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Effect of *N*-Cyclohexyl Linoleamide on Cholesterol Metabolism in Rats* (32639)

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The cyclohexylamine amide of linoleic acid (Clinolamide; Linolexamide) has been found to lower serum and liver cholesterol levels and to reduce the severity of atherosclerosis in cholesterol-fed rabbits (1,2). The hypocholesteremic effect of this compound has been attributed in part to inhibition of cholesterol absorption (3), but there are no other published reports concerning its effects on other aspects of cholesterol metabolism. We have investigated the effect of *N*-cyclohexyl linoleamide upon cholesterol metabolism in normocholesteremic rats fed a cholesterol-free semisynthetic diet. The results of this experimental series are the basis of this report.

Methods and Materials. All rats used were males of the Wistar strain. Groups of equal starting weights were used for each experiment: Average starting weights in the biosynthesis experiments were 171 gm, 200 gm, 174 gm, and 160 gm, in experiments 1, 2, 3, and 4, respectively; in the cholesterol oxidation experiment, average starting weights were 171 gm. The rats were maintained for 3 weeks on a diet consisting of mixed cereal, 70 (Pablum, Mead Johnson Co.); wheat germ, 7; skim milk powder, 22; and vitamin mix, 1. The test compound (0.3 or 0.6%) was added to the diet at the expense of the cereal. This diet has been used in other feeding experiments involving drugs and is readily accepted by the rats (4,5).

At the end of the feeding period, the animals were killed by exsanguination. In the cholesterol biosynthesis experiments, liver slices (0.5 gm) were incubated for 3 hours under 100% oxygen in 5 ml phosphate buffer (pH 7), containing 0.006 *M* $MgCl_2$, 0.03 *M* nicotinamide and either 1 μC of sodium acetate-1-^{14}C or 0.5 μC of mevalonic acid-2-^{14}C. In one series, 1.5×10^{-5} moles (10 mg) of *N*-cyclohexyl linoleamide in 0.1 ml of ethanol was added to 0.5 gm of normal rat liver in order to test the *in vitro* effect of this compound on cholesterol biosynthesis. The reaction was stopped by the addition of 5 ml of alcoholic KOH; the sterol was then extracted with petroleum ether and isolated as the digitonide for radioactive assay by liquid scintillation spectrometry (6).

In several experiments the basic solution remaining after extraction of the nonsaponifiable material was acidified and the fatty acids extracted with ether. The ether extracts were dried over anhydrous Na_2SO_4 and an aliquot taken for radioactive assay. A 1-gm aliquot of each liver was taken for cholesterol analysis. In 2 experiments 1 kidney and 1

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TABLE I. Autopsy Data on Rats Fed *N*-Cyclohexyl Linoleamide (L) for 21 Days.

Group	Number	Wt. gain (gm)	Liver wt. (gm)	Liver body wt. (%)	Cholesterol		Serum + liver cholesterol pool (mg)
					Liver (mg/100 gm)	Serum (mg/100 ml)	
Expt. 1							
L (0.3)[a]	6	61	10.2	4.39	356 ± 56[b]	40.1 ± 4.0[b]	39.1
Control	6	52	10.0	4.48	384 ± 21	33.2 ± 4.3	40.6
Expt. 2							
L (0.3)	8	100	11.8	4.07	243 ± 19	35.8 ± 3.6	31.8
Control	7	92	11.1	3.81	241 ± 21	34.4 ± 3.3	29.8
Expt. 3							
L (0.3)	8	57	9.2	4.00	159 ± 19	25.2 ± 4.5	15.8
Control	8	83	9.5	3.79	155 ± 18	30.5 ± 7.9	17.0
Expt. 4							
L (0.3)	7	88	9.2	3.72	144 ± 25	31.3 ± 3.1	15.6
L (0.6)	8	79	8.9	3.74	145 ± 14	34.2 ± 3.1	15.3
Control	7	91	9.4	3.75	160 ± 20	24.7 ± 2.9	16.9
Expt. 5							
L (0.3)	8	39	8.4	4.00	378 ± 29	40.4 ± 5.0	34.3
Control	6	67	9.0	3.81	403 ± 9	38.0 ± 1.9	40.0

[a] Dosage (%).
[b] Standard error.

gm of skin were also taken for cholesterol analysis.

Oxidation of cholesterol-26-^{14}C by rat liver mitochondria was carried out using previously detailed methods (7,8). Incubations were carried out in stoppered 125-ml Erlenmeyer flasks containing center wells. The incubation mixture consisted of 1 ml of mitochondrial preparation; 1 ml of a solution containing ATP (25 mg), NAD (5 mg), AMP (8 mg), reduced glutathione (15 mg), sodium citrate monohydrate (30 mg), magnesium nitrate hexahydrate (10 mg), potassium penicillin G (2000 U) and streptomycin sulfate (1 mg); 5 ml of labeled substrate in 0.25 *M* tris(hydroxymethyl)aminomethane, HCl, pH 8.5; and 5 ml of boiled supernatant. The center wells contained 2.0 ml of 2.5 *N* NaOH. Incubations were carried out for 18 hours at 37°C. The $^{14}CO_2$ trapped in the base was precipitated as $Ba^{14}CO_3$ and the radioactivity assayed by liquid scintillation spectrometry. Serum and tissue cholesterol levels were determined using the method of Mann (9). Radioactive substrates were purchased from the New England Nuclear Corp., Boston, Massachusetts, and the cholesterol-26-^{14}C was purified prior to use by chromatography on alumina. The *N*-cyclohexyl linoleamide was generously supplied by Dr. S. Yamaoka, Sumitomo Chemical Co., Osaka, Japan.

Results and Discussion. From the autopsy findings presented in Table I, it is evident that *N*-cyclohexyl linoleamide did not exert a significant effect on weight gain; however, liver size in the drug-fed animals was somewhat larger than it was in the controls. There were no significant differences between the serum and liver cholesterol levels of the control rats and rats fed either 0.3 or 0.6% of Clinolamide. Calculation of the serum plus liver cholesterol pools, however, revealed that, generally, this pool was somewhat smaller in the rats fed the test compound. These findings suggest an inhibition of reabsorption of endogenous cholesterol even in rats maintained on a cholesterol-free diet. Duncan *et al.* (10) have shown that, despite their lower serum cholesterol levels, the total body cholesterol in hyperthyroid rats is greater than that in hypo- or euthyroid rats. The difference in body cholesterol levels in these animals is

TABLE II. Influence of *N*-Cyclohexyl Linoleamide (L) Feeding on Hepatic Cholesterogenesis in Rats (% of Incorporation of Substrate).

Group	Number	Cholesterol		Fatty acids
		Acetate-1-^{14}C	Mevalonate-2-^{14}C	acetate-1-^{14}C
Expt. 1				
L (0.3%)	6	1.06 ± .28[b]	2.36 ± .38	—
Control	6	1.86 ± .48	2.28 ± .24	—
Expt. 2				
L (0.3%)	8	0.98 ± .24	3.26 ± .52	2.10 ± .39
Control	7	1.02 ± .22	2.86 ± .60	2.31 ± .48
Expt. 3				
L (0.3%)	7	0.98 ± .16	1.84 ± .26	5.97 ± .66
L ($1.5 \times 10^{-5} M$)[a]	6	0.28 ± .04	—	—
Control	8	0.82 ± .08	1.92 ± .26	5.47 ± .48
Expt. 4				
L (0.3%)	6	1.96 ± .24	3.02 ± .42	4.65 ± .69
L (0.6%)	6	1.60 ± .19	2.82 ± .30	5.97 ± 1.05
Control	6	2.40 ± .14	3.68 ± .24	7.20 ± 1.02

[a] Added to normal liver slices.

[b] Standard error.

due to an accumulation of skin cholesterol in the hyperthyroid rats. Analysis of the cholesterol content of 1 kidney and 1 gm of skin from each animal was carried out in experiments 3 and 4. In experiment 3, the average kidney weights were 1.33 and 1.14 gm for groups L and C, respectively; kidney and skin cholesterol levels (mg/100 gm) were 231±15 and 109±10 in group L, and 305± 20 and 123±4 in group C. In experiment 4, average kidney weights were: 1.04 gm for group L (0.3%); 0.93 gm for group L (0.6%); and 0.96 gm for group C. Kidney and skin cholesterol levels (mg/100 gm) for group L (0.3%) were 500±16 and 101±12; for group L (0.6%), 548±27 and 105±7 and for the control group, 542±35 and 112±9. The data suggest a slight depletion of kidney and skin cholesterol in rats fed 0.3% of Clinolamide for 3 weeks.

The results of the cholesterol biosynthesis experiments presented in Table II show that in the first 2 experiments there are no significant differences between the drug-treated and control groups in the conversion of either acetate-1-^{14}C or mevalonate-2-^{14}C to cholesterol or of conversion of acetate-1-^{14}C to fatty acids. The addition of 1.55×10^{-5} *M* of *N*-cyclohexyl linoleamide to normal rat liver slices (Expt. 3) significantly inhibits the conversion of acetate-1-^{14}C to cholesterol [L (.3%) vs L (1.5×10^{-5} *M*), $.01 > p > .001$; Control vs L (1.5×10^{-5} *M*), $p < .001$]. In this experiment, we again did not observe any differences between the control group and group L (0.3%) in conversion of acetate to cholesterol or fatty acid or of mevalonate to cholesterol. In our earlier experiments on the effect of Clinolamide upon experimental atherosclerosis in rabbits (2), we had found that a dose of 300 mg/day lowered serum and liver cholesterol levels but did not affect the severity of atherosclerosis. However, if the dose of Clinolamide was increased to 600 mg/day, inhibition of atherosclerosis was also observed. Consequently, in experiment 4, we maintained groups of rats on 0.3% and 0.6% of this compound. It may be seen from Table II that biosynthesis of cholesterol from acetate-1-^{14}C was significantly inhibited [L (0.6%) vs control, $.01 > p > .001$], and inhibition of conversion of mevalonate-2-^{14}C to cholesterol was significant below the 5% level [L (0.6%) vs control, $.05 > p > .02$]. For the overall series (27 experiments), conversion of sodium acetate-1-^{14}C to cholesterol

TABLE III. Oxidation of Cholesterol-26-^{14}C to $^{14}CO_2$ by Liver Mitochondrial Preparations from Rats Fed 0.3% *N*-Cyclohexyl Linoleamide.

Preparation	Oxidation (%)[a]	
	Control	0.3% *N*-Cyclohexyl linoleamide
1	23.7	39.1
2	20.9	28.0
3	2.7	22.7
4	8.3	8.2
5	2.9	10.1
6	8.7	10.0
7	—	2.6
8	—	6.9
Average	11.2	16.0
SD	8.2	16.4
SE	3.7	6.2

[a] $^{14}CO_2$ (as Ba $^{14}CO_3$)/cholesterol-26-^{14}C × 100. Corrected for mg N.

was somewhat higher in the control series than in the group fed 0.3% Clinolamide (1.45±0.17% vs 1.22±0.15%), but the difference was not significant. Cholesterogenesis from mevalonic acid-2-^{14}C was 2.63±0.24% in the control group and 2.64±0.23% in the drug-treated group. Gas-liquid chromatography showed that cholesterol was the major liver sterol of both control and treated groups. The only other component seen in the gas chromatogram was a small quantity of phytosterol; this material was similar to that isolated from the nonsaponifiable material of the dietary wheat germ.

Liver slices from control rats converted 4.57±0.56% of the sodium acetate-1-^{14}C to fatty acids as compared with a conversion of 4.12±0.49% by liver slices of rats fed 0.3% Clinolamide (averages of 21 experiments), but the difference was not significant.

Mitochondrial preparations from rats fed 0.3% *N*-cyclohexyl linoleamide oxidized more cholesterol-26-^{14}C to $^{14}CO_2$ than did similar preparations from control rats (Exp. 5). Because of the variability of oxidation observed in experiments of this type, the differences are not significant. However, as can be seen from Table III, in only 4 out of 6 experiments was more than 10% oxidation observed with mitochondrial preparations from control rats; whereas with mitochondrial preparations from treated rats, oxidation of 10% or more was observed in 5 of 8 experiments.

Summary. Male Wistar rats were fed 0.3% *N*-cyclohexyl linoleamide (Clinolamide; Linolexamide) for 21 days. In a series of 5 experiments it was found that the drug did not significantly affect serum, liver, or kidney cholesterol levels. The livers of the Clinolamide-fed rats were larger (% body weight) than those of the controls. The serum plus liver cholesterol pool was generally higher in the control animals as was skin cholesterol. Synthesis of cholesterol from either sodium acetate-1-^{14}C or mevalonic acid-2-^{14}C by liver slices of rats fed 0.3% Clinolamide was not affected. Liver slices from rats fed 0.6% of the test compound synthesized significantly less cholesterol from either substrate than did slices from control rats. Addition of *N*-cyclohexyl linoleamide (1.5×10^{-5} *M*) to normal rat liver slices significantly depressed cholesterol synthesis from acetate-1-^{14}C. Liver mitochondrial preparations from rats fed 0.3% of Clinolamide do not oxidize significantly more cholesterol-26-^{14}C to $^{14}CO_2$ than do similar preparations from control rats.

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