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QUANTITATIVE ASPECTS OF CYCLOHEXIMIDE INHIBITION OF AMINO ACID INCORPORATION*

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Abstract—Quantitative data have been obtained on the inhibitory effects of cycloheximide on ^{14}C-leucine incorporation into the proteins of a variety of rat tissues. Of fourteen organ systems examined, all were significantly affected under the test condition with 0·5 mg/kg of the drug given i.p., i.v. or intragastrically. As little as 0·05 mg/kg i.p. of cycloheximide affected certain organs; most tissues had some inhibition at 0·25 mg/kg with increasing effects up to 4 mg/kg, which was the maximum dose tested. When the labelled amino acid was injected 0·5 hr after 0·5 mg/kg of cycloheximide and the rats were sacrificed 4 hr later, maximum inhibition of leucine incorporation was noted. The inhibition began to decrease within 3 hr after cycloheximide administration and disappeared at 6–12 hr. Cycloheximide was found to be absorbed from the stomach in the pylorus-ligated rats and to exert a systemic effect on leucine incorporation. There was no consistent difference between males and females in response to cycloheximide. Acetoxycycloheximide was more potent than cycloheximide on an equal weight basis and was more active in females than in males. Cycloheximide inhibited amino acid incorporation by liver slices and intestine segments at concentrations of 1 and 10 μg/ml respectively.

CYCLOHEXIMIDE, an antibiotic isolated from *Streptomyces griseus*,[1] has been found to inhibit protein synthesis *in vivo*[1–3] and *in vitro*[4] in a variety of organisms, including mammalian species.[2, 3] Its inhibitory action is believed to be specific for protein and not to involve significantly DNA or RNA synthesis.[1] Quantitative measurements have been made of the activity of this inhibitor on leucine incorporation into rat tissues *in vivo* and *in vitro* with respect to dosage, duration of action, route of administration and sex.

MATERIALS AND METHODS

Adult rats of both sexes (Charles River or Carworth Farm CD strain) were randomly divided into groups and fasted overnight. Cycloheximide dissolved in 0·9% NaCl (0·25 mg/ml) was given intraperitoneally, intravenously (via tail vein with the animal under light ether anesthesia) or intragastrically with a plastic catheter; the drug was given in single, graded doses; saline was administered to controls. In a study of possible local effects on the stomach, the pylorus was ligated under ether anesthesia and cycloheximide or saline was given intragastrically immediately after closure of the abdominal wall; in this experiment, a mixture of ^{14}C-labeled amino acids was used. In all other experiments, 5 μc *dl*-leucine-1-^{14}C (specific activity, 25·8 mc/m-mole) dissolved in 1 ml of 0·9% NaCl was given i.p. at various intervals after

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the drug and the animals were sacrificed 4 hr later. Proteins of various tissues were isolated and their specific activity determined as previously described.[5] Liver slices approximately 0·5 mm thick were prepared with a Stadie–Riggs microtome. Everted small intestine was cut into rings about 1 mm long. Two or three pieces of randomly selected liver slices weighing 30–40 mg or ten intestinal rings were incubated in 10 ml of Krebs–Ringer bicarbonate buffer containing 10 mg dextrose and 1 μc *dl*-leucine-1-^{14}C with or without cycloheximide at 37° for 1 hr under 95% oxygen and 5% CO_2. The reaction was stopped by addition of trichloroacetic acid to a final concentration of 5%, and the labeled proteins were then isolated.[5]

RESULTS

Leucine incorporation into the proteins of plasma and 13 different tissues was found to be significantly reduced with cycloheximide at 0·5 mg/kg (Table 1). The most

TABLE 1. CYCLOHEXIMIDE AND LEUCINE INCORPORATION *IN VIVO* INTO VARIOUS TISSUE PROTEINS

Cyclo-heximide (mg/kg)	Specific activities (dpm/mg; mean ± S.E.)					
	Plasma	Kidneys	Liver	Muscle	Spleen	Brain (whole)
0 (6)*	944 ± 53	437 ± 22	666 ± 44	39 ± 1·8	497 ± 15	214 ± 11
0·5 (6)*	392 ± 14	116 ± 10	269 ± 9	26 ± 1·1	279 ± 23	149 ± 7
% diff.†	58·5	73·5	59·6	33·3	43·9	30·4
		Heart	Lung	Stomach	Small intestine	Large intestine
0		224 ± 11	417 ± 30	1500 ± 95	1205 ± 65	846 ± 52
0·5		110 ± 6	236 ± 10	594 ± 27	719 ± 27	451 ± 24
% diff.†		50·9	43·4	60·4	40·3	46·7

* Number of rats is shown in parentheses; females of 200 g, sacrificed 4 hr after leucine-1-^{14}C or 7 hr after cycloheximide.
† $P < 0·01$ between control and treated groups for each tissue.

marked effects were noted in kidney, liver, stomach and plasma; muscle and brain were least affected. As the dosage was increased from 0·01 to 4 mg/kg i.p., there was increasing inhibition (Fig. 1). Significant differences between treated and control groups occurred with 0·05 mg/kg in liver and kidneys; all organs tested, except muscle and pancreas (not shown), were inhibited at 0·25 mg/kg.

Acetoxycycloheximide was found to be more potent than cycloheximide. On an equal weight basis in female rats, the degree of inhibition brought about by acetoxycycloheximide at 0·25 mg/kg was almost the same as that with cycloheximide somewhere in the dosage range of 0·5 to 4 mg/kg.

Leucine incorporation was not significantly different between the sexes as the result of cycloheximide treatment. However, acetoxycycloheximide was much more inhibitory in female rats than in males ($P < 0·01$; Fig. 2).

The effect of time on the inhibiting effect of cycloheximide on leucine incorporation was studied by measurements at increasing intervals after drug injection. A marked effect was noted in the three tissues illustrated in Fig. 3 and also in muscle and kidney

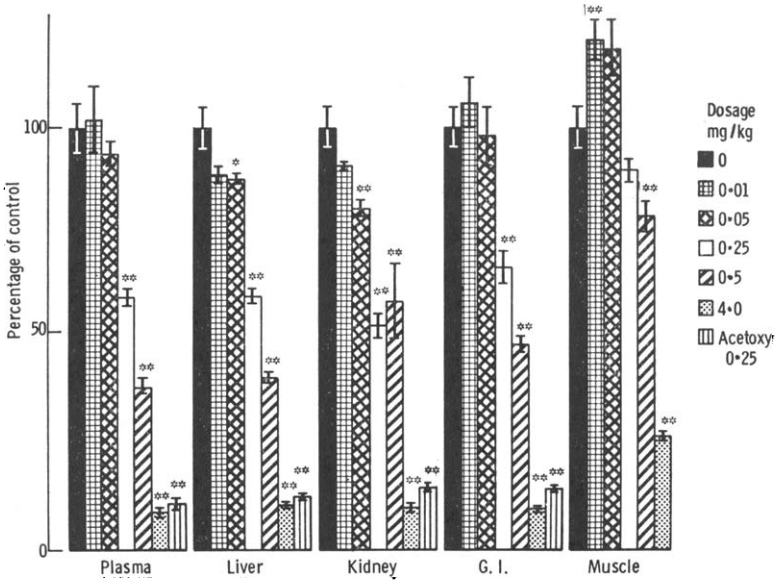

FIG. 1. Effect of varying dosages of cycloheximide on leucine incorporation into proteins of various organs. G. I. = stomach, small and large intestines after removal of luminal contents. Female rats (six per group), about 200 g, fasted overnight were sacrificed 4 hr after leucine-1-^{14}C and 7 hr after injection of cycloheximide at the dosages indicated. Acetoxycycloheximide (Acetoxy) was given at a single dosage. The mean ± standard error of the mean of the experimental groups is expressed as a percentage of the mean value of the controls. *P < 0·05; ** P < 0·01 in comparing test group with controls.

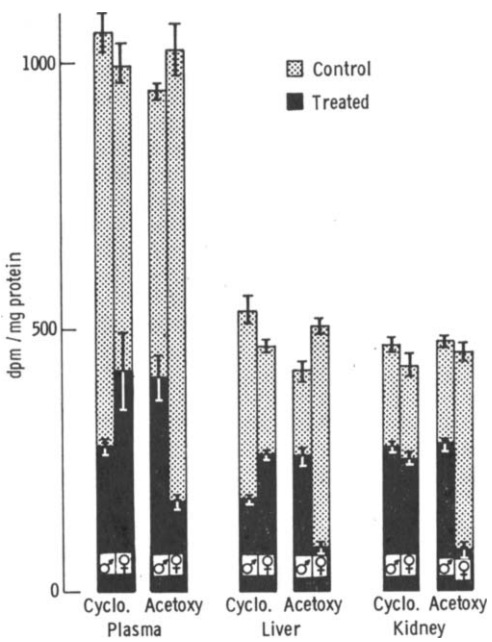

FIG. 2. Influence of sex on drug inhibition of leucine incorporation. Rats weighing 200–250 g (six per group) were sacrificed 4 hr after leucine-1-^{14}C and 7 hr after the i.p. injection of cycloheximide (Cyclo; 0·5 mg/kg) or acetoxycycloheximide (Acetoxy; 0·25 mg/kg). I = Mean ± S.E. of mean.

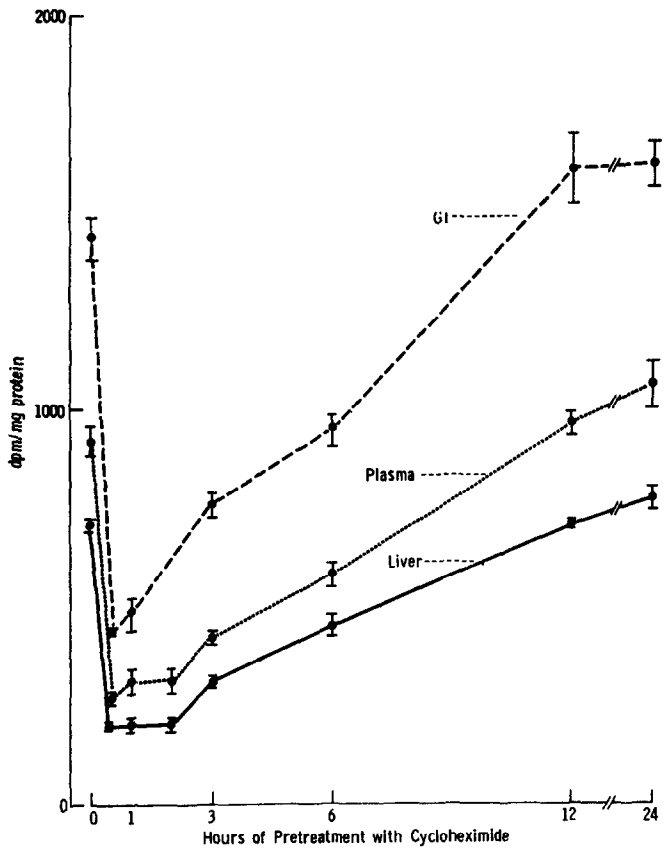

FIG. 3. Duration of inhibitory effect of cycloheximide on leucine incorporation into proteins of plasma, liver and GI tract (stomach and intestines without luminal contents). Female rats (six per interval group), 200 g, were given leucine-1-^{14}C at the intervals noted ranging from 0·5 to 24 hr after i.p. injection of cycloheximide (0·5 mg/kg). They were sacrificed 4 hr after leucine administration. I = Mean ± S.E. of mean.

when the amino acid was given 30 min after intraperitoneal cycloheximide; this was near maximal, since the inhibition at this time was not significantly different from that after 1 or 2 hr. Thereafter, the inhibition gradually decreased and disappeared by 12 hr. When leucine was given 30 min after intragastric cycloheximide, incorporation was inhibited markedly at relatively small doses in seven tissues examined. Muscle was not affected significantly at 0·05 mg/kg, but plasma, liver, kidney, stomach, and small and large bowel were inhibited by 20–30 per cent; at 0·2 mg/kg, all these tissues were inhibited by 60–70 per cent and muscle by 50 per cent.

Cycloheximide was approximately equally effective when given intragastrically or intravenously with similar responses to graded doses (Fig. 4). The inhibition appeared to be quite similar to that obtained by the intraperitoneal route in other experiments (Fig. 1). The effect on the gastrointestinal tract was no greater when cycloheximide was given intragastrically than when given by other routes. Systemic inhibitory effects were noted when the drug was given intragastrically (0·5 mg/kg) in rats in whom the pylorus had been ligated (Fig. 5).

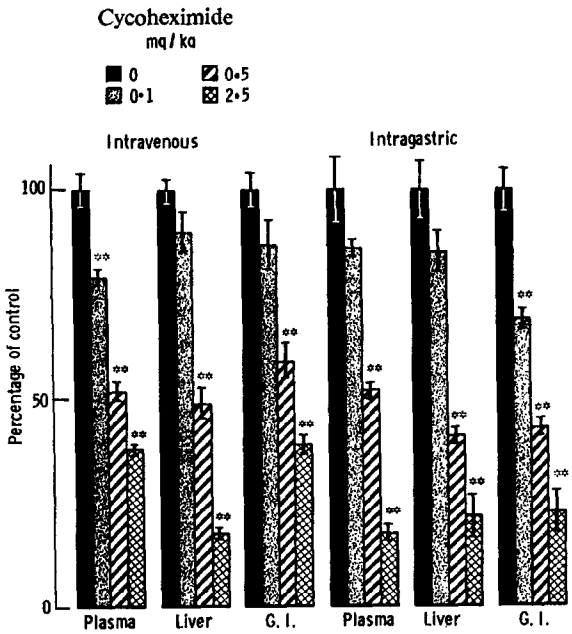

FIG. 4. Effect of cycloheximide on leucine incorporation into plasma and various organ proteins when given in varying dosages by intravenous or intragastric routes. G. I. = stomach and intestines after removal of luminal contents. Female rats, 200 g (four per group), were sacrificed 4 hr after leucine and 7 hr after cycloheximide at the dosage indicated. The control data (in dpm/mg protein) for plasma, liver and G.I. tract were, respectively, for i.v. route: 933 ± 36, 700 ± 24, 1125 ± 50, and for the intragastric route, the figures were: 1121 ± 95, 800 ± 56, 1417 ± 68. I = Mean ± S.E. of mean.

** $P < 0.01$ between control and test group.

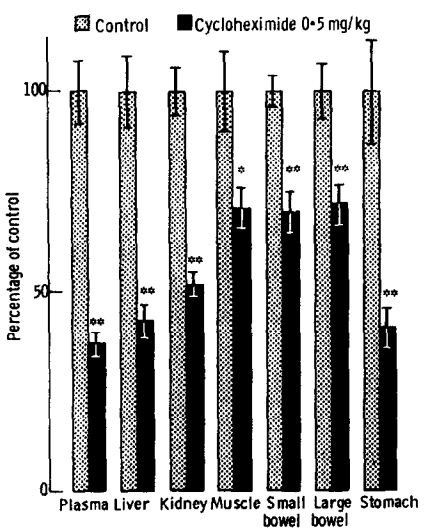

FIG. 5. Effect of cycloheximide on leucine incorporation into protein of plasma and various organs when given intragastrically to pylorus-ligated rats. Five control and six experimental rats were given saline or cycloheximide (0·5 mg/kg), respectively, 0·5 hr before a ^{14}C-labeled amino acid mixture (2 μc in 0·4 ml saline) was injected via penile vein. Rats were sacrificed 4·5 hr after cycloheximide. I = Mean ± S.E. of mean. * $P < 0.05$; ** $P < 0.01$.

Liver slices from cycloheximide-treated rats given 0·5 mg/kg 3 hr prior to sacrifice incorporated leucine at 105 ± 10·3 dpm/mg of protein as compared to 212 ± 35·0 dpm/mg in the controls (six experiments per group; $P < 0·05$). Cycloheximide added *in vitro* caused an inhibition of amino acid incorporation in liver slices and intestinal segments obtained from unfasted normal rats. Intestine was inhibited at 1 μg/ml and liver at 10 μg/ml (Table 2).

TABLE 2. EFFECT OF CYCLOHEXIMIDE ON LEUCINE INCORPORATION *IN VITRO**

Cycloheximide (μg/ml)	Specific activities of proteins (dpm/mg; mean ± S.E.)	
	Liver slices	Intestinal segments
0 (5)	2083 ± 280	2092 ± 369
1 (4)	1585 ± 191	1179 ± 164†
10 (4)	771 ± 114‡	948 ± 178†
100 (5)	338 ± 55‡	214 ± 38‡

* Liver slices weighing 50 mg and 10 everted intestinal rings were incubated in 10 ml Krebs–Ringer bicarbonate buffer, pH 7·0, containing 1 mg dextrose and 0·1 μc *dl*-leucine-1-^{14}C/ml. Incubation was carried out for 60 min at 37° under 95% oxygen and 5% CO_2. The number of determinations is shown in parentheses.
† $P < 0·05$.
‡ $P < 0·01$.

TABLE 3. BLOOD UREA NITROGEN LEVELS IN CYCLOHEXIMIDE TREATED RATS*

Cycloheximide (mg/kg)	Route		
	Intraperitoneal (mg/100 ml)	Intragastric (mg/100 ml)	Intravenous (mg/100 ml)
0	13·2 ± 0·7 (5)	11·5 ± 0·9 (5)	12·1 ± 0·4 (4)
0·01	15·3 ± 0·4 (5)†		
0·05	12·1 ± 1·2 (5)	9·6 ± 0·3 (4)	12·7 ± 0·6 (4)
0·25	15·5 ± 1·6 (5)†		
0·5		16·9 ± 1·3 (5)‡	16·3 ± 0·9 (4)‡
2·5	31·6 ± 4·1 (5)‡	32·3 ± 4·3 (4)‡	42·7 ± 2·8 (4)‡

* Number of rats is shown in parentheses; females about 200 g, fasted overnight 7 hr after cycloheximide. Results expressed as mean ± S.E.
† $P < 0·05$, comparing test group against control group.
‡ $P < 0·01$, comparing test group against control group.

The cycloheximide-treated animals appeared to be alert and active with no change in body temperature and no mortality during the experimental periods. Watery diarrhoea and gastric distention with presence of bile-tinged material were noticed at the time of sacrifice (usually 7 hr after drug administration) in the animals given either cycloheximide in doses above 2·5 mg/kg or acetoxycycloheximide at 0·25 mg/kg; lesser degrees of gastric distention were apparent at lower doses in proportion to amount. Blood urea nitrogen was within normal limits wth 0·5 mg/kg or less and was elevated in animals given 2·5 mg/kg, regardless of the route of administration (Table 3). Slight acidosis was also noticed in the rats given cycloheximide at

2·5 mg/kg i.p. (Table 4); this is perhaps secondary to impaired renal function. There were no abnormalities on histological examination under light microscopy of hematoxylin-eosin stained sections of liver, kidney and thyroid glands from animals treated with 0·5 mg/kg of cycloheximide and sacrificed after 7 hr. There were no changes in the parietal cell population or in the intestinal villi, except at dosages above 2·5 mg/kg of cycloheximide or 0·25 mg of acetoxycycloheximide when a mild degree of karyorrhexis was noticed in the crypts of the intestinal villi. Even with cycloheximide at 2·5 mg/kg, treated animals recovered rapidly and at 24 hr there were no demonstrable intestinal mucosal changes (H and E stain) or abnormalities in absorptive functions.

TABLE 4. ACID–BASE BALANCE IN CYCLOHEXIMIDE-TREATED RATS*

Cycloheximide (mg/kg)	pH	pCO_2 (mm Hg)	CO_2 (mEq/L)
0 (5)	7·41 ± 0·18	38·0 ± 0·4	24·3 ± 0·2
2·5 (5)	7·38 ± 0·10	29·5 ± 0·3†	17·7 ± 0·2†

* Arterial blood collected anaerobically from the aorta under Nembutal anesthesia, 7 hr after a single i.p. dose of cycloheximide and determined by microelectrode with Radiometer (Copenhagen) pH meter. Results expressed as mean ± S.E. Number of rats is shown in parentheses; females of 200 g.
† $P < 0·01$.

DISCUSSION

Our results confirm various reports[2, 3] that cyclohexamide is a potent and rapidly acting inhibitor of protein synthesis and that acetoxycycloheximide is more potent than cycloheximide on an equal weight basis, particularly in female rats. Dosages of these agents used by various investigators have differed markedly. Most have been appreciably higher than those found effective in this study and this, as well as species susceptibility, may account for certain differences in results. Trakatellis *et al.*[3] noted inhibition for 24 hr of incorporation of leucine into liver protein *in vivo* in mice given 4 and 80 mg/kg of cycloheximide. This is in contrast to the more transient effect noted in our study with much lower dosage in the rat. Jondorf[6] observed that the marked and rapid inhibition of 0·5–5·0 mg/kg on leucine incorporation into rat liver microsomal proteins had almost completely disappeared after 24 hr. A range of dosage of cycloheximide from 0·25 to 50 mg/kg has been reported in this and in other studies *in vivo* in the rat.[7–10] Acetoxycycloheximide has been used in dosages from 0·25 to 5 mg/kg in various species.[2, 11–13]

The route of administration of cycloheximide appears to be relatively unimportant in inducing systemic effects in protein synthesis. This drug appears to be rapidly and efficiently absorbed from the intestinal tract and peritoneal cavity. The experiment with pylorus ligation indicates good absorption from the stomach. Despite the absorption through the gastric mucosa, amino acid incorporation into stomach protein was not preferentially inhibited, as one might expect if direct exposure to a high concentration of the drug were a critical factor.

While these experiments involved a 4-hr interval between leucine administration and sacrifice, which permitted additional time for cycloheximide to act, it is clear

that onset of action is rather rapid and drug effect is limited to 12–16 hr in the dosage used. Similar findings by Jondorf[6] have been referred to above. Six hr after administration of cycloheximide at 1·5 mg/kg, the complete mitosis arrest of mouse intestinal villi, which had been noted at 1·5 hr after cycloheximide, was no longer present.[14]

The greater sensitivity of females to acetoxycycloheximide-induced inhibition of leucine incorporation noted in this study is consistent with a report of greater mortality in this sex with this drug.[15] However, in our studies of the effect of cycloheximide or acetoxycycloheximide on intestinal absorption of lipids,[16] vitamin B_{12}[17] or iron,[18] the degree of malabsorption induced in both sexes by a given dose was approximately the same. On the other hand, iodine uptake by the thyroid* was decreased in female rats but not in males given cycloheximide (0·5 mg/kg, i.p.). Acetoxycycloheximide (0·25 mg/kg, i.p.) markedly reduced renal function in females, but not in males.* The biochemical basis for these sex differences is unknown.

While the exact mechanism of cycloheximide is not completely known, it is a useful tool in exploring the biological functions related to protein synthesis because of its rapid, potent, reproducible and reversible action. However, larger doses of this group of drugs may cause secondary changes in organ function (renal damage, gastric stasis, malabsorption and shock) which may, in turn, directly or indirectly affect metabolic and physiologic activities under investigation. As noted in Tables 3 and 4, azotemia and acid–base changes occurred with 2·5 mg per kg of cycloheximide. Studies *in vivo* of a particular drug function require a range of dosages and ancillary studies necessary to assist in ruling out possible interferences.

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