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Effect of Benzyl N-Benzyl Carbethoxyhydroxamate on Cholesterol Metabolism in the Rat.* (30995)

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Berger and his coworkers(1) have reported that benzyl N-benzyl carbethoxyhydroxamate (W-398) will reduce serum and liver lipids and degree and extent of atherosclerosis when administered to cholesterol-fed rabbits at a level of 2% of the diet. This compound (0.25-0.50%) has also been found to lower serum cholesterol levels of weanling rats fed a hypercholesteremic diet. We have investigated the effect of W-398 upon cholesterol metabolism in normocholesteremic rats fed a sterol-free semi-synthetic diet. The results of our experiments are reported below.

Methods. All rats used were males of the Wistar strain and their average weight at the beginning of each experiment was 185 ± 5 g. The rats were maintained on a diet consisting of infant cereal (Pablum, Mead Johnson Co.) (70), wheat germ (7) skim milk powder (21) and vitamin mix (2). The diet provides 20% protein, 11% fat and 62% carbohydrate, and is readily accepted by the rats. The experimental compound was added to the diets at the expense of the cereal.

The rats were kept on the diets for 3 weeks, at which time they were weighed, injected with either sodium acetate-1-^{14}C (1 μc/100 g or mevalonic acid-2-^{14}C (0.5 μc/100 g) and killed by exsanguination 4 hours after administration of the radioactive substrate. The livers were homogenized in chloroform-methanol 2:1 and an aliquot of the dried extract was taken for cholesterol determination. Another aliquot of each extract was taken for saponification and separation of the cholesterol, whose radioactivity was determined by liquid scintillation spectrometry (2). Serum and liver cholesterol levels were determined by the method of Mann(3). In the experiment in which cholesterol biosynthesis was studied *in vitro*, liver slices (0.5 g) were incubated for 3 hours under 100% oxygen in 5 ml phosphate buffer, pH 7 containing 0.006 M $MgCl_2$ and 0.03 M nicotinamide and 1 μc of either sodium acetate-1-^{14}C or mevalonic acid-2-^{14}C. The reaction was stopped by addition of hot alcoholic KOH and the cholesterol isolated and analyzed for radioactivity. In this experiment a one gram aliquot of each liver was taken for cholesterol analysis. Radioactive substrates were purchased from New England Nuclear Corp., Boston, Mass. Benzyl N-ben-

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TABLE I. Autopsy Data on Rats Fed W-398 (2.0 or 0.5%) for 21 Days.

Group	No.	Wt change (g)	Liver wt (g)	Liver as % body wt	Cholesterol: Liver (mg/100 g)	Cholesterol: Serum (mg/100 ml)	Serum + liver cholesterol pool (mg)
Exp 1							
W (2%)	6	−41	7.7	5.38	504 ± 68*	52.2 ± 6.1	41.1
Control	6	+50	9.4	3.86	213 ± 14[a]	57.3 ± 4.8	24.2
Exp 2							
W (2%)	7	−43	8.5	6.03	627 ± 53	59.1 ±12.2	55.8
Control	9	+83	10.3	3.90	279 ± 52[a]	43.5 ± 3.8	32.2
Exp 3							
W (2%)	4	−40	10.1	6.96	1540 ± 81	—	—
Control	4	+48	10.6	4.39	380 ± 28[a]	—	—
Exp 4							
W (.5%)	10	+48	11.5	4.79	251 ± 37	61.4 ± 3.8	33.3
Control	9	+69	10.5	4.18	132 ± 7[b]	66.7 ± 5.2	18.4

* Standard error.

[a] $p < .001$; [b] $.01 > p > .001$.

zyl carbethoxyhydroxamate (W-398) was generously provided by Dr. F. M. Berger, Wallace Laboratories, Cranbury, N. J.

Results and discussion. The autopsy findings are presented in Table I. It is evident that when fed as 2% of the diet, W-398 resulted in dramatic weight losses (about 21% of the starting weight in 3 weeks). Even when fed at the 0.5% level, W-398 caused some retardation of weight gain. The other striking finding was the effect of W-398 upon liver size and liver cholesterol content. In the 3 experiments in which 2% W-398 was added to the diet, the liver size in proportion to body size was greatly increased. In Experiment 4, when W-398 was fed at a lower dosage (0.5%) the livers of the drug fed rats were still larger in size than those of the controls. The increased liver volume was also reflected in the highly significant increase in liver cholesterol observed even in rats fed only 0.5% of W-398. Serum cholesterol levels were not significantly affected when the drug was fed at either level. The α and β serum lipoprotein cholesterol levels were determined after precipitation of the β lipoproteins by the dextran sulfate method (4,5) and were found to be the same (40% of the serum cholesterol present in the α lipoprotein) for both control and drug groups.

The effect of the experimental compound on cholesterol biosynthesis is presented in Table II. In the *in vivo* experiments(1,2,4) the conversion of either acetate or mevalonate to cholesterol was always greater in the rats fed W-398. Douglas(6) has reported that when W-398 was added to rat liver homogenates at concentrations of 7×10^{-4} M it reduced the conversion of acetate to cholesterol but did not affect the biosynthesis

TABLE II. Influence of W-398 on Hepatic Cholesterogenesis in Rats (% Conversion of Substrate to Cholesterol).

	Group	No.	Acetate-1-^{14}C	No.	Mevalonate-2-^{14}C
Exp 1 (*in vivo*)†	W (2%)	6	.19 ± .06*	—	—
	Control	6	.11 ± .03	—	—
Exp 2 (*in vivo*)†	W (2%)	4	.23 ± .07	3	6.52 ±2.20
	Control	5	.13 ± .04	4	2.76 ± .36
Exp 3 (*in vitro*)‡	W (2%)	4	.55 ± .08	4	1.38 ± .08
	Control	4	.81 ± .19	4	1.39 ± .31
Exp 4 (*in vivo*)†	W (.5%)	5	.14 ± .04	5	1.56 ± .19[a]
	Control	5	.07 ± .001	4	.81 ± .12

* Standard error.

[a] $p = .01$.

† Dosage: acetate 1 μc/100 g; mevalonate .5 μc/100 g.

‡ Dosage: 1 μc each substrate/incubation.

of cholesterol from mevalonate. In our hands (Exp. 3) liver slice preparations from rats fed W-398 for 3 weeks gave essentially the same results. Gas-liquid chromatography revealed no sterol other than cholesterol in the livers of rats fed W-398.

These data show that benzyl N-benzyl carbethoxyhydroxamate (W-398) had a profound effect upon cholesterol metabolism in the intact rat fed a cholesterol free diet. When fed as 2% of the diet, W-398 did not affect the serum cholesterol level but caused a significant increase in the liver cholesterol level. The result was a large increase in the serum-liver cholesterol pool(7), despite the fact that rats fed 2% W-398 for 3 weeks exhibited a marked loss in body weight. The weight loss was not due to anorexia, since the rats maintained on W-398 consumed about the same quantity of food as did the controls. Berger *et al*(1) commented on the fact that rats fed 2% W-398 did not gain weight, but presented no data.

In view of the large accumulation of cholesterol in the livers of rats fed W-398, the data on *in vivo* cholesterogenesis are surprising. It has been demonstrated(8,9) that cholesterol feeding strongly inhibits hepatic cholesterol synthesis in rats; however, rats fed either 0.5 or 2.0% of W-398 convert more injected substrate (acetate-1-^{14}C or mevalonate-2-^{14}C) to cholesterol than do controls. If the data were calculated in terms of cholesterol radioactivity per gram of liver, the difference would be even more pronounced. The findings suggest that the liver cholesterol of the rats fed W-398 may be compartmentalized in such a way as not to interfere with biosynthesis or that, due to the weight loss resulting from ingestion of W-398, there was not enough endogenous substrate present to dilute the injected precursor.

The one *in vitro* biosynthesis experiment yielded data which correlate well with the findings of Douglas(6), who added the drug to a normal liver homogenate. In our hands, liver slices from rats fed 2% W-398 converted 32% less acetate to cholesterol than did slices from control rats, but there was no effect on mevalonate utilization. Douglas found a 50% reduction in acetate incorporation in the presence of W-398. Possibly the presence of the drug in the liver affected the *in vitro* utilization of acetate or the large amount of cholesterol present in the livers of the W-398-fed rats exerted the expected inhibitory effect on cholesterol biosynthesis.

The data suggest that in rats fed W-398, the relative liver enlargement and the large increases in liver cholesterol were due to an increased rate of cholesterol biosynthesis, coupled with an inability to transfer the sterol from the liver.

Summary. In 3 experiments, male Wistar rats (185 g) were fed 2% benzyl N-benzyl carbethoxyhydroxamate (W-398) for 3 weeks. The rats exhibited marked weight loss (losing 40 g while the controls were gaining 50-80 g); livers of the drug-fed group were larger (5.4-7.0% body wt) compared with the controls (3.9-4.4% body wt) and liver cholesterol levels were significantly elevated (avg of 3 experiments: drug—858 mg/100 g and control—279 mg/100 g). The compound had no effect upon serum cholesterol levels. In one experiment, in which W-398 was fed as 0.5% of the diet, the rats gained 30% less weight than did the normals, their livers were larger and their liver cholesterol levels were significantly higher than were those of the control rats. The data suggest a continuing synthesis of cholesterol in rats fed W-398 but an apparent inability to transfer it from the liver.

When either acetate-1-^{14}C or mevalonate-2-^{14}C was injected into the drug-treated rats they converted more (but not significantly more) of the substrate to cholesterol than did the controls. Liver slices from W-398-treated rats converted less acetate-1-^{14}C to cholesterol than did liver slices from control rats.

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Collagen Formation and Endochondral Ossification in Estrogen Treated Mice.* (30996)

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The ability of estrogen to stimulate endosteal bone formation in the skeleton of the mouse has been demonstrated many times since the original report of Gardner and Pfeiffer(1). It is not known why this response to estrogen occurs, nor why it appears to be specific (among mammals) to mice(2). The reaction begins in the metaphyses of growing long bones and eventually extends along the entire shaft. Ultimately, if treatment is prolonged, the marrow elements may be completely displaced by the centripetal growth of bone trabeculae. Vaes and Nichols (4) demonstrated *in vitro* that bone collagen formation, as measured by uptake of radioglycine, was enhanced in estrogen-primed mice. Autoradiographic studies with tritiated thymidine(4) in mice have shown that estrogen treatment increases the rate at which metaphyseal and endosteal osteoblasts are formed from undifferentiated reticular cells in the marrow. While, after estrogen treatment, the increase in osteoblast numbers may be adequate to explain the enhanced collagen synthesis observed *in vitro,* it is not known whether the hormone increases the ability of the individual osteoblasts to form collagen. To resolve this question, further studies of bone formation in mice have been pursued by high resolution autoradiography following administration of H^3-proline and H^3-glycine at different times after estrogen treatment.

Materials and methods. Male, CF_1 albino mice, 30 days of age, were injected with one subcutaneous dose of 1 mg estradiol valerate (Delestrogen)† to stimulate endosteal bone formation. The controls were not treated with estrogen. Tritiated-proline (1 μc/g body weight)‡ was administered intraperitoneally to 15 control and 15 estrogen-treated mice 24 hours after hormone treatment, and 3 animals from each series were sacrificed by decapitation thereafter at 1, 4, 8, 16 and 24 hours.

A second series of mice was injected subcutaneously with H^3-glycine (0.5 μc/g body weight)§ on the seventh day after estrogen treatment when the endosteal bone reaction had been well established. Four untreated control mice and 4 estrogen-treated mice were sacrificed by decapitation 1, 2, 4, 12, and 24 hours after isotope administration. The times at which the labeled amino acids were administered were based on data(3) which indicated (a) that the differentiation of osteoblasts from the precursor cell population was increased as early as 6 hours after estrogen treatment, and (b) that the population of

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‡ L-Proline-3,4,-H^3 Hydrochloride (Lot 64-191-3), New England Nuclear Corp., Specific Activity = 371 mC/mmole.

§ Glycine-1-C^{14}, New England Nuclear Corp., Specific Activity = 0.66 mC/mmole.