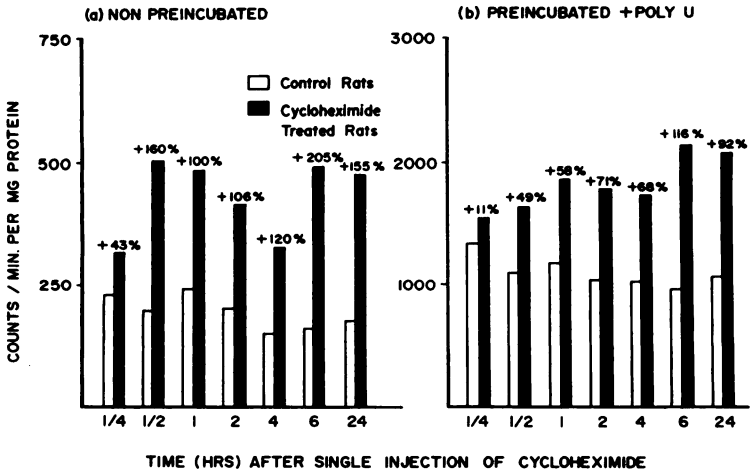

FIG. 5. *The rate of development of the cycloheximide-induced stimulation of amino acid incorporation in vitro after administration of the drug in vivo*

Experimental conditions are given in Methods.

TABLE 2
Short-term effects of administering lethal dose levels of cycloheximide to rats on the amino acid incorporation into protein in vitro

Cycloheximide (mg/kg)	Time[a] (hr)	Nonpreincubated[b]	Preincubated + PolyU[b]
—	1	473	1737
5	1	734 (+ 56%)[c]	2294 (+ 31%)
10	1	836 (+ 77%)	2695 (+ 54%)
5	2	1095 (+ 132%)	3244 (+ 86%)
10	2	988 (+ 109%)	2951 (+ 68%)

[a] Time refers to interval between intraperitoneal injection and killing of animals. All animals were starved for 24 hr before sacrifice.

[b] Values are stated as counts per minute per milligram of protein.

[c] Figures in parentheses represent percentage changes from control values, which are set at 100%.

poration system within the first hour after injection. Microsomes isolated from cycloheximide-treated rats have a higher amino acid incorporating ability than controls in the whole time range, ¼–24 hr, after administration of cycloheximide. The short-term effectiveness of cycloheximide in this respect leads us to the conclusion that the drug itself, rather than one of its metabolites, may be responsible for the observed enhancement of the *in vitro* protein synthesizing activity.

It was of interest to test the short-term effectiveness of larger doses of cycloheximide in enhancing the amino acid incorporation of the microsomal system. Consequently, cycloheximide was administered at 5 and 10 mg/kg levels to rats which were killed 1 hr and 2 hr later. It is readily discerned in Table 2 that these lethal doses of cycloheximide stimulate the L-(^{14}C)-phenylalanine incorporation in the endogenous RNA or polyU-dependent system *in vitro*.

Figure 6 shows that the cycloheximide effect on the amino acid incorporation *in vitro* is a combination of two separate effects. In experiments to determine how long the effects of one injection of cycloheximide persisted, we isolated the microsomal fractions from liver at daily intervals from 1 to 5 days and compared the endogenous RNA-dependent and the preincubated, polyU-dependent, amino acid incorporation with the appropriate controls. The enhancement of L-phenylalanine incorporation in the endogenous system gradually diminished and returned to the control level at day 5 or 6. In contrast, however, the sensitivity of microsomes to

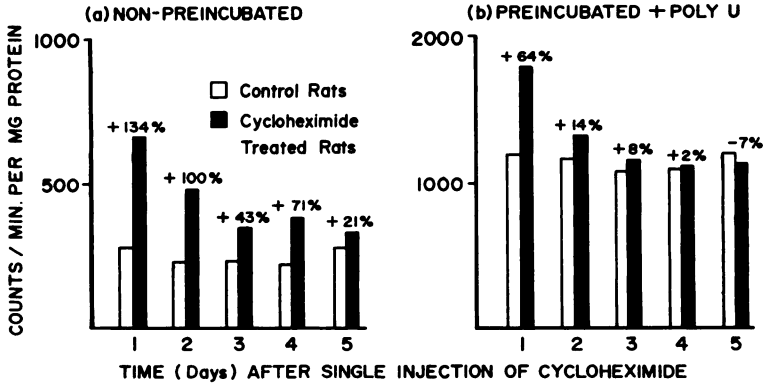

FIG. 6. *The persistence (in the time range 1–5 days) of the cycloheximide-induced stimulation of amino acid incorporation in vitro after a single injection of the drug*

added messenger RNA returned to the level of the controls on day 3. This suggests that the cycloheximide effect is related to changes in the endogenous RNA of the liver microsomal fraction and to a lesser extent, in terms of percentage change and duration, to the sensitivity of the ribosomes to added messenger RNA.

Effect of Chronic Administration of Cycloheximide on the Growth Rate of Rats

Figure 7 shows the effect of chronic daily intraperitoneal administration of cycloheximide (1 mg/kg) or water on the growth of groups of six male Sprague-Dawley rats maintained on Purina Chow and water ad libitum. It is clear that there is a dramatic

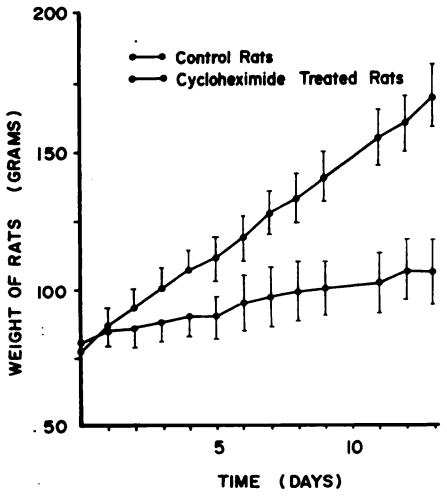

FIG. 7. *Comparison of the growth rate of groups of six control and cycloheximide-treated (1 mg/kg intraperitoneally, daily) rats*

Average weights and standard deviations were determined daily as indicated.

decline in the growth rate of the cycloheximide-treated rats compared with the controls receiving daily injections of water only. This suggests that overall there might be a decline in the synthesis of functional protein in cycloheximide-treated rats. At the end of the experiment on day 13 the control animals weighed 72 ± 14 g and the cycloheximide-treated rats weighed 112 ± 13 g. The animals were then killed and the cycloheximide-treated rats weighed There was a slight decrease in the weight of the livers from cycloheximide-treated rats (6.4 ± 0.4 g) compared with controls (7.7 ± 0.6 g) but there was an increase in their liver:body weight ratio from 4.4% in the controls to 5.8% in the cycloheximide-treated animals. Microsomal fractions isolated from the livers of rats chronically treated with cycloheximide had a greater amino acid incorporating activity than the controls in the *in vitro* system (Jondorf, unpublished observation).

Influence of Cycloheximide Treatment on the Hexobarbital Sleeping Time

It was of interest to examine the effect that *in vivo* administration on cycloheximide might have on the drug metabolizing enzymes of the liver in view of the finding that treated animals had a lower growth rate than controls. This implied that the treated animals might have a lower capacity for synthesizing functional protein overall. In experiments with groups of ten Sprague-Dawley rats of the same age, sex, and weight (45 g males) the hexobarbital sleeping time was measured (34) in control and cycloheximide-treated groups injected with 1 mg/kg 24 hr prior to hexobarbital (100 mg/kg). Cycloheximide-treated animals under these conditions slept for 156 ± 32 min. Controls slept for 74 $\pm$ 16 min.

In the corresponding experiments with animals pretreated with water or cycloheximide for 1, 2, or 6 hr before treatment with hexobarbital, the prolongation of the sleeping time in the cycloheximide-treated rats was less than 10%. This contrasts with the 110% prolongation of the sleeping time observed after 24 hr pretreatment with cycloheximide.

The response to hexobarbital is an indicator of the activity of the drug-metabolizing enzymes in the liver microsomes. On the assumption that pretreated and control animals recover their righting reflexes at comparable plasma levels of hexobarbital (34, 35), there is an inverse relationship between the drug metabolizing enzymes and the duration of action of the

drug. It would appear, therefore, that the rate of metabolism of hexobarbital is impaired in rats pretreated with cycloheximide for 24 hr.

Influence of Cycloheximide Treatment on the Drug Metabolizing Enzymes

On incubating the 15,000 *g* supernatant fractions from livers of control and cycloheximide-treated rats (1 mg/kg) under the conditions described in the section on Methods, with drug metabolizing enzyme substrates that are indicators of *N*-demethylating, aromatic ring hydroxylating, and azo-link reducing activities, respectively, and measuring the appearance of metabolites, we obtained the data shown in Table 3. The data refer to animals killed 24 hr after injection and are given in millimicromoles per milligram of protein per 0.5 hr. We found that there were no time course differences in the drug metabolism *in vitro* by 15,000 *g* supernatant fractions from control and cycloheximide-treated rats. *N*-Demethylation and aromatic ring hydroxylation proceeded linearly for 45 min and thereafter began to plateau.

The microsomal fractions of livers of cycloheximide-treated rats had a lower capacity for *N*-demethylation and azo-reduction than the controls, although hydroxylation was hardly affected. The drug metabolizing activity of hepatic subcellular material from cycloheximide-treated rats was lowered by about 50% compared with the controls. Such a diminution in activity correlates in inverse proportion with the observation of a prolonged hexobarbital sleeping time of intact cycloheximide-treated (24 hr) animals.

In parallel experiments with animals killed 6 hr after injection, the *N*-demethylation *in vitro* of monomethyl-4-amino antipyrine and of aminopyrine in the cycloheximide-treated subcellular fractions was lowered by only 8% compared with the controls. Aromatic ring hydroxylating activity was identical in cycloheximide-treated and control animals.

TABLE 3

Effect of cycloheximide treatment of rats on the metabolism of foreign compounds by hepatic microsomes

All incubations were carried out in duplicate as described under Methods, with pooled tissue samples from ten control and ten cycloheximide-treated rats, respectively, starved for the 24-hr period between injection and sacrifice. Incubation mixtures contained 15,000 *g* supernatant tissue preparations equivalent to 0.375 g of liver (37.5 mg of protein). Cycloheximide treatment did not alter the amount of protein per gram of liver in the 15,000 *g* supernatant fraction by more than 5%. Values represent mμmoles of metabolite formed by 1 mg protein in 0.5 hr. During this time the reaction rate is linear.

Substrate	Metabolite	Formation of metabolite (mμmoles/mg protein per 0.5 hr)		%
		Control	Cycloheximide	Control
Monomethyl-4-amino antipyrine	Formaldehyde	18	10	55
Aminopyrine	Formaldehyde	23	9.5	41
Aniline	*p*-Aminophenol	8.7	8.3	95
Neoprontosil[a]	Sulfanilamide	203 ± 13[a]	138 ± 7[a]	68

[a] Determinations were carried out in collaboration with Dr. P. Hernandez. Aliquots of individually redispersed microsomal pellets from four control and four cycloheximide-treated rats were incubated under nitrogen for 0.5 hr in the system cited under Methods. Results are expressed as mμmoles/mg microsomal protein per 0.5 hr.

Effect of SKF-525A Treatment in Vivo on the Amino Acid Incorporation in Vitro

The selective inhibition of drug metabolizing enzymes brought about by administration of cycloheximide to rats is reminiscent of the action of a variety of other inhibitors recently reviewed by Netter

(36). It was considered necessary to test the effect of the best known of these inhibitors, SKF-525A, on the amino acid incorporation system. Animals were treated with SKF-525A at a dose level of 25 mg/kg daily for 1, 2, or 3 days. Microsomal fractions prepared from the excised livers and incubated under our standard conditions had the same amino acid incorporation (endogenous RNA and polyU-dependent) as the controls. The similarity of biochemical events induced by cycloheximide and SKF-525A at the hepatic microsomal level is, therefore, restricted to the inhibition of the rate of biotransformation of drugs. A fuller study of the possible role of SKF-525A in the control of protein synthesis is at present being conducted in our laboratory.

DISCUSSION

The administration of cycloheximide to rats *in vivo* stimulates the amino acid incorporation *in vitro* of the subsequently isolated microsomal fraction of the liver (12–14) relative to the controls. We have now shown that the increased amino acid incorporation is not due to a shift in the $MgCl_2$ requirement of the *in vitro* system. Furthermore, the stimulation of the amino acid-incorporating system is not due to a stabilization of endogenous microsomal RNA. The response to cycloheximide is localized in the microsomal fraction and is apparently unrelated to changes in the 105,000 *g* supernatant fraction of the liver cells.

The investigations described in this paper on the time response to cycloheximide reveal that there is an increase in the *in vitro* amino acid incorporation within the first hour after cycloheximide administration.

It is of considerable interest that the effects of a single intraperitoneal injection of cycloheximide persist for several days. Two effects are involved in the microsomal response. Both the endogenous RNA-dependent amino acid incorporation and the preincubated, exogenous RNA-dependent incorporation are stimulated. The first of these two effects seems to be more pronounced in terms of percentage increase and in its persistence for 5–6 days.

Our findings with cycloheximide contrast with those of Wettstein *et al.* (7), Ennis and Lubin (8), Bennett *et al.* (9), and Colombo *et al.* (10, 11). These workers added cycloheximide in varying amounts directly to the incubation mixtures in various *in vitro* systems and obtained results that indicated quite clearly that cycloheximide inhibited protein synthesis under these conditions.

We feel that there is no doubt that cycloheximide administration to rats has a different effect on the subsequently isolated hepatic microsomal fractions than the mere addition of cycloheximide to the microsomal incubation mixtures. Consequently, the drug has to be considered as belonging to the group of compounds that stimulate specific increases in microsomal amino acid incorporation into protein.

Recent review articles on drugs and chemicals which, when injected into experimental animals, stimulate the activities of the microsomal drug metabolizing enzymes in the liver (37–39) have dealt in part also with the correlation that exists between the stimulation of microsomal drug metabolizing enzymes and *de novo* hepatic protein synthesis (39). Thus, 3-methylcholanthrene stimulates both the activity of the rat liver microsomal drug metabolizing enzymes (40–43) and the capacity for incorporating amino acids into protein *in vitro* (15, 40, 42)). Both the stimulation of drug-metabolizing enzyme activity and amino acid incorporation can be prevented by the coadministration of inhibitors of protein synthesis at the microsomal level or of agents like actinomycin D which block DNA-primed RNA synthesis (41–43).

Phenobarbital, when administered to rats, also brings about the stimulation of drug-metabolizing activity (41, 44) and amino acid incorporation (15, 32, 45). The stimulating effect on the drug-metabolizing enzymes is prevented by the simultaneous administration of puromycin (41, 44) or of actinomycin D (44).

In some respects the cycloheximide

effect on the stimulation of amino acid incorporation in the microsomal system *in vitro* resembles that of phenobarbital or 3-methylcholanthrene, but there are differences in the level of drug which have to be administered to induce the effect, and there are differences dependent on the hormone status of the experimental animals under investigation (13).

Cycloheximide does not however belong to the group of compounds which stimulate hepatic microsomal drug-metabolizing activity. Although treatment of rats with 3-methylcholanthrene or phenobarbital stimulated the drug-metabolizing activity of the subsequently isolated microsomal fractions of the liver, pretreatment of animals with cycloheximide had no such stimulating effect.

For an understanding of the mechanism of cycloheximide action, one has to differentiate between the increased synthesis of possibly nonfunctional protein in an *in vitro* system, and the impairment of existing functional protein *in vivo*.

It then becomes possible to reconcile our findings with those of Young *et al.* (5) with amino acid incorporation into rabbit liver protein of control and cycloheximide-treated animals *in vivo*. Such studies with labeled amino acid indicated that amino acids were incorporated into functional newly synthesized liver protein to a lesser extent in cycloheximide-treated animals than in controls. The studies of Gorski and Axman (6) with a rat uterus system also showed that cycloheximide treatment prevented uptake of amino acid into functional protein. Similar considerations apply to the inhibition of β-galactosidase formation in *Staphylococcus aureus* (46) and tryptophan pyrrolase formation in rat liver (47) under the influence of cycloheximide. Our results on the slowing of the overall growth rate of rats and on the inhibition of drug-metabolizing enzymes after cycloheximide administration would tend to fall in the same category as the incorporation studies *in vivo* just cited, for they indicate that cycloheximide may impair functional protein synthesis and activity.

An unusual stimulation of functional enzyme protein was reported, however, by Fiala and Fiala (47), where cycloheximide appeared to act *in vivo* by a pseudohormonal mechanism in the induction of tyrosine α-ketoglutarate transaminase, an enzyme not associated with the microsomal fraction of the liver (48). Whether the action of cycloheximide is pseudohormonal in a more general way, or is dependent on the formation of artificial messenger RNA which would code for more efficient uptake of amino acid into trichloroacetic acid-precipitable material *in vitro* without necessarily increasing the formation of protein with coordinated functional properties *in vivo*, as previously discussed (13), remains to be determined.

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