Prevention of cholesterol-induced gallstones by hyodeoxycholic acid in the prairie dog

Anil K. Singhal, Bertram I. Cohen, Erwin H. Mosbach,[1] Mizuho Une, Richard J. Stenger, Charles K. McSherry, Patricia May-Donath, and Thomas Palaia

Departments of Surgery and Pathology, Beth Israel Medical Center and Mount Sinai School of Medicine, New York, NY

Abstract Prairie dogs of both sexes were fed a semisynthetic diet containing 0.35% cholesterol for a period of 8 weeks. This lithogenic diet induced cholesterol gallstones in ten "lithogenic control animals", five males and five females. Three animals maintained with a high glucose, fat-free diet did not develop gallstones although the cholesterol saturation of their bile approached unity. The formation of gallstones was prevented in four out of five males and all five females fed the lithogenic diet plus 0.1% hyodeoxycholic acid (30 mg per kg body weight per day). The biles of the prairie dogs receiving hyodeoxycholic acid were abnormally colored, cloudy, and highly saturated with cholesterol but contained neither cholesterol crystals nor gallstones (with the exception of one male). Feeding the relatively hydrophilic bile acid, hyodeoxycholic acid, was associated with an increase in hepatic microsomal HMG-CoA reductase activity. Cholesterol 7α-hydroxylase, on the other hand, was inhibited by the administered bile acid. The dietary hyodeoxycholic acid was transformed, in part, to 3α,6β-dihydroxy-5β-cholanoic acid and hyocholic acid. It is concluded that hyodeoxycholic acid and its metabolites did not prevent the induced cholelithiasis by causing a decrease in the concentration of biliary cholesterol. Instead, this hydrophilic bile acid apparently increases the amount of cholesterol in the bile, probably in the form of a liquid crystalline mesophase. Hyodeoxycholic acid apparently prevents gallstones by preventing the nucleation and aggregation of cholesterol crystals. The lithogenic diet induced moderate to marked bile duct proliferation together with portal fibrosis and inflammatory infiltration. The addition of hyodeoxycholic acid to the lithogenic diet reduced all of the portal tract changes.—**Singhal, A. K., B. I. Cohen, E. H. Mosbach, M. Une, R. J. Stenger, C. K. McSherry, P. May-Donath, and T. Palaia.** Prevention of cholesterol-induced gallstones by hyodeoxycholic acid in the prairie dog. *J. Lipid Res.* 1984. **25:** 539–549.

Supplementary key words animal model • prairie dog (*Cynomys ludovicianus*) • cholesterol cholelithiasis • 3α,6β-dihydroxy-5β-cholanoic acid

There currently exist two well-established animal models of cholesterol cholelithiasis, a hamster model first described by Dam and Christensen (1) and a prairie dog model, extensively studied by Brenneman et al. (2). In both models gallstones are produced by dietary manipulation. In Dam's model, gallstones are induced in young, growing hamsters fed a fat-free diet high in glucose. In this species, as opposed to the rat, the lack of essential fatty acids, together with the high glucose content of the diet, stimulated hepatic cholesterol biosynthesis (3, 4). For this reason, the formation of lithogenic bile and gallstones has been ascribed to an overproduction of cholesterol by the liver. It was recently reported, however, that in the hamster model more than 90% of the biliary cholesterol was not derived from newly synthesized molecules. The authors nevertheless hypothesized that "there may be a general correlation between hepatic or total body cholesterol synthesis and the rate at which sterol is secreted in the bile" (5).

Dam and co-workers (6, 7) tested the ability of different dietary bile acids to prevent gallstones in his model and found that most bile acids (CA, DA, LA) had no effect on the experimentally induced disease while CDA actually aggravated it. Of all of the bile acids studied, only HDA prevented stone formation although the bile had an abnormal appearance (7). It was subsequently found that HDA did not act by reducing the cholesterol saturation of the bile (8). Apparently, stone formation failed to take place because cholesterol was present largely in liquid crystalline dispersion with lecithin. According to Salvioli, Igimi, and Carey (9), these "observations are consistent with the transformation of solid cholesterol monohydrate into liquid crystals or a prevention of a liquid → solid cholesterol monohydrate transition which occurs as part of initial nucleation events."

In the prairie dog model, lithogenic bile and gallstones are induced by the administration of a semisynthetic diet containing added cholesterol. Depending upon the cho-

Abbreviations: CA, cholic acid; DA, deoxycholic acid; LA, lithocholic acid; HDA, hyodeoxycholic acid; CDA, chenodeoxycholic acid; UDA, ursodeoxycholic acid; HCA, hyocholic acid; GLC, gas–liquid chromatography; GLC–MS, gas–liquid chromatography–mass spectrometry.

[1] To whom reprint request should be addressed at: Department of Surgery, Beth Israel Medical Center, 10 Nathan D. Perlman Place, New York, NY 10003.

lesterol content of the diet, gallstones form within 3 weeks with 1.2% dietary cholesterol, within 8 weeks with 0.4% cholesterol, and within 6 months with 0.2% cholesterol (10). In this model the effect of added dietary bile acids has not been studied very extensively. It is known, however, that CDA and UDA administered at levels of 15 to 30 mg/kg body weight per day reduced the cholesterol saturation of bile and inhibited stone formation wholly or in part (10, 11). It has been suggested that CDA and UDA exerted their preventative effect by reducing the intestinal absorption of cholesterol (which is an essential component of the lithogenic diet) (10, 11).

We have now investigated the effect of HDA at a dose of 30 mg/kg body weight per day in male and female prairie dogs receiving a lithogenic diet containing 0.35% cholesterol. Although the biles of the animals become highly saturated with cholesterol, the formation of gallstones and of biliary cholesterol crystals was prevented in nine out of ten animals. In this species, appreciable amounts of the administered HDA were transformed into 3α,6β-dihydroxy-5β-cholanoic acid and HCA.

MATERIALS AND METHODS

Animals and diets

Prairie dogs (*Cynomys ludovicianus*, trapped in the wild), 18 males and 18 females, were purchased from Fur and Feather Game Farm, Green Bay, WI. The animals were housed in individual rabbit cages and underwent a 2-week quarantine period during which they received Purina rat chow and water ad libitum. The animals were then weighed and divided into six groups. Group I, five males and five females, fat-free diet; group II, six males, semisynthetic diet; group III, five males, lithogenic diet; group IV, five females, lithogenic diet; group V, five males, lithogenic diet + 0.1% hyodeoxycholic acid, 30 mg per kg body weight per day; group VI, five females, lithogenic diet + 0.1% hyodeoxycholic acid, 30 mg per kg body weight per day.

The fat-free diet was Dam's lithogenic diet (used in his hamster model of cholesterol cholelithiasis) and contained: glucose, 75%; casein, 20%; mineral mix, 4%; and vitamin mix, 1% (1). The semisynthetic diet (Teklad, Madison, WI) consisted of: sucrose, 56.6%; corn starch, 13.9%; soy protein, 20.2%; corn oil, 1.6%; cellulose, 2.6%; mineral mix (Teklad #17082), 4%; and vitamin mix (Teklad #40060), 1%. This diet contained 0.08% cholesterol. The lithogenic diet was identical to this semisynthetic diet but contained in addition 0.2% cholesterol from egg yolk powder and 0.15% crystalline cholesterol (Sigma Chemical Co., St. Louis, MO). Total cholesterol content of this diet was 0.35%. The prairie dogs were fed the pelleted diets and drinking water ad libitum for a period of 8 weeks. The animals were kept on an alternating 12-hr-light and 12-hr-dark schedule.

At the beginning of week 7, 2-day pools of feces were collected quantitatively from each animal. During the last 24 hr of the 8-week experimental period the animals were starved and were then anesthetized with 100 mg of ketamine hydrochloride (Bristol Labs, Syracuse, NY) and 20 mg of xylazine (Haver-Lockhart, Shawnee, KS). The animals were killed by exsanguination and blood was collected for the determination of serum cholesterol. The gallbladder was exposed and bile was obtained from the gallbladder by syringe. The gallbladder was opened and examined for the presence of gallstones. One drop of bile was placed under a polarizing microscope (Olympus model MCHAP) to determine the presence of cholesterol crystals and liquid crystals. The liver was excised and weighed, and aliquots were taken for cholesterol analysis and for the preparation of microsomes. Small slices of liver were fixed in Millonig's buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin, periodic acid-Schiff, trichrome, and reticulin stains. All the procedures outlined above have been described (11). The pathologist was unaware of the previous dietary treatment of the animals from which liver specimens were obtained.

Gas–liquid chromatography and gas–liquid chromatography–mass spectrometry

Gas–liquid chromatography of cholesterol in bile, liver, and serum and of fecal neutral and acidic steroids was carried out on a Hewlett-Packard 5830 gas chromatograph as described previously (11). Biliary bile acids were analyzed as methyl ester acetates on 0.5% OV-210 and 0.5% SP-525 columns. Relative retention times on 0.5% OV-210 were: lithocholic acid, 0.47; deoxycholic acid, 1.0; chenodeoxycholic acid and hyodeoxycholic acid, 1.31; 3α,6β-dihydroxy-5β-cholanoic acid, 1.53; cholic acid and hyocholic acid, 2.47; and 3α,7α-dihydroxy-12-keto-5β-cholanoic acid, 3.47. The bile acids not separated on OV-210 were resolved on SP-525. Relative retention times: lithocholic acid, 0.72; deoxycholic acid, 1.0; chenodeoxycholic acid, 1.31; hyodeoxycholic acid and 3α,6β-dihydroxy-5β-cholanoic acid, 1.85; cholic acid, 1.66; hyocholic acid, 2.2; 3α,7α-dihydroxy-12-keto-5β-cholanoic acid, 3.09.

Positive identification of sterols and bile acids in bile and feces was carried out with a Hewlett-Packard 5992B mass spectrometer using the following conditions: 3-ft glass column, 4 mm OD, 2 mm ID, packed with 3% SP-2250 on 100/120 mesh Supelcoport; column temperature, 260°C; injector temperature, 265°C; source pressure 2×10^{-6} torr; source temperature, 140°C.

Bile acids and reference compounds

Hyodeoxycholic acid (Sigma Chemical Co., St. Louis, MO) and hyocholic acid (Canada Packers, Inc., Toronto, Canada) were analyzed as the methyl ester trimethylsilylether derivatives by GLC on an SE-30 column and were found to be better than 95% pure.

3α,6β-Dihydroxy-5β-cholanoic acid was synthesized from methyl hyodeoxycholate by oxidizing the latter with CrO_3-acetic acid at −10°C (12). The crude methyl 3α-hydroxy-6-keto-5β-cholanoate was purified by silica gel column chromatography, crystallized, and reduced with $NaBH_4$ in methanol. The reaction product consisted of a mixture of methyl hyodeoxycholate and 3α,6β-dihydroxy-5β-cholanoate in a proportion of 2 to 8. The epimeric mixture was recrystallized three times from ethyl acetate–acetone yielding chromatographically pure methyl 3α,6β-dihydroxy-5β-cholanoate (12, 13). The identity of this compound (and of the biological product, see below) was confirmed by GLC–MS of the TMS derivative. The following major fragments (m/z) were obtained: 460 [100%, M-90]; 445 [12%, M-(90 + 15)]; 405 [52%, M-(90 + ring A)]; 370 [58%, M-180]; 355 [17%, M-(180 + 15)].

3α,7α-Dihydroxy-12-keto-5β-cholanoic acid (Steraloids, Wilton, NH) was used as recovery standard for the determination of biliary and fecal bile acids.

Determination of fecal steroids

These procedures have been described previously and were validated for the prairie dog (11, 14).

Determination of cholesterol in serum and liver

Determinations were carried out as described previously (14).

Biliary lipid composition

Gallbladder bile obtained at the time of killing was centrifuged immediately at 2000 *g* for 10 min and aliquots of the supernatant solution were used for biliary lipid analysis as described previously (15). The lithogenic indices of the bile samples were calculated from Carey's tables (16); corrections for HDA were not available.

Enzyme assays

Liver microsomes were prepared in the cold room at 4°C (17). HMG-CoA reductase was measured by a modified micro procedure (17, 18). Cholesterol 7α-hydroxylase was determined as described previously (19) except that the extraction of the sterols from the incubation mixture and their separation by TLC were carried out under nitrogen. Experiments were carried out to establish optimal assay conditions with respect to incubation time, pH, protein, and co-factor concentrations. The conditions previously determined for the hamster (17) were found to be applicable to the prairie dog.

Statistical evaluation

The data are reported as mean ± SEM. Student's *t*-test or chi-square were employed to determine statistical significance (20).

RESULTS

The effects of different regimens on the occurrence of gallstones and biliary cholesterol crystals, and on liver and serum cholesterol concentrations are summarized in **Table 1.** The lithogenic diet with and without added

TABLE 1. Cholesterol concentrations and incidence of gallstones and biliary cholesterol crystals in prairie dogs

Group and Regimen	No. and Sex of Animals	Cholesterol Concentration		Incidence of	
		Serum	Liver	Gallstones	Cholesterol Crystals
		mg/dl	*mg/g wet wt.*		
I Fat-free diet	2M; 1F	172 ± 21[a]	2.7 ± 0.4[a]	0/3[a]	0/3[a]
II Semisynthetic diet[b]	6M	145 ± 16[a]	2.9 ± 0.2[a]	0/6[a]	0/6[a]
III Lithogenic diet	5M	787 ± 133	6.5 ± 0.7	5/5	5/5
IV Lithogenic diet	5F	879 ± 107	5.5 ± 0.4	5/5	5/5
V Lithogenic diet + hyodeoxycholic acid	5M	158 ± 9[a]	1.9 ± 0.2[a]	1/5[a]	1/5[a]
VI Lithogenic diet + hyodeoxycholic acid	5F	208 ± 15[a,c]	2.0 ± 0.3[a]	0/5[a]	0/5[a]

The prairie dogs received the different diets for a period of 8 weeks. Compositions of the diets are described in the Materials and Methods section. The dose of hyodeoxycholic acid was 0.1% of the diet or approximately 30 mg per kg body weight per day. The fat-free diet is not strictly comparable to the semisynthetic diet since the latter is not fat-free and contains a different sugar (sucrose) and protein (soy protein).

[a] Differs from groups on lithogenic diet (III and IV) ($P < 0.05$).

[b] This group (II) was not studied simultaneously with the other five groups (11).

[c] Differs from group V (males) ($P < 0.01$).

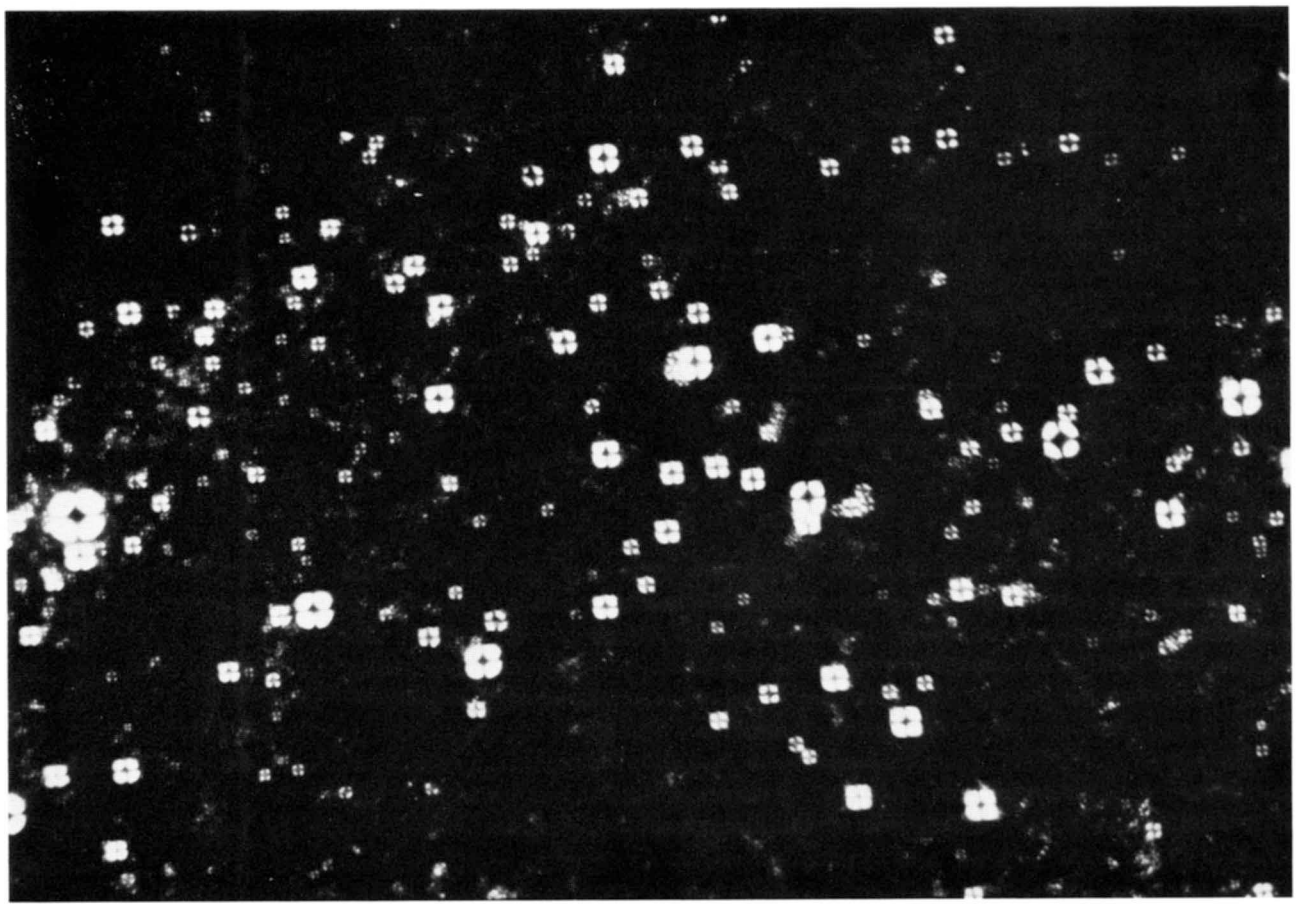

Fig. 1. Gallbladder bile of a prairie dog fed lithogenic diet plus hyodeoxycholic acid examined at autopsy by polarizing light microscopy. The presence of liquid crystals is indicated by the typical "Maltese crosses" (×100).

0.1% HDA was administered to both male and female prairie dogs in order to detect possible sex differences. None of the animals on the fat-free diet or on the low cholesterol semisynthetic diet developed gallstones, and their serum and liver cholesterol levels were similar to those of previously studied control animals on rodent

TABLE 2. Activities of rate-limiting enzymes and fecal steroid excretion in prairie dogs

Group and Regimen	No. and Sex of Animals	Hepatic HMG-CoA Reductase	Hepatic Cholesterol 7α-Hydroxylase	Fecal Steroid Excretion[a]		Fecal Output
				Sterols	Bile Acids	
		pmol/mg protein/min		*mg/g dry feces*		*g/day*
I Fat-free diet	2M; 1F	9.0 ± 9.0[b]	6.3 ± 2.0[b]	3.3 ± 0.36	1.24 ± 0.35	1.62 ± 0.27
II Semisynthetic diet	6M	50.6 ± 10.9[c]	31.1 ± 5.3	4.4 ± 0.50	1.80 ± 0.42	1.70 ± 0.20
III Lithogenic diet	5M	0[b]	42.9 ± 13.1	27.4 ± 1.7[b]	4.69 ± 1.30[d]	2.33 ± 0.68
IV Lithogenic diet	5F	0[b]	37.0 ± 8.7	23.3 ± 3.4[b]	6.30 ± 2.54[d]	1.59 ± 0.41
V Lithogenic diet + hyodeoxycholic acid	5M	280 ± 97[c,d]	9.1 ± 2.6[c]	38.7 ± 1.8[b,c]	10.47 ± 0.82[b]	2.40 ± 0.39
VI Lithogenic diet + hyodeoxycholic acid	5F	118 ± 26[c,d]	2.1 ± 0.5[c,e]	33.9 ± 4.3[b]	9.98 ± 2.81[b]	2.84 ± 0.42

The fecal steroid excretion was determined during the 7th week of the 8-week feeding period. Compositions of the diets are described in the Materials and Methods section. The dose of HDA was 0.1% of the diet or approximately 30 mg per kg body weight per day. The microsomal enzymes were assayed at the end of the 8-week experimental period.

[a] Nine animals (four males and five females) were used for fecal steroid analysis.
[b] Differs from group II, $P < 0.01$.
[c] Differs from group III, $P < 0.01$.
[d] Differs from group II, $P < 0.05$.
[e] Differs from group V, $P < 0.01$.

TABLE 3. Effect of diet and hyodeoxycholic acid on biliary lipid concentrations in prairie dogs

Group and Regimen	No. and Sex of Animals	Biliary Total Lipid Conc.	Biliary Lipids			Lithogenic Index[a]
			Cholesterol	Phospholipids	Bile Acids	
		g/dl		*mol percent*		
I Fat-free diet	2M; 1F	6.2 ± 2.0[b]	3.4 ± 0.3[b]	9.6 ± 0.8	87.1 ± 1.0	0.94 ± 0.13[c]
II Semisynthetic diet	6M	2.0 ± 0.4	1.4 ± 0.7	7.6 ± 0.4	91.0 ± 0.7	0.42 ± 0.12
III Lithogenic diet	5M	4.8 ± 1.8	5.5 ± 1.8[c]	7.5 ± 2.9	87.0 ± 4.6	1.36 ± 0.20[c]
IV Lithogenic diet	5F	3.1 ± 0.9	5.0 ± 0.7[c]	10.5 ± 2.8	84.6 ± 3.4	1.65 ± 0.22[c]
V Lithogenic diet + hyodeoxycholic acid	5M	5.2 ± 0.9[b]	9.4 ± 3.2[c]	12.4 ± 1.8	78.2 ± 4.9	1.95 ± 0.42[c]
VI Lithogenic diet + hyodeoxycholic acid	5F	7.7 ± 0.4[b]	10.9 ± 1.4[c,d]	10.3 ± 0.7	78.8 ± 2.4	1.55 ± 0.30[c]

[a] Calculated from the critical tables of Carey (16).
[b] Differs from group II, $P < 0.05$.
[c] Differs from group II, $P < 0.01$.
[d] Differs from group IV, $P < 0.01$.

chow (serum cholesterol, 187 mg/dl; liver cholesterol, 2.7 mg/g wet weight). In contrast, all ten animals, male or female, receiving the high (0.35%) cholesterol, lithogenic diet (groups III and IV) developed biliary cholesterol crystals and gallstones. As observed previously, administration of the lithogenic diet led to large increases in serum and liver cholesterol concentrations (11). The incorporation of 0.1% HDA into the lithogenic diet, equivalent to a bile acid intake of 30 mg/kg body weight per day, prevented the development of cholesterol gallstones in nine of the ten animals tested (groups V and VI). One of the male animals in group V had cholesterol crystals in gallbladder bile and a single small stone was detected. The gallbladder biles of all other animals in groups V and VI contained numerous liquid crystal spherulites as indicated by the presence of typical "Maltese crosses" under the polarizing microscope (**Fig. 1**). The biles of all animals in this group were a darker green and appeared cloudy in comparison with the controls fed the lithogenic diet. The administration of HDA prevented the elevations of serum and liver cholesterol usually seen in prairie dogs on high cholesterol intakes (11). In the males (group V) bile acid feeding resulted in significantly lower serum cholesterol concentrations than in the females (group VI).

The data summarized in **Table 2** present an attempt to find possible correlations between the activities of the rate-limiting enzymes of cholesterol/bile acid biosynthesis, namely, HMG-CoA reductase and cholesterol 7α-hydroxylase, and fecal steroid output. Administration of the fat-free diet and of the high cholesterol lithogenic diet resulted in very low reductase activities. The increased fecal neutral sterol output on the lithogenic diet probably reflects the effect of the administered cholesterol. Unexpectedly, in the groups fed lithogenic diet plus HDA, HMG-CoA reductase activity was near normal

TABLE 4. Effect of diet and hyodeoxycholic acid on biliary bile acid composition of prairie dogs

Group and Regimen	No. and Sex of Animals	LA	DA	CDA	HDA	6β-HDA	CA	HCA
I Fat-free diet	2M; 1F	0.1 ± 0.1	2.0 ± 1.2	29.1 ± 9.5			68.8 ± 8.4	
II Semisynthetic diet	6M	0.3 ± 0.2	5.1 ± 0.9	15.3 ± 1.6			79.3 ± 3.6	
III Lithogenic diet	5M	0.3 ± 0.1	0.9 ± 0.4	41.9 ± 2.4[a]			57.0 ± 2.6[a]	
IV Lithogenic diet	5F	0.9 ± 0.6	2.2 ± 1.3	45.5 ± 8.8[a]			51.5 ± 7.8[a]	
V Lithogenic diet + hyodeoxycholic acid	5M		0.7 ± 0.2	4.3 ± 0.7[a,b]	44.4 ± 6.5	26.9 ± 6.3	17.9 ± 4.6[a,b]	5.9 ± 1.8
VI Lithogenic diet + hyodeoxycholic acid	5F		2.1 ± 0.3	5.2 ± 0.9[a,b]	30.3 ± 1.3[c]	20.5 ± 1.7	41.1 ± 1.9[a,c]	0.8 ± 0.7[c]

Prairie dogs were maintained with the pertinent diets for 8 weeks. Compositions of the diets are described in the Materials and Methods section. The dose of HDA was 0.1% of the diet or approximately 30 mg/kg body weight per day. Bile acid composition was determined by analyzing two aliquots of a given bile sample on two different GLC systems. The following abbreviations are used: LA, lithocholic acid; DA, deoxycholic acid; CDA, chenodeoxycholic acid; HDA, hyodeoxycholic acid; 6β-HDA, 3α,6β-dihydroxy-5β-cholanoic acid; CA, cholic acid; HCA, hyocholic acid.
[a] Differs from group II, $P < 0.05$.
[b] Differs from groups III and IV, $P < 0.01$.
[c] Differs from group V, $P < 0.05$.

TABLE 5. Semiquantitative assessment of liver pathology

Group and Regimen	Numbers of Prairie Dogs by Category																				
	Parenchymal Lipid					Portal Fibrosis					Bile Duct Proliferation										
	0	±	+	++	+++	++++	0	±	+	++	+++	++++	0	±	+	++	+++	++++
II Semisynthetic diet (M)[a]	4	2					4	2					4	2				
III Lithogenic diet (M)		3		2					1	2	2				1	2	2	
IV Lithogenic diet (F)		2	1	2						2	1	2				2	1	2
V Lithogenic diet + hyodeoxycholic acid (M)		3	1	1				4	1					4	1			
VI Lithogenic diet + hyodeoxycholic acid (F)		5						4	1					3	2			

The experiments were carried out as detailed in Table 1. The pathological changes range from 0 = no change to ++++ = severe involvement.
[a] Sex of animals in parentheses.

levels in the males, 280 pmol/mg protein per min (chow controls had an activity of 237 pmol/mg protein per min), and approximately 50% of normal in the females. Because of large experimental variations, the difference between the male and female prairie dogs was not significant (group V vs. group VI, $P = 0.1$). These two groups had a considerably higher fecal sterol output than the corresponding control groups (III and IV), but only the difference among the males was significant.

It is known from previous studies (11) that in the prairie dog (as in the rat) cholesterol feeding stimulates cholesterol 7α-hydroxylase activity and bile acid output. Similar data were obtained in the present experiments, but only the increase in bile acid output of the males on lithogenic diet (group III) was significant.

The animals on the fat-free (cholesterol-free) diet had very low cholesterol 7α-hydroxylase activities, 6.3 pmol/mg protein per min, compared to the animals on the semisynthetic diet which averaged 31.1 units (controls on rodent chow averaged 32 units). However, the differences in fecal bile acid output and enzyme activity between groups I and II were not significant. It is evident that adult prairie dogs maintained with semisynthetic diets have low daily bile acid productions, 2–2.5 mg per day for animals weighing about 1 kg. In contrast to our previous results with CDA and UDA which exerted relatively small inhibitory effects on cholesterol 7α-hydroxylase (11), HDA feeding strongly inhibited the enzyme activity in both sexes. This effect was most pronounced in the females. Since the bile acid pool of the animals was not labeled, the presumed decrease in bile acid production could not be measured. The HDA-fed prairie dogs excreted approximately the amount of bile acid expected from the administered dose.

The data summarized in **Table 3** illustrate the effect of the different diets on biliary lipid composition and the lithogenic index of bile. As compared to the animals of group II (semisynthetic, low cholesterol diet) all groups exhibited increased proportions of cholesterol in biliary lipids. The group of prairie dogs on the fat-free diet (group I) had a lithogenic index close to unity, but the gallbladder biles contained neither cholesterol crystals nor gallstones (Table 1). Although nine of the ten animals treated with HDA had no gallstones, they developed the greatest concentration of cholesterol in biliary lipids and their biles were highly lithogenic. In the females the biliary cholesterol content of the bile acid-treated groups was significantly higher than those of the controls receiving lithogenic diet alone (group IV vs. group VI).

The biliary bile acid compositions of the animals are listed in **Table 4** and confirm two observations made previously (11). First, prairie dog bile has low levels of secondary bile acids and, second, the CA/CDA ratio is lowered in cholesterol-fed animals. Hyodeoxycholic acid

was evidently absorbed by the prairie dog and was present in bile exclusively as the taurine conjugate (44% of total biliary bile acids in the males, 30.3% in females ($P < 0.01$)). It was further observed that considerable proportions of the administered HDA were transformed into 3α,6β-dihydroxy-5β-cholanoic acid (21–27%) so that HDA and its metabolite were the predominant biliary bile acids.

In the males some 7α-hydroxylation of HDA took place since the bile contained 5.9% hyocholic acid. In the biles of both males and females fed HDA, CA was present in relatively large concentrations. The latter was the most abundant bile acid in the females of group VI (41.1% of total biliary bile acids).

In all except one of the ten prairie dogs fed the lithogenic diet, histologic evaluation of the livers revealed moderate to marked bile duct proliferation, together with portal fibrosis and inflammatory infiltration (**Table 5**). This effect was somewhat more prominent in the females. The livers also displayed a mild to moderate amount of micro- and macrovacuolar lipid accumulation in the parenchyma (**Fig. 2**). Bile stasis was not evident by histologic methods in any of these animals. All of the portal tract changes were markedly reduced by the addition of HDA to the lithogenic diet (**Fig. 3**), although the parenchymal steatosis was not much altered (Table 5). In many of these prairie dogs, the portal tracts were virtually normal (**Fig. 4**).

DISCUSSION

Dam's hamster model of cholesterol cholelithiasis and the prairie dog model employed in the present investigation differ in several respects. In the hamster model, the fat-free diet proves very debilitating in the young growing animals, and the number of animals surviving until the end of the experimental period is frequently disappointingly small (21, 22). The fat-free diet strongly stimulates cholesterol biosynthesis in liver and extrahepatic tissues, although it has not been proved that the newly synthesized sterol molecules enter the bile and render it lithogenic (5). The biliary bile acids are predominantly conjugated with glycine.

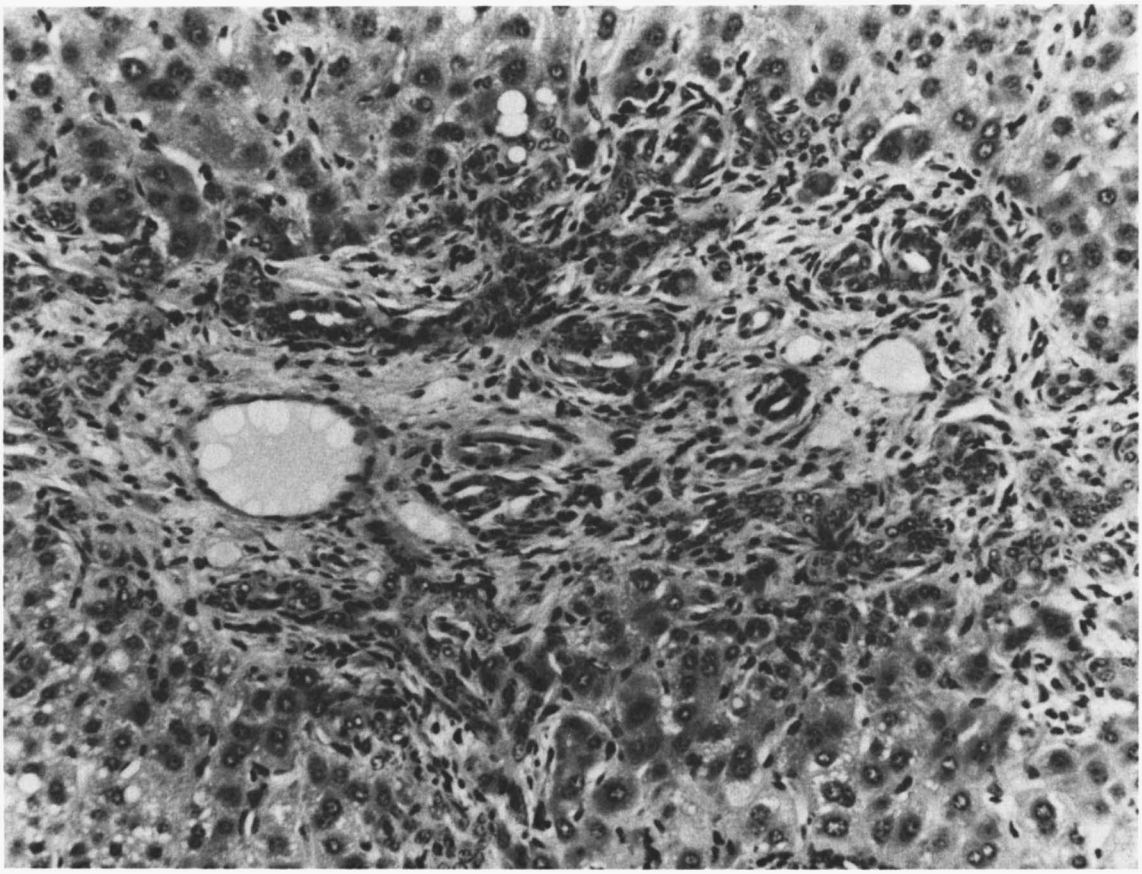

Fig. 2. Liver from a prairie dog fed lithogenic diet. This microscopic field displays an expanded portal tract with evidence of moderate bile duct proliferation, inflammatory infiltration, and reactive fibrosis. The contiguous parenchyma reveals regenerative features and scattered cytoplasmic lipid vacuoles (H&E stain, ×175).

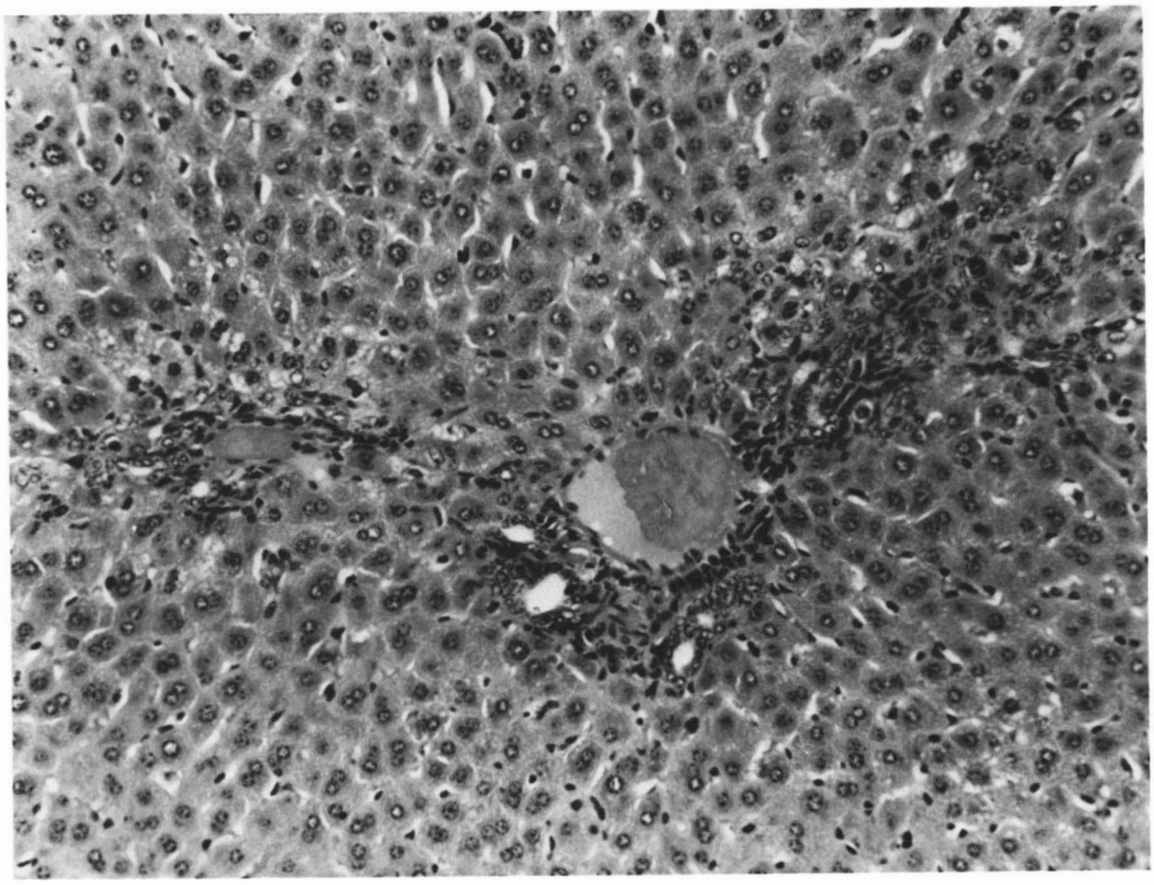

Fig. 3. Liver from a prairie dog fed lithogenic diet supplemented with HDA. The portal tract displays mild bile duct proliferation, a modest inflammatory reaction, and minimal fibrosis. The parenchyma reveals minimal microvacuolar steatosis (H&E stain, ×175).

In the prairie dog model, the lithogenic stimulus is in the form of excess dietary cholesterol administered in a semisynthetic diet that contains adequate amounts of essential fatty acids. The animals tolerate this diet without apparent ill effects and, as opposed to the hamster model, young animals gain weight throughout the experimental period. It is not known whether the semisynthetic diet employed in prairie dog studies is essential for the production of gallstones. The biliary bile acids are exclusively present as taurine conjugates.

Bile acid metabolism in the two species is similar, although daily synthesis in the hamster is about 10 times greater than in the prairie dog (23). However, bile acid composition is similar, with CA predominating and the CA/CDA ratio ranging from 3:1 to 5:1 (11, 21). In both species, secondary bile acids are present in very low proportions, presumably because the animals possess active hepatic bile acid 7α-hydroxylases (24, 25). Interestingly, the effect of HDA feeding is identical in both models. Administration of this bile acid in the lithogenic diet prevents the induced cholelithiasis yet increases the mol % cholesterol in biliary lipids. In both species, hepatic HMG-CoA reductase activity was greatly stimulated by dietary HDA (21).

The mechanism whereby dietary HDA prevents the formation of gallstones appears to be identical in the two models. Studies in the hamster by Wheeler (8) and in this laboratory (21) indicate that HDA does not prevent cholelithiasis by inhibiting biliary cholesterol synthesis or secretion. Indeed, it has been observed in acute experiments that this bile acid and its glycine conjugate tend to render the bile more supersaturated with cholesterol than the lithogenic diet alone.[2] In the present study, the biles of the groups treated with HDA were more lithogenic than those of animals on the lithogenic diet alone, although the differences were not always significant. In the hamster model, the increase in biliary cholesterol saturation during the administration of HDA and other hydrophilic bile acids (e.g., UDA) was much more pronounced, but only in the case of HDA was this associated

[2] Cohen, B. I., A. K. Singhal, C. K. McSherry, M. A. Rothschild, and E. H. Mosbach. Unpublished observations.

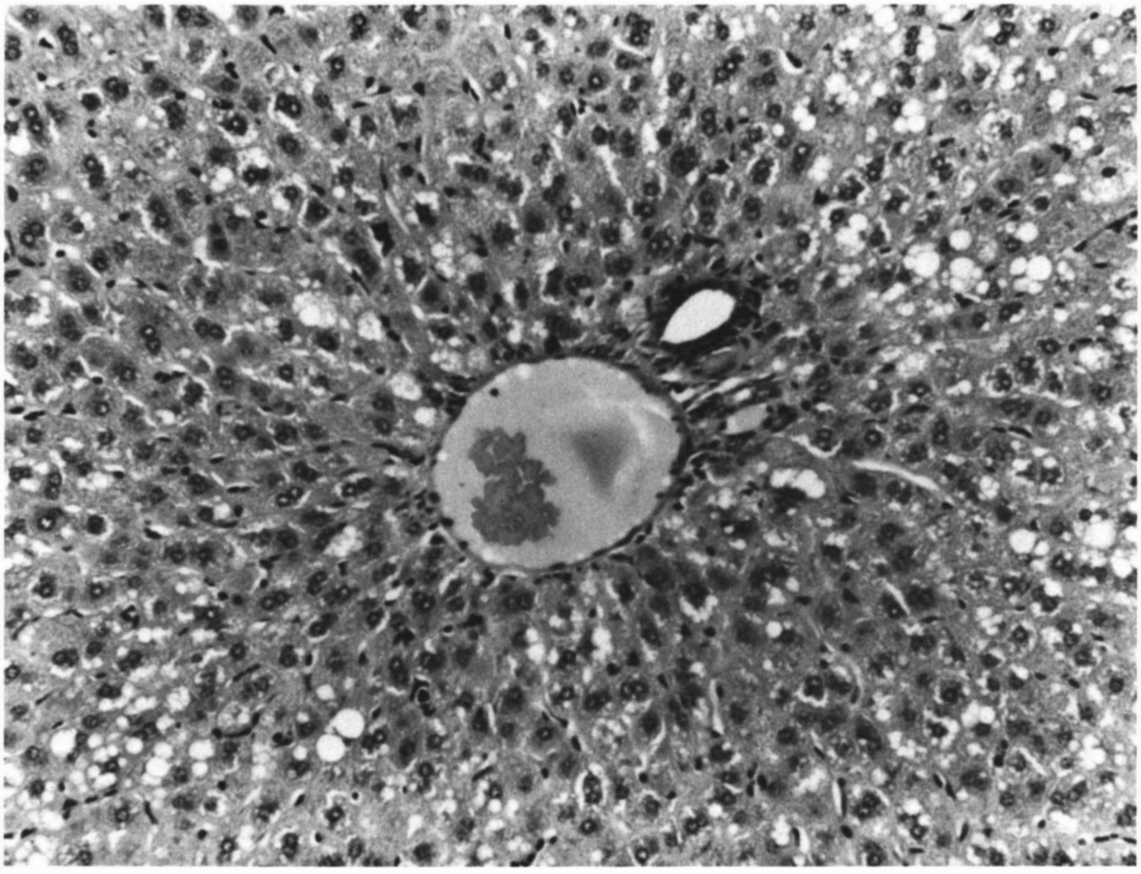

Fig. 4. Liver from a prairie dog fed lithogenic diet supplemented with HDA. The portal tract is essentially normal, and the contiguous parenchyma exhibits a spotty micro- and macrovacuolar steatosis (H&E stain, ×175).

with a 50% increase of hepatic microsomal HMG-CoA reductase activity (21).

In the prairie dog model the administered bile acid, HDA, might have been expected to prevent the cholesterol-induced cholelithiasis since it apparently interfered with the absorption of dietary cholesterol. This is suggested, though not proved, by the finding that increases in serum and liver cholesterol were prevented by 0.1% dietary HDA. In addition, the inhibition of hepatic HMG-CoA reductase activity observed in the animals on the lithogenic diet alone was prevented in the two groups receiving the dietary bile acid. It is possible that the increase of HMG-CoA reductase activity, together with the decrease of cholesterol 7α-hydroxylase observed in these groups rather than cholesterol feeding was either the primary or an additional stimulus leading to supersaturation of the bile with cholesterol.

In any case, the absence of cholesterol crystals and gallstones in the gallbladders of nine out of ten animals studied, in the presence of highly saturated bile, suggests that the administered bile acid prevented the transition of cholesterol from the liquid crystalline phase to crystalline cholesterol monohydrate. Therefore, like other hydrophilic bile acids studied in vitro, HDA acted as an antinucleating agent in the hamster and prairie dog models. Salvioli et al. (9) have predicted on the basis of studies in vitro that the administration of HDA and other hydrophilic bile acids should dissolve existing gallstones, and this is now under investigation.

In the prairie dog model it is conceivable that hyodeoxycholic acid is not the sole active agent. The bile of the animals fed this bile acid contained appreciable concentrations of the administered hyodeoxycholic acid, as well as 3α,6β-dihydroxy-5β-cholanoic acid and hyocholic acid. The 6β-hydroxy acid was probably formed by bacterial oxidation of HDA to 3α-hydroxy-6-keto-5β-cholanoic acid followed by hepatic or bacterial 6β-reduction. Hyocholic acid was presumably produced by the hepatic 7α-hydroxylation of the administered HDA (24).

These studies have demonstrated that a high incidence of gallstones can be produced in both male and female prairie dogs fed a semisynthetic diet containing 0.35% cholesterol for 8 weeks. There was no pronounced sex difference either in the production of gallstones or the

prevention by dietary (0.1%) HDA. The bile of prairie dogs fed a high glucose, fat-free diet similar to the diet used in Dam's hamster model had a significantly higher lithogenic index (0.94) than controls fed a low-cholesterol diet, but no gallstones were found. In the prairie dog, the fat-free diet appeared to reduce hepatic HMG-CoA reductase activity to very low levels (as previously observed in the rat (5)). It is possible, however, that if the fat-free diet had been administered to younger, rapidly growing animals (as in the hamster model of Dam), an essential fatty acid deficiency might have been produced leading to increased enzyme activity and, perhaps, to increased cholesterol saturation of the bile.

The presence of portal tract changes, including bile duct proliferation, inflammatory infiltration, and fibrosis, confirms our previous observations on the effects of this lithogenic diet in prairie dogs (11). This experimental model has proved to be both reliable and reproducible. The striking amelioration of the portal tract pathology due to the addition of HDA to the lithogenic diet clearly indicates that this experimental model can serve as a useful index of the effects of various agents upon the development of liver pathology. The model also affords an excellent opportunity to determine the mechanisms by which various agents might effect a beneficial result.

Our data suggest that the prairie dog is a useful model for the testing of cholelitholytic agents. This species not only responds to the known gallstone-dissolving bile acids, CDA and UDA, but also exhibits in vivo the typical effects of a hydrophilic cholanoic acid, namely, HDA, predicted from in vitro experiments. It will be of interest to find out whether HDA and other hydrophilic bile acids are useful not only in the prevention of gallstones but also in their dissolution.

This work was supported in part by USPHS grant HL-24061 from the National Heart, Lung, and Blood Institute.

Manuscript received 27 October 1983.

REFERENCES

1. Dam, H., and F. Christensen. 1952. Alimentary production of gallstones in hamsters. *Acta Pathol. Microbiol. Scand.* **30:** 236–242.
2. Brenneman, D. E., W. E. Connor, E. L. Forker, and L. DenBesten. 1972. The formation of abnormal bile and cholesterol gallstones from dietary cholesterol in the prairie dog. *J. Clin. Invest.* **51:** 1495–1503.
3. Jensen, B., and H. Dam. 1966. Cholesterol metabolism in hamsters reared on diets with different effects on gallstone formation. 1966. *Biochim. Biophys. Acta.* **125:** 367–374.
4. Muroya, H., S. Ryokuero, and Y. Hikasa. 1968. Stimulation of hepatic cholesterol biosynthesis by dietary glucose, and its relation to cholesterol gallstone formation in the hamster. *Arch. Biochem. Biophys.* **124:** 12–17.
5. Turley, S. D., D. K. Spady, and J. M. Dietschy. 1983. Alteration of the degree of biliary cholesterol saturation in the hamster and rat by manipulation of the pools of preformed and newly synthesized cholesterol. *Gastroenterology.* **84:** 253–264.
6. Prange, I., E. Søndergaard, and H. Dam. 1973. Alimentary production of gallstones in hamsters. 26. The influence of orally ingested lithocholic, cholic, dehydrocholic and deoxycholic acid on gallstone production compared with the influence of chenodeoxycholic acid. *Z. Ernährungswiss.* **12:** 92–107.
7. Dam, H., I. Prange, and E. Søndergaard. 1972. Alimentary production of gallstones in hamsters. 24. Influence of orally ingested chenodeoxycholic and hyodeoxycholic acid on formation of gallstones. *Z. Ernährungswiss.* **11:** 80–94.
8. Wheeler, H. O. 1973. Biliary excretion of bile acids, lecithin, and cholesterol in hamsters with gallstones. *Gastroenterology.* **65:** 92–103.
9. Salvioli, G., H. Igimi, and M. C. Carey. 1983. Cholesterol gallstone dissolution in bile. Dissolution kinetics of crystalline cholesterol monohydrate by conjugated chenodeoxycholate-lecithin and conjugated ursodeoxycholate-lecithin mixtures: dissimilar phase equilibria and dissolution mechanisms. *J. Lipid Res.* **24:** 701–720.
10. Doty, J. E., L. DenBesten, J. J. Roslyn, H. A. Pitt, S. L. Kuchenbecker, and V. Porter-Fink. 1982. Interaction of chenodeoxycholic acid and dietary cholesterol in treatment of cholesterol gallstones. *Am. J. Surg.* **143:** 48–54.
11. Cohen, B. I., A. K. Singhal, R. J. Stenger, P. May-Donath, J. Finver-Sadowsky, C. K. McSherry, and E. H. Mosbach. 1984. Effects of chenodeoxycholic acid and ursodeoxycholic acid on lipid metabolism and gallstone formation in the prairie dog. *Hepatology.* **4:** 300–307.
12. Hoehn, W. H., J. Linsk, and R. B. Moffett. 1946. 3-Keto 6(β) hydroxycholanic acid and 3(α) hydroxy-6-keto cholanic acid. *J. Am. Chem. Soc.* **68:** 1855–1857.
13. Ratliff, R. L., J. T. Matschiner, E. A. Doisy, Jr., S. L. Hsia, S. A. Thayer, W. H. Elliott, and E. A. Doisy. 1961. Partial synthesis of the new metabolite of deoxycholic acid, 3α,6β,12α-trihydroxy cholanic acid. *J. Biol. Chem.* **236:** 685–687.
14. Cohen, B. I., R. F. Raicht, and E. H. Mosbach. 1974. Effect of dietary bile acids, cholesterol and β-sitosterol upon formation of coprostanol and 7-dehydroxylation of bile acids by rats. *Lipids.* **9:** 1024–1029.
15. McSherry, C. K., J. E. Deitrick, P. S. May, W. Niemann, K. P. Morrissey, R. H. Palmer, and F. Glenn. 1977. Biliary lipid metabolism in the pregnant baboon. *Surg. Gynecol. Obstet.* **144:** 727–733.
16. Carey, M. C. 1978. Critical tables for calculating the cholesterol saturation of native bile. *J. Lipid Res.* **19:** 945–955.
17. Singhal, A. K., J. Finver-Sadowsky, C. K. McSherry, and E. H. Mosbach. 1983. Effect of cholesterol and bile acids on the regulation of cholesterol metabolism in hamster. *Biochim. Biophys. Acta.* **752:** 214–222.
18. Shapiro, D. J., J. L. Nordstrom, J. J. Mitschelen, V. W. Rodwell, and R. T. Schimke. 1974. Micro assay for 3-hydroxy-3-methylglutaryl-CoA reductase in rat liver and in L-cell fibroblasts. *Biochim. Biophys. Acta.* **370:** 369–377.
19. Shefer, S., S. Hauser, and E. H. Mosbach. 1968. 7α-Hydroxylation of cholestanol by rat liver microsomes. *J. Lipid Res.* **9:** 328–333.

20. Snedecor, G. W., and W. G. Cochran. 1946. Statistical Methods. 4th ed. Iowa State University Press, Ames, IA. 56–81; 182–184.
21. Singhal, A. K., B. I. Cohen, J. Finver-Sadowsky, C. K. McSherry, and E. H. Mosbach. 1984. Role of hydrophilic bile acids and of sterols on cholelithiasis in the hamster. *J. Lipid Res.* **25:** 564–570.
22. Dam, H., and F. Christensen. 1962. Alimentary production of gallstones in hamsters. 10. The effect of orally ingested bile acids on development of cholesterol gallstones in hamsters fed a fat-free diet with glucose as carbohydrate component. *Z. Ernährungswiss.* **2:** 154–159.
23. Singhal, A. K., N. K. N. Ayengar, P. S. May, C. K. McSherry, and E. H. Mosbach. 1983. Cholesterol metabolism in two strains of hamsters. *In* Dietary Fats and Health. E. G. Perkins and W. J. Visek, editors. American Oil Chemists' Society, Champaign, IL. 267–277.
24. Cohen, B. I., A. K. Singhal, J. Mongelli, M. A. Rothschild, C. K. McSherry, and E. H. Mosbach. 1983. Hydroxylation of secondary bile acids in the perfused prairie dog liver. *Lipids.* **18:** 909–912.
25. Emerman, S., and N. B. Javitt. 1967. Metabolism of taurolithocholic acid in the hamster. *J. Biol. Chem.* **242:** 661–664.