Effect of Hyocholic Acid on Cholesterol and Bile Acid Metabolism in the Mouse.* (29273)

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Previous experiments in this and other laboratories have shown that hyodeoxycholic acid effectively prevents accumulation of blood and liver cholesterol in mice fed diets supplemented with cholesterol(1,2), and lowers tissue cholesterol concentrations in mice fed standard laboratory diets(2,3). This lowering results from an increase in excretion of fecal neutral sterols of hepatic origin(4).

Since hyodeoxycholic acid, which has a more pronounced effect in lowering cholesterol levels than other bile acids tested, is unique among them in having a 6α-hydroxyl group, it was of interest to study effects of additional bile acids with such a group. Hyocholic acid was therefore investigated.

It is possible that the effects of an administered bile acid may be due to its metabolic products. Intestinal flora are known to alter the structure of bile acids present in the intestinal content. Howe and Bosshardt(5) have shown that lithocholic acid is an active hypocholesteremic agent through its conversion to unidentified products by intestinal bacteria. Therefore we determined the effects of hyocholic acid on sterol metabolism in normal mice, and in mice whose intestinal bacteria were limited by antibiotics.

Methods. One hundred female albino Webster strain mice, weighing 20-25 g, were divided into 5 equal groups, and fed the following diets *ad lib*:

I. Lipid-free diet (LFD)†
II. LFD + 0.5% hyocholic acid‡
III. LFD + 0.5% hyocholic acid + 1% cholesterol
IV. LFD + 0.5% hyocholic acid + 1% cholesterol + 0.5% cholic acid
V. LFD + 1% cholesterol + 0.5% cholic acid.

There was no significant difference in food intake or body weight in any of the 5 groups during the studies. Half of each dietary group (10 mice) were treated with antibiotic by adding 2% succinylsulfathiazole plus 0.4% oxytetracycline§(5) to the diet.

After 3 weeks each animal received an intraperitoneal injection of 5 μc sodium acetate-1-C^{14} dissolved in 0.9% saline. One hour later the animals were sacrificed by cervical fracture, and blood, liver and carcass preserved for analysis. Serum cholesterol concentrations were determined by the Sperry and Webb method(6). Liver and carcass cholesterol were isolated as digitonides by methods developed in this laboratory(7). The digitonides were analyzed by application of the Lieberman-Burchard reaction, or plated for counting by the filtration tower technique. Samples were counted in a windowless gas flow counter.

The small intestinal contents from each group were extracted with normal butanol and hydrolyzed with 7 N sodium hydroxide at 15 lbs pressure for 3 hours. Nonsaponifiable material was removed by extracting the alkaline hydrolysate with petroleum ether. The aqueous phase was acidified to pH 1.0, and extracted again with petroleum ether to remove fatty acids. Free bile acids were then extracted from the aqueous phase with ethyl ether. Bile acids were separated by thin layer chromatography, using a moving phase consisting of 96% ethyl acetate and 4% glacial

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† 25% vitamin free casein, 55% sucrose, 16% powdered cellulose, 4% Phillips-Hart salt mixture; supplemented with the following vitamins in mg/10 kilo: 40.4 thiamin hydrochloride, 60.8 riboflavin, 151 calcium pantothenate, 1008 niacin, 40.4 pyridoxine hydrochloride, 5.12 2-methyl-1, 4-naphthoquinone, 30.2 α-tocopherol, 20.3 folic acid, 2.02 biotin, 20,200 choline chloride, 202-p-aminobenzoic acid, 10,100 inositol, 1,250,000 units vit. A.

‡ Generous gift from Canada Packers Ltd., Toronto, Canada.

§ Generous gift from Chas. Pfizer and Co., Inc., New York.

TABLE I. Effects of Hyocholic Acid, Cholesterol and Cholic Acid on Tissue Total Cholesterol Concentrations in Normal and Antibiotic Treated* Mice.

	Group I, No dietary supplement	Group II, Hyocholic acid, .5%	Group III, Cholesterol, 1% + hyocholic acid, .5%	Group IV, Cholesterol, 1% + hyocholic acid, .5% + cholic acid, .5%	Group V, Cholesterol, 1% + cholic acid, .5%
			Serum cholesterol, mg/100 ml		
Untreated	153 ± 14	121 ± 20	119 ± 5	116 ± 18	426 ± 74
Antibiotic treated	108 ± 9	137 ± 9	207 ± 33	228 ± 116	484 ± 10
			Liver cholesterol, mg/g†		
Untreated	5.78 ± 1.72	6.28 ± 1.58	11.5 ± 2.07	4.86 ± 1.01	53.5 ± 18.6
Antibiotic treated	6.22 ± 1.12	4.24 ± .41	33.0 ± 6.80	36.3 ± 9.85	75.0 ± 9.2
			Carcass‡ cholesterol, mg/g†		
Untreated	2.64 ± .45	2.59 ± .67	2.60 ± .25	3.15 ± .45	3.87 ± .87
Antibiotic treated	2.54 ± .39	3.04 ± .30	3.27 ± .14	3.27 ± .57	3.78 ± .32

* 2% succinylsulfathiazole + 0.4% oxytetracycline.
† Wet weight.
‡ Body minus liver, blood and intestinal contents.
± standard deviations.

acetic acid (v/v). Bile acids were detected and identified on the developed chromatograms by spraying with a reagent made by dissolving 2 g anhydrous ferric chloride in 83 ml of anhydrous normal butanol and mixing with 15 ml concentrated sulfuric acid(8).

Results and discussion. Table I shows the effects of diets supplemented with combinations of hyocholic acid, cholesterol and cholic acid on tissue cholesterol concentrations in serum, liver and carcass of untreated and antibiotic-treated mice.

In serum of untreated mice, hyocholic acid lowered cholesterol concentrations and prevented accumulation of this sterol when diets were supplemented with cholesterol or cholesterol plus cholic acid. In liver tissue hyocholic acid had no effect by itself (Group II), but there was a small increase in cholesterol concentrations when 1% cholesterol was also added (Group III). Comparison of Groups IV and V shows that hyocholic acid prevented the β-sterol accumulation that otherwise resulted from feeding the combination of cholesterol and cholic acid. In both serum and liver tissue, treatment with antibiotics reversed the hypocholesteremic effects of hyocholic acid to a great extent (Groups III and IV).

In mice receiving no antibiotic, carcass cholesterol concentrations were somewhat higher in groups fed diets supplemented with cholesterol-cholic acid combinations (IV and V) than in groups not receiving cholic acid (I, II, and III). Hyocholic acid, either by itself (Group II) or in combination with cholesterol (Group III), had no effect on carcass cholesterol levels. On the other hand, in mice treated with antibiotics, hyocholic acid caused a small but significant increase in carcass cholesterol concentrations (Groups II and III).

Table II shows the effects of hyocholic acid on the incorporation of acetate-1-C^{14} into tissue cholesterol in normal and antibiotic-treated mice.

In untreated mice, hyocholic acid (Group II) reduced the acetate-1-C^{14} incorporation in serum and liver to half that in the unsupplemented group (1); while the cholesterol and hyocholic acid dietary combination (Group III) reduced C^{14} incorporation to a small fraction of that in Group I. As expected, addition of cholesterol and cholic acid (Group V) reduced the incorporation to zero. When this diet (V) was further supplemented with hyocholic acid (Group IV), the incorporation was restored to only a small degree, in spite of the fact that cholesterol concentrations in serum and liver were below the level of that in the unsupplemented group (Table I). None of the diets affected the incorporation of C^{14} in the mouse carcass.

Antibiotics with no dietary supplement (Group I) reduced acetate-1-C^{14} incorporation in serum, but had no effect on liver

TABLE II. Effects of Hyocholic Acid, Cholesterol and Cholic Acid on Incorporation of Sodium Acetate-1-C^{14} into Tissue Total Cholesterol in Normal and Antibiotic Treated* Mice.

	Group I, No dietary supplement	Group II, Hyocholic acid, .5%	Group III, Cholesterol, 1% + hyocholic acid, .5%	Group IV, Cholesterol, 1% + hyocholic acid, .5% + cholic acid, .5%	Group V, Cholesterol, 1% + cholic acid, .5%
	Serum cholesterol-×-C^{14}, counts/min./ml				
Untreated	262 ± 61	128 ± 41	36 ± 13	125 ± 43	0
Antibiotic treated	140 ± 62	129 ± 6	0	0	0
	Liver cholesterol-×-C^{14}, counts/min./g tissue†				
Untreated	1,734 ± 670	857 ± 434	213 ± 75	382 ± 212	0
Antibiotic treated	1,530 ± 604	1,060 ± 490	0	0	0
	Carcass‡ cholesterol-×-C^{14}, counts/min./g tissue†				
Untreated	341 ± 77	304 ± 130	302 ± 106	370 ± 110	243 ± 185
Antibiotic treated	167 ± 56	179 ± 54	104 ± 27	138 ± 25	137 ± 13

* 2% succinylsulfathiazole + 0.4% oxytetracycline.
† Wet weight.
‡ Body minus liver, blood and intestinal contents.
± standard deviations.

β-sterol-C^{14}. They had no significant effect on C^{14} incorporation into serum and liver β-sterol-C^{14} in animals fed hyocholic acid by itself (Group II), but in Groups III and IV, receiving cholesterol and hyocholic acid combinations, C^{14} incorporation was reduced to zero. In all dietary groups (I → V), antibiotic treatment reduced acetate-1-C^{14} incorporation in carcass to about the same extent.

From the above findings, it appears that the cholesterol reducing effects of hyocholic acid are due largely to bacterial metabolites. To investigate the nature of these, the bile acid spectrum of small intestinal content was determined by thin layer chromatography. The samll intestinal bile acids of a normal mouse were (Fig. 1) predominantly cholic acid, small amounts of deoxycholic acid, and trace amounts of chenodeoxycholic and trihydroxy acids migrating slightly faster than cholic acid. As expected, treatment with antibiotic eliminated deoxycholic acid. In the absence of antibiotics, feeding hyocholic acid completely eliminated cholic acid; hyocholic acid and small amounts of hyodeoxycholic acid were found. When antibiotics were administered together with hyocholic acid, about 75% of the cholic acid was restored, and hyodeoxycholic acid could not be detected. When 0.5% hyodeoxycholic acid was added to the diet, either in the presence of or in the absence of antibiotics, cholic acid could not be detected in the intestinal content.

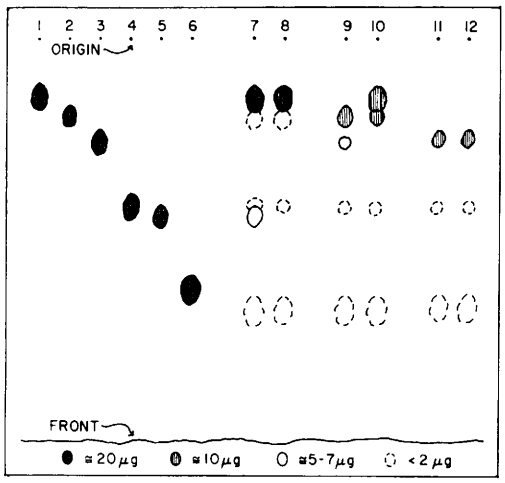

FIG. 1. Tracing of a chromatogram of bile acids isolated from mouse small intestinal content. Reference bile acids with detection colors: (1) cholic, green-black; (2) hyocholic, green; (3) hyodeoxycholic, black; (4) chenodeoxycholic, purple-black; (5) deoxycholic, brown; (6) lithocholic, purple. Intestinal bile acid spectrum; (7) no dietary supplement, no antibiotic; (8) same, antibiotic treated; (9) hyocholic acid-fed, no antibiotic; (10) same, antibiotic treated; (11) hyodeoxycholic acid-fed, no antibiotic; (12) same, antibiotic treated.

From the above data, it appears that hyocholic acid is partially converted to hyodeoxycholic acid by the intestinal flora of the mouse. The hypocholesteremic effect of hyocholic acid must be almost entirely due to its conversion to hyodeoxycholic acid, which has been shown to effectively prevent accu-

mulation of cholesterol and promote a rapid elimination of accumulated tissue β-sterol. It would seem that the presence of a 6α-hydroxyl group in the cholanic acid molecule has no special significance with respect to the hypocholesteremic properties of the acid.

It was of interest that no cholic acid could be detected in the small intestinal content of hyocholic and hyodeoxycholic acid-fed mice. Two possible mechanisms may explain this observation: (a) hyodeoxycholic acid may interfere with some early step in the conversion of cholesterol to cholic acid, or (b) hyodeoxycholic acid may bring about such a rapid excretion of hepatic sterols that oxidation is bypassed. A rapid excretion of hepatic sterol, resulting from administration of hyodeoxycholic acid, has been shown previously(4).

Summary. Hyocholic acid effectively prevented accumulation of dietary cholesterol in mice fed diets supplemented with cholesterol but had little or no effect on tissue cholesterol concentrations in mice fed normal diets. Treating mice with antibiotics largely reversed the effect of hyocholic acid, and permitted the accumulation of dietary cholesterol in hyocholic acid-treated mice. It was demonstrated that the hypocholesteremic effect is probably mediated by hyodeoxycholic acid, which arises from the action of intestinal bacteria on hyocholic acid. In animals which received hyocholic or hyodeoxycholic acid, cholic acid disappeared from the bile acid spectrum of the small intestine. Treatment with antibiotics restored cholic acid to the bile acid spectrum of hyocholic acid-treated mice but not to that of hyodeoxycholic acid-treated mice.

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Subcellular Transplantation Antigen Derived from Dog Spleen Cells Cultivated *in vitro*.* (29274)

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Previous work from this laboratory demonstrated that transplantation antigenic activity could be detected in the medium in which rabbit spleen cells had been cultivated in tissue culture(1,2). The antigenic activity was not removed by Millipore filtration but could be recovered by ultracentrifugation. When injected intradermally into a recipient rabbit the antigenic material was consistently able to induce transplantation immunity as indicated by the accelerated rejection of a test skin homograft from the donor of the cultured spleen. That the antigenic activity was specific for the spleen donor could be shown by histologic comparison of test skin homografts from the spleen donor and from an indifferent donor applied to the same antigen recipient, and by the fact that the antigenic preparation had no effect when injected back into the spleen donor. Following Millipore filtration the antigenic material was obtained as a fine suspension which was non-toxic upon intravenous infusion. This rabbit transplantation antigen (or antigens) appeared to be significantly more stable than several trans-

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