MOLECULAR PHARMACOLOGY, 7, 269-279

Effect of Cycloheximide on the Mixed-Function Oxidase System of Rat Liver

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(Received January 7, 1971)

SUMMARY

The effect of cycloheximide treatment on the mixed-function oxidase system of rat liver was studied at 12 and 24 hr after administration of the antibiotic. Cycloheximide caused an alteration in the sedimentation properties of the endoplasmic reticulum. A "fluffy" fraction containing appreciable amounts of drug-metabolizing activity, as well as cytochrome P-450, was recovered with the microsomes from the 15,000 × *g* supernatant fraction from the livers of treated animals but was never observed in control preparations. Also, there was an increase in the microsomal protein level of treated animals that was apparently unrelated to drug-metabolizing activity; therefore, total protein was not a suitable reference standard for expressing the activity of this enzyme system. However, liver weight was relatively constant during the experimental period, and was used as the reference standard in the present study.

N-Demethylation of ethylmorphine was significantly decreased in the microsomal preparations from drug-treated rats. This decrease in enzymatic activity was not the consequence of a cycloheximide-induced change in the sedimentation properties of the cellular particles, because a comparable loss of demethylase activity was observed in the corresponding liver homogenates. The apparent Michaelis constant, K_m, did not change with drug treatment, but V_{max} was significantly lower. The reduction of demethylase activity did not result directly from the effect of cycloheximide on the drug-metabolizing enzymes, but may reflect a quantitative change in enzyme levels due to the inhibitory effect of cycloheximide on protein synthesis.

The change in drug-metabolizing activity produced by cycloheximide treatment did not correlate with a change in the NADPH–cytochrome *c* reductase activity, the level of cytochrome P-450, or the magnitude of the type I or type II binding spectrum. However, the rate of reduction of cytochrome P-450 in the presence of ethylmorphine was significantly decreased, and this decrease closely paralleled the effect of drug treatment on the rate of metabolism. The data suggest that the rate-limiting step in the *N*-demethylation of ethylmorphine is the cytochrome P-450 reductase activity.

This investigation was supported by United States Public Health Service Pharmacology Training Grant 5T1-GM 153 and Research Grant NB-04553.

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INTRODUCTION

The administration of various inhibitors of protein synthesis, such as puromycin (1), cycloheximide (2), actinomynic D (3), ethionine (4), and thioacetamide (5), has been shown to decrease the activity of the microsomal enzyme system involved in drug metabolism. For example, Jondorf *et al.* (2) observed that 24 hr after injection of cycloheximide there was a significant prolongation of hexobarbital sleep time, and approximately a 50% decrease in the rate of *N*-demethylation of aminopyrine and monomethyl-4-aminoantipyrine and in the reduction of azosulfamide (Neoprontosil). Such a change in drug metabolism produced by inhibitors of protein synthesis could result from a direct interaction of the agent with some component of the microsomal enzyme system or could be a consequence of the inhibitory effect of the drug on protein synthesis *in vivo*, thereby reducing the activity or level of some constituent in the reaction sequence.

The purpose of the present study was to determine the nature of the inhibition of the activity of the mixed-function oxidase system by cycloheximide. The results suggest that the decreased rate of drug metabolism produced by cycloheximide is a consequence of a decrease in the NADPH–cytochrome P-450 reductase activity, and that this reaction is the rate-limiting step in the metabolism of ethylmorphine.

MATERIALS AND METHODS

Preparation of tissue. Male Sprague-Dawley rats (130–150 g) were fasted for 24 hr prior to death but were allowed water ad libitum. At 12 or 24 hr before death, the rats received cycloheximide (Sigma) dissolved in 0.9% NaCl solution, 2 mg/kg of body weight, intraperitoneally.) As previously reported (6, 7), cycloheximide treatment caused watery diarrhea approximately 3 hr after administration, and gastric distension was noted in some animals killed at 24 hr; otherwise the rats appeared active and alert. Control animals received 0.9% NaCl solution only.

Control and cycloheximide-treated rats were killed by a blow on the head and decapitation. All animals were killed between 9 and 11 a.m. to minimize any effects due to diurnal variation in drug-metabolizing activity. The livers were perfused *in situ* via the hepatic vein by intermittent injections of 10 ml of ice-cold 1.15% KCl solution (approximately 60 ml); then they were excised and placed in a beaker containing the same ice-cold KCl solution. After removal of connective tissue, the livers were weighed and placed in a beaker containing fresh KCl solution. All subsequent procedures involving tissue manipulations were performed in an ice bath. The livers were cut into small pieces and homogenized in the KCl solution (1:4, w/v) in a glass homogenizer with a motor-driven Teflon pestle.

The cell debris, cell nuclei, and mitochondria were isolated by centrifugation at 15,000 × *g* for 20 min in a Beckman model L-2 centrifuge. Microsomes were prepared, except where indicated, from the decanted 15,000 × *g* supernatant fraction by centrifugation for 1 hr at 105,000 × *g*. The resulting microsomal pellet was resuspended either in ice-cold 1.15% KCl solution or in 0.05 M Na_2HPO_4–KH_2PO_4 buffer, pH 7.4. Protein was measured by the method of Lowry *et al.* (8), with human serum albumin as the reference standard.

Metabolism studies. For assay of the *N*-demethylation of ethylmorphine, the following reaction mixture, adjusted to pH 7.4, was used in open 50-ml Erlenmeyer flasks: $MgCl_2$, 10 μmoles; $NADP^+$ (Sigma), 2 μmoles; glucose 6-phosphate (Sigma), 20 μmoles; 2 units of glucose 6-phosphate dehydrogenase (Boehringer); semicarbazide hydrochloride, 37.5 μmoles; Na_2HPO_4–KH_2PO_4 buffer, pH 7.4, 0.2 mmole; various concentrations of ethylmorphine hydrochloride (Mallinckrodt) dissolved in 1.15% KCl solution; 1 ml of homogenate or microsomal suspension equivalent to 250 mg of wet liver; and sufficient 1.15% KCl solution to give a final volume of 5 ml. Reactions were started by the addition of the tissue preparation, and all assays were carried out in a constant temperature water bath shaker (120 oscillations/min) at 37° for 10 min. The rates of metabolism of ethylmorphine by various amounts of microsomes from both the con-

trol and cycloheximide-treated animals were linear with respect to cofactor requirements. Formaldehyde produced from the demethylation of ethylmorphine was measured by the modification by Anders and Mannering (9) of the method of Nash.

Cytochrome P-450 assay and estimation of type I and type II spectral changes. The method used for the determination of cytochrome P-450 was a modification of that described by Omura and Sato (10). Microsomal suspensions (1 ml, equivalent to 250 mg of liver) in 1.15% KCl solution were diluted 1:3 with 0.1 M Na_2HPO_4–KH_2PO_4 buffer solution, pH 7.4. Carbon monoxide (Matheson) was bubbled through the sample cuvette for 30 sec, and then the difference spectrum between 500 and 400 nm was measured with a Cary model 11 recording spectrophotometer in order to determine whether hemoglobin was present. A few milligrams of sodium dithionite were added to both the sample and reference cuvettes, and the absorption spectrum was recorded. The quantity of cytochrome P-450 was calculated from the absorbance difference ($A_{450} - A_{490}$) and the molar extinction coefficient of 91 mM^{-1} cm^{-1}.

For the estimation of type I and type II spectral changes, the microsomal suspension in 1.15% KCl solution was diluted with 0.3 M Na_2HPO_4–KH_2PO_4 buffer solution, pH 7.4, to a final protein concentration of 1 or 2 mg/ml. The type I or type II spectral change produced by the addition of hexobarbital (Winthrop Laboratories) or aniline (Eastman Organic Chemicals), respectively, to the microsomal suspension in the sample cuvette was obtained from the difference spectrum recorded between 500 and 350 nm. The final concentrations of hexobarbital (2.5 mM) or aniline (6.7 mM) in the sample cuvette gave maximal absorbance changes. The magnitudes of the spectral changes caused by hexobarbital and aniline are expressed as the absorbance differences ($A_{trough} - A_{490}$) and ($A_{peak} - A_{490}$), respectively.

Cytochrome c reductase and cytochrome P-450 reductase activity measurements. NADPH–cytochrome *c* reductase activity was determined by a modification of the method of Williams and Kamin (11). Each cuvette contained 1 ml of a microsomal suspension (equivalent to 100 mg of liver) in 0.05 M sodium potassium phosphate buffer solution (pH 7.4), 1 ml of 3 mM KCN solution, and 1 ml of a 0.15 mM oxidized cytochrome *c* (Sigma, type III) solution. The sample cuvette was equipped with a plunger assembly (American Instrument Company) containing 50 μl of a 0.05 M NADPH (Sigma, type II) solution. After the samples had been equilibrated to 37° (approximately 12 min) in the Cary spectrophotometer, the plunger was quickly depressed three times, and the increase in absorbance at 550 nm was recorded on the chart paper (chart speed, approximately 60 cm/min). The number of micromoles of cytochrome *c* reduced per minute was calculated from the initial linear phase of the curve and from the molar extinction coefficient of 18.5 mM^{-1} cm^{-1} (12).

The rate of reduction of cytochrome P-450 was determined essentially as described by Sasame and Gillette (13). The microsomal pellets were resuspended in 0.05 M sodium potassium phosphate buffer solution, pH 7.4, such that 1 ml of the suspension was equivalent to 500 mg of liver. The sample and reference cuvettes, each containing 3 ml of the microsomal suspension, were gassed for 5 min with carbon monoxide that had been bubbled through a deoxygenating solution (0.5% sodium dithionite and 0.05% sodium anthraquinone-2-sulfonate in 0.1 M NaOH solution). The plunger assembly, containing 50 μl of a 0.05 M NADPH solution, was fitted to the sample cuvette, and carbon monoxide was passed through the inlet of the cuvette for an additional 3 min. The sample cuvette was then sealed, and both cuvettes were transferred to the Cary spectrophotometer. After the samples had been equilibrated to 37° (approximately 12 min), a baseline of equal absorbance was established between 500 and 400 nm, the wavelength was set at 450 nm, and the plunger was quickly depressed three times. The increase in absorbance at 450 nm was recorded with time (chart speed, approximately 61 cm/min). After 5 min the plunger assembly was removed, a few milligrams of sodium dithionite were added to both the sample and reference cuvettes, and the absorption spectrum was

recorded between 500 and 400 nm. The difference in absorbance between 450 and 490 nm was used for the estimation of total cytochrome P-450 content. All reaction velocities were calculated from the initial linear portion of the curve and the extinction coefficient for the carbon monoxide–cytochrome P-450 complex noted above.

RESULTS

Effect of cycloheximide treatment on cellular fractionation by differential centrifugation. Centrifugation of hepatic homogenates from control and cycloheximide-treated rats produced grossly dissimilar fractions. The 15,000 × *g* fractionation of both homogenates yielded a pellet and a supernatant portion, but the preparations from cycloheximide-treated animals also contained a layer of flocculent material at the pellet-supernatant interface. This "fluffy" layer was most prominent in preparations from rats that had been treated with drug 24 hr prior to death, and it was never observed in the fractionation of control hepatic homogenate. When the 15,000 × *g* supernatant portion from cycloheximide-treated animals was decanted for the isolation of the hepatic microsomes, the "fluffy" layer was also decanted; on the other hand, the decanted supernatant from control animals was generally free of any visible particulate material. The difference between the two supernatant preparations was clearly manifested in both the size and appearance of their respective microsomal pellets, particularly 24 hr after drug administration. The pellet from treated animals was larger than that from controls, which yielded a compact, reddish, gelatinous pellet. The treated microsomal pellet appeared to consist of two components; the major one consisted of lightly packed material surrounding a component that resembled the control microsomal pellet.

Table 1 shows that microsomal protein levels per gram of liver from control and drug-treated animals were not equivalent at 12 and 24 hr. At both time intervals there was a significant increase in the microsomal protein obtained from animals receiving cycloheximide. All the values for microsomal protein in Table 1 are considerably lower than some values previously reported (e.g., refs. 14 and 15); however, the lower values are probably the result of recovering microsomes from a 15,000 × *g* rather than the generally employed 9000 × *g* supernatant. A greater centrifugal force was used to ensure that the supernatant fraction from which the microsomes were isolated would be essentially free of mitochondria. The variation in fractionation procedure may account for the lower protein content of the microsomal pellets obtained in the current study, but this would not explain the differences in the protein levels between control and drug-treated animals.

TABLE 1

Effect of cycloheximide treatment on liver wet weight and microsomal protein concentration

Values represent the means ± standard errors of 20–34 rats.

Treatment	Time after treatment	Liver weight	Microsomal protein
	hr	*g*	*mg/g liver, wet wt*
Control	12	6.4 ± 0.2	8.5 ± 0.5
Cycloheximide (2 mg/kg)	12	6.3 ± 0.2	10.4 ± 0.4[a]
Control	24	6.0 ± 0.1	6.1 ± 0.4
Cycloheximide (2 mg/kg)	24	7.1 ± 0.2[a]	9.4 ± 0.5[a]

[a] Significantly different at $p < 0.05$ from the corresponding control value as determined by a two-way analysis of variance.

It is possible that the greater protein level of the microsomal pellets from cycloheximide-treated animals was a consequence of the inclusion of the "fluffy" material in the decanted 15,000 × *g* supernatant fraction. Experiments were performed in which the 15,000 × *g* supernatant portion from drug-treated animals was aspirated by a needle and syringe, rather than decanted, in order to avoid including the "fluffy" material. In these experiments the microsomal protein content from cycloheximide-treated animals was approximately the same as the control protein value, which was relatively constant for microsomes prepared from both supernatant fractions. Thus, the increased micro-

TABLE 2

Rate of ethylmorphine metabolism by microsomes obtained from decanted and aspirated 15,000 × g supernatant fraction

Values represent the means ± standard errors of four to nine rats. Results are expressed as micromoles of formaldehyde formed from ethylmorphine per gram of liver per hour or per nanomole of cytochrome P-450; the concentration of ethylmorphine in the incubation mixture was 2.0 mM.

Treatment	Time after treatment	*N*-Demethylase activity in microsomes		*N*-Demethylase activity per nmole of cytochrome P-450	
		Decanted 15,000 × *g* supernatant	Aspirated 15,000 × *g* supernatant	Decanted 15,000 × *g* supernatant	Aspirated 15,000 × *g* supernatant
	hr				
Control	12	3.9 ± 0.3	3.4 ± 0.5	0.82 ± 0.05	0.84 ± 0.08
Cycloheximide (2 mg/kg)	12	2.6 ± 0.3[a] (33%)[b]	1.4 ± 0.1[a] (59%)	0.43 ± 0.03	0.47 ± 0.01
Control	24	3.4 ± 0.4	3.3 ± 0.6	1.00 ± 0.05	0.97 ± 0.15
Cycloheximide (2 mg/kg)	24	1.5 ± 0.1[a] (56%)	0.6 ± 0.1[a] (82%)	0.38 ± 0.03	0.31 ± 0.04

[a] Significantly different at $p < 0.05$ from the corresponding control value as determined by a two-way analysis of variance.

[b] Numbers in parentheses represent the percentage decrease from control values.

somal protein level observed in the cycloheximide-treated animals may be attributed to the "fluffy" layer present in the decanted 15,000 × *g* supernatant fraction.

Microsomal protein is commonly employed as a reference standard to express drug-metabolizing activity. However, in the present study liver weight, as shown in Table 1, appears to be a more constant parameter than microsomal protein; therefore, equivalent liver weight is used as the reference standard for the comparison of drug-metabolizing activities of treated and untreated animals.

Effect of cycloheximide treatment on metabolism of ethylmorphine in vitro. The effect of drug treatment on the rate of *N*-demethylation of ethylmorphine by hepatic microsomal preparations is reported in Table 2. As can be seen, cycloheximide treatment markedly affected the rate of ethylmorphine metabolism by microsomes from both decanted and aspirated supernatants. Although the drug-metabolizing activities of control microsomal pellets from both the decanted and aspirated preparations were approximately the same, the activity of the pellet obtained from cycloheximide-treated animals depended on whether the "fluffy" layer was included in the 15,000 × *g* supernatant fraction. Inclusion of the "fluffy" material yielded microsomes with greater drug-metabolizing activity than those obtained from the aspirated samples. Moreover, other experiments showed that the "fluffy" material contained cytochrome P-450, accounting for the observation that cytochrome P-450 levels were greater in microsomes from decanted than from aspirated supernatants. However, as indicated in Table 2, at a given time interval the ratios of drug-metabolizing activity to cytochrome P-450 level were approximately the same in the microsomes derived from the decanted and aspirated 15,000 × *g* supernatant fractions. A similar ratio of enzyme activity to cytochrome level implies that the same relationship exists between these two parameters in the microsomes prepared from both the decanted and aspirated supernatant fractions. Therefore, the lower drug-metabolizing activity in the microsomes from aspirated samples was due to the exclusion of the "fluffy" layer, which contains enzymatic activity. Because a substantial amount of drug-metabolizing activity and cytochrome P-450 was present in the "fluffy"

material, subsequent experiments were performed with the decanted supernatant as the only source of the microsomal fraction. Under these conditions, the *N*-demethylase activity of microsomes from treated animals was inhibited 33 and 56% at 12 and 24 hr, respectively. These results are essentially in agreement with the inhibitory effect of cycloheximide treatment on the *N*-demethylation of aminopyrine and monomethyl-4-aminoantipyrine reported by Jondorf *et al.* (2).

The differences between the cellular fractions from control and cycloheximide-treated animals obtained in the present study suggested that drug treatment affected the sedimentation properties of the fragments of the endoplasmic reticulum. Because the observed decrease in the rate of ethylmorphine metabolism might have been the consequence of an alteration in the sedimentation properties of the microsomes, *N*-demethylase activity was determined for the liver homogenate of control and cycloheximide-treated animals (Table 3). The metabolism of ethylmorphine by homogenates from drug-treated animals was 28 and 49% lower than control values at 12 and 24 hr, respectively. This degree of inhibition is roughly equivalent to the inhibition of *N*-demethylase activity determined in the microsomes isolated from the decanted 15,000 × *g* supernatant obtained from cycloheximide-treated animals (Table 2). Although there is an absolute difference between the rates of ethylmorphine metabolism by the homogenates and by the microsomes, the ratio of the homogenate activity to the microsomal activity remained relatively constant at a given time for each treated group and its control. Therefore, the decrease in the rate of ethylmorphine *N*-demethylation measured in the microsomal fraction from cycloheximide-treated animals was not the consequence of an alteration in the sedimentation properties of the microsomes.

TABLE 3

Effect of cycloheximide treatment on protein concentration and demethylase activity of whole liver homogenate

Values represent the means ± standard errors of five rats. Assays for ethylmorphine *N*-demethylase activity were performed with 1 ml of liver homogenate (equivalent to 250 mg of liver, wet weight); the concentration of ethylmorphine in the incubation medium was 2.0 mM. Results are expressed as micromoles of formaldehyde formed from ethylmorphine per gram of liver per hour. The incubation procedure is described under MATERIALS AND METHODS.

Treatment	Time after treatment	Liver protein	Ethylmorphine metabolism
	hr	*mg/g liver, wet wt*	
Control	12	125 ± 6	8.5 ± 0.4
Cycloheximide (2 mg/kg)	12	115 ± 5	6.1 ± 0.3[a] (28%)[b]
Control	24	119 ± 3	9.1 ± 0.2
Cycloheximide (2 mg kg)	24	140 ± 8[a]	4.6 ± 0.3[a] (49%)

[a] Significantly different at $p < 0.05$ from the corresponding control value as determined by a two-way analysis of variance.

[b] Numbers in parentheses represent the percentage decrease from control value.

Table 3 also includes the protein level of the homogenates from control and cycloheximide-treated animals. At 24 hr the homogenate from drug-treated animals contained a significantly increased amount of protein, which might explain the concomitant change in microsomal protein. However, a comparison of homogenate and microsomal protein levels (Tables 1 and 3) indicates that the increased microsomal protein level accounts for only a small fraction of the increase in homogenate protein; hence, the major proportion of the increased protein in the homogenate from cycloheximide-treated animals must represent non-microsomal material.

Effect of cycloheximide treatment on kinetic parameters of ethylmorphine metabolism. The effect of cycloheximide treatment on the Michaelis constant, K_m, and the maximal velocity, V_{max}, for the *N*-demethylation reaction was determined (Table 4). (Because the microsomal enzymes studied have not been isolated and purified, these values are only "apparent" values.) At neither 12 nor 24 hr after injection of cycloheximide was there a significant difference between the

TABLE 4

Effect of cycloheximide treatment on K_m and V_{max}

The incubation conditions are described under MATERIALS AND METHODS; the concentration of ethylmorphine in the incubation mixture was varied from 0.4 to 2.0 mM. The K_m and V_{max} values were calculated by the method of Wilkinson (16) and represent the means ± standard errors of six rats.

Treatment	Time after treatment	K_m	V_{max}
	hr	[S] × 10^4 *M*	*μmoles formaldehyde/g wet liver/hr*
Control	12	3.1 ± 0.4	4.6 ± 0.5
Cycloheximide (2 mg/kg)	12	2.6 ± 0.4	3.2 ± 0.5[a]
Control	24	3.5 ± 0.5	3.0 ± 0.4
Cycloheximide (2 mg/kg)	24	3.8 ± 0.5	1.7 ± 0.2[a]

[a] Significantly different at $p < 0.05$ from the corresponding control value as determined by a two-way analysis of variance.

apparent K_m values for the metabolism of ethylmorphine by microsomal preparations from control and treated animals. The apparent K_m obtained in both groups of animals essentially agrees with the previously reported Michaelis constant for the *N*-demethylation of ethylmorphine by control animals (17, 18). In accord with the lower rate of ethylmorphine demethylation noted above, the V_{max} values for drug-treated animals were significantly lower than those for controls.

The decrease in enzyme activity is compatible with the view that drug treatment reduced the amount of enzyme as a result of decreased protein synthesis. However, the lowered demethylase activity might also be the consequence of direct microsomal inhibition by cycloheximide or one of its metabolites. The present experiments do not define the cause of the decrease in enzyme activity, but other experiments, involving the addition of cycloheximide (0.01–1.0 mM) to incubation mixtures *in vitro*, have demonstrated that the drug does not directly depress demethylase activity. Therefore, the most reasonable cause for the cycloheximide-induced decrease in enzyme activity still appears to be the depression of protein synthesis.

Effect of cycloheximide treatment on level of cytochrome P-450, magnitude of type I and type II spectral changes, and NADPH–cytochrome c reductase activity. Changes in the rate of drug metabolism have frequently been correlated with the level of cytochrome P-450 (19, 20), the magnitude of the absorption spectrum produced by various drugs (21–23), and the NADPH–cytochrome *c* reductase activity (24–26). Tables 5 and 6, however, indicate that the decrease in ethylmorphine metabolism in cycloheximide-treated animals was not paralleled by a corresponding change in any of these parameters. There was no significant variation in the amount of cytochrome P-450 in the microsomal preparations from control and drug-treated animals; and although at 12 hr the magnitude of the type I absorption spectrum produced by hexobarbital was diminished, no difference in the type I spectrum was apparent at 24 hr. With respect to the type II spectrum, there was no significant quantitative difference between the two groups of animals at either of the experimental times. The low demethylase activity of the cycloheximide-treated animals was also not the consequence of a decrease in NADPH–cytochrome *c* reductase activity; in fact, the increase in reductase activity observed at 12 hr, when demethylase activity was markedly depressed, suggests that NADPH–cytochrome *c* reductase is not the rate-determining step of ethylmorphine metabolism in the treated animals.

Effect of cycloheximide treatment on NADPH–cytochrome P-450 reductase activity. The values for the NADPH–cytochrome P-450 reductase activity in microsomal preparations from control and treated rats are presented in Table 6. At both 12 and 24 hr, the reductase activity in the drug-treated animals was significantly lower than in control animals. At 12 hr there was approximately a 50% decrease in the rate of reduction of cytochrome P-450, and at 24 hr a 70% decrease was observed.

Gigon *et al.* (27, 28) reported that type I

TABLE 5

Effect of cycloheximide treatment on level of cytochrome P-450 and magnitude of type I and type II spectral changes

Values represent the means ± standard errors of 10 or 11 rats. The buffer was 2.5 mM hexobarbital for type I spectra and 6.7 mM aniline for type II spectra.

Treatment	Time after treatment	Cytochrome P-450[a]	Spectral change	
			Type I	Type II
	hr	*nmoles/g wet liver*	*ΔA/g liver, wet wt*	
Control	12	5.1 ± 0.3	0.088 ± 0.007	0.046 ± 0.010
Cycloheximide (2 mg/kg)	12	4.6 ± 0.4	0.063 ± 0.012[a]	0.056 ± 0.007
Control	24	4.5 ± 0.4	0.081 ± 0.008	0.056 ± 0.008
Cycloheximide (2 mg/kg)	24	4.7 ± 0.3	0.079 ± 0.009	0.054 ± 0.003

[a] Significant at $p < 0.05$ as compared with the corresponding control as determined by a two-way analysis of variance.

TABLE 6

Effect of cycloheximide treatment on rate of reduction of cytochromes c and P-450

Values represent the means ± standard errors of 5–10 determinations, each of which was replicated two to four times. Each determination was made from a microsomal preparation obtained from the pooled livers of two animals.

Treatment	Time after treatment	NADPH–cytochrome *c* reductase	NADPH–cytochrome P-450 reductase
	hr	*μmoles/min/g wet liver*	*nmoles/min/g wet liver*
Control	12	1.03 ± 0.14	38.8 ± 2.2
Cycloheximide (2 mg/kg)	12	1.55 ± 0.14[a]	18.1 ± 2.0[a] (53%)[b]
Control	24	1.25 ± 0.08	40.0 ± 2.4
Cycloheximide (2 mg/kg)	24	1.30 ± 0.10	11.9 ± 1.7[a] (70%)

[a] Significantly different from the control value at $p < 0.05$, as determined by a two-way analysis of variance.

[b] Numbers in parentheses represent the percentage decrease from control values.

compounds, such as ethylmorphine, enhance the rate of cytochrome P-450 reduction; therefore, NADPH–cytochrome P-450 reductase activity was determined in the absence and presence of ethylmorphine. In accord with these observations, the data shown in Table 7 illustrate the stimulatory effect of ethylmorphine on the rate of reduction of cytochrome P-450 in both control and cycloheximide-treated animals. Ethylmorphine enhanced the rate of reduction of cytochrome P-450 of the control preparations approximately 45%, and in treated animals the reduction was stimulated by 77% at 12 hr and 120% at 24 hr. Although these percentage increases are markedly different, the absolute increase in the rate of reduction was approximately the same for all groups of animals, 17 nmoles of cytochrome P-450 reduced per minute per gram of liver. If the effect of cycloheximide treatment on the reductase activity is evaluated in the presence of ethylmorphine, there is a 37% decrease at 12 hr and a 45% decrease at 24 hr.

DISCUSSION

Cycloheximide was used as the inhibitor of protein synthesis in the present study because a previous report (2) had indicated that administration of the antibiotic results

TABLE 7

Effect of ethylmorphine on rate of reduction of cytochrome P-450 in microsomes from control and cycloheximide-treated rats

Values represent the means ± standard errors of five determinations, each of which was replicated twice. Each determination was made from a microsomal preparation obtained from the pooled livers of two animals.

Treatment	Time after treatment	Ethylmorphine (0.4 mM)	NADPH-cytochrome P-450 reductase	
			Activity	Change produced by ethylmorphine
	hr		*nmoles/min/g wet liver*	
Control	12	−	43.3 ± 1.7	
		+	62.8 ± 2.1	19.5
Cycloheximide (2 mg/kg)	12	−	21.8 ± 2.4	
		+	39.1 ± 1.7	17.3
Control	24	−	45.9 ± 2.7	
		+	61.1 ± 4.0	15.2
Cycloheximide (2 mg/kg)	24	−	15.2 ± 2.5	
		+	34.1 ± 3.0	18.9

in approximately a 50% depression of drug-metabolizing activity in 24 hr. Although investigations of the inhibitory effect of cycloheximide on protein synthesis in the rat *in vivo* have utilized doses as high as 50 mg/kg (6, 29), the study by Verbin *et al.* (30) illustrates that doses as low as 1.5 mg/kg are capable of producing essentially complete inhibition of protein synthesis. Furthermore, Jondorf (31) noted that the intensity and duration of the inhibition of protein synthesis by low doses of cycloheximide are dose-dependent. Therefore, because as great an inhibitory effect as possible during the 24-hr experimental period was desired, the largest nonlethal dose of the antibiotic was sought. Rats can tolerate high doses of cycloheximide for a short period of time (29); however, the LD_{50} has been reported to be 2.5 mg/kg (32, 33). In the present study, the animals generally survived a dose of 2 mg/kg for at least 24 hr. This dose should result, according to the work of Jondorf (31), in substantial inhibition of protein synthesis for a 24-hr experimental period.

Cellular fractions obtained by differential centrifugation of hepatic homogenates from control and cycloheximide-treated rats were distinctly different; the 15,000 × *g* fraction of the homogenate from treated animals contained a "fluffy" layer that was never observed in control preparations. As shown in Table 2, the "fluffy" material possessed a considerable amount of drug-metabolizing activity; in addition, cytochrome P-450 was present in this material. Because drug-metabolizing activity and cytochrome P-450 are generally associated with the membranes of the endoplasmic reticulum that sediment in the microsomal fraction, drug treatment seems to cause some change in the sedimentation properties of these fragmented membranes. Cycloheximide treatment can produce substantial structural alterations of the endoplasmic reticulum (30, 34), and when hepatic tissue is homogenized such alterations may give rise to the particles comprising the "fluffy" layer.

Another difference between the control and cycloheximide-treated animals was the level of microsomal protein. Cycloheximide-treated animals had a greater amount of microsomal protein than did control animals at both 12 and 24 hr after injection of the antibiotic. In view of the inhibitory effect of cycloheximide on protein synthesis, the in-

crease in protein is paradoxical, but may be related to the reported proliferation of the endoplasmic reticulum associated with cycloheximide treatment (34).

Administration of cycloheximide markedly decreased the rate of *N*-demethylation of ethylmorphine by both the homogenate and the microsomal fraction from treated animals, and the only enzyme activity of the mixed-function oxidase system that was consistently lower in the cycloheximide-treated animals was the reduction of cytochrome P-450, in both the presence and absence of ethylmorphine. In other studies, the rate of reduction of cytochrome P-450 has been correlated with the rate of drug metabolism (35, 36). With respect to the effect of cycloheximide, the best correlation with the decrease in the *N*-demethylation of ethylmorphine is given by the reduction of the cytochrome P-450–ethylmorphine complex. Gigon *et al.* (28) found that the cytochrome P-450–drug complex was more readily reducible in male than in female rats; however, in the cycloheximide experiments, the reducibility of the cytochrome P-450–drug complex did not change, as indicated by the similar values for the increase in rate of reduction of cytochrome P-450 in both control and treated animals.

The correlation of the decreased rate of metabolism with the decreased rate of reduction of the cytochrome P-450–ethylmorphine complex suggests that this is the rate-limiting reaction in the *N*-demethylation of ethylmorphine in the treated animals. Although the kinetic constants obtained for the enzyme system represent only apparent values, the similarity between the K_m values for control and treated animals also indicates that in both groups the rate of ethylmorphine metabolism may be governed by the same rate-determining reaction. The decrease in V_{max} value without a change in K_m is compatible with the interpretation that cycloheximide blocked the synthesis of the enzyme involved in the reduction of cytochrome P-450; and the data in Tables 6 and 7 suggest that the half-life of the reductase activity is approximately 12 hr, though the actual half-life is probably less than this value. Nevertheless, this represents the shortest half-life estimated for any of the components of the mixed-function oxidase system (37–41), and may constitute further evidence that the cytochrome P-450 reductase activity normally regulates the rate of ethylmorphine metabolism (42). The fact that the rate-limiting step appears to be the reduction of cytochrome P-450 agrees with the results of other approaches to this problem (28, 35, 36). However, because only ethylmorphine has been used in work relating the rate of reduction of cytochrome P-450 to the rate of drug metabolism, it is not known whether this relationship is applicable to other drug oxidation reactions.

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