Experimental cholelithiasis in the rabbit induced by cholestanol feeding: effect of neomycin treatment on bile composition and gallstone formation

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ABSTRACT   Fed cholestanol is converted by the rabbit to 5α-bile acids which coprecipitate with the normally occurring 5β-bile acids to form gallstones composed of calcium and sodium glycoallodeoxycholate and glycodeoxycholate. The present study shows that oral administration of large doses of neomycin prevents gallstone formation in the cholestanol-fed rabbit and reduces the elevated concentration of allodeoxycholic acid in bile, with a reciprocal increase in allocholic acid concentration. The reduction in the concentration of allodeoxycholic acid and in the incidence of gallstones is proportional to the dose of neomycin; at a concentration of allodeoxycholic acid below about 20% of total bile acids, gallstone formation does not occur. Neomycin probably exerts its action by modifying the anerobic intestinal flora which dehydroxylate allocholic acid to allodeoxycholic acid; if so, this suggests that both hepatic and bacterial transformations are essential steps in the pathogenesis of cholestanol-induced cholelithiasis.

The bile of rabbits on a normal diet contains allodeoxycholic acid (5% of total bile acids). A similar decrease in allodeoxycholic acid concentration and reciprocal increase in allocholic acid concentration is observed when neomycin is administered to rabbits on a normal diet.

KEY WORDS   5α-cholestan-3β-ol metabolism · rabbit · 3α,12α-dihydroxy-5α-cholanoic acid · 3α,7α,12α-dihydroxy-5α-cholanoic acid · experimental cholelithiasis · gallstones · 7α-dehydroxylation · bile composition · bile acids · in vivo · intestinal microorganisms · neomycin

Iɴ ᴛʜᴇ ᴄᴏɴᴠᴇʀsɪᴏɴ ᴏꜰ ᴄʜᴏʟᴇsᴛᴇʀᴏʟ to bile acids, the $\Delta^{5,6}$-double bond of cholesterol is saturated stereospecifically: mammalian bile acids are 5β-steroids, possessing a *cis* A/B ring juncture (1). Cholestanol, the saturated 5α-, A/B *trans*, homologue of cholesterol is well absorbed by rabbits and, like cholesterol, is converted to bile acids. The bile acids so formed are 5α-, A/B *trans* or "allo" bile acids (2).

Formation of substantial amounts of these abnormal 5α-bile acids in the cholestanol-fed rabbit is associated with the occurrence of crystalline gallstones composed largely of sodium and calcium salts of glycine-conjugated dihydroxy bile acids. An important component of these stones is allodeoxycholic acid, the 5α-epimer of deoxycholic acid (2). Sodium glycoallodeoxycholate (5α) in solution is more easily precipitated by calcium ions than is sodium glycodeoxycholate (5β), and the difference in solubility properties of these two geometrical isomers

affords a possible explanation for the formation of gall-stones (3).

Lindstedt demonstrated that deoxycholic acid is not a primary bile acid, i.e. derived from cholesterol in the liver, but is formed by the bacterial dehydroxylation of cholic acid in the intestinal lumen (4, 5). Other studies have shown that not only deoxycholic acid, but a great variety of bile acids are formed by bacterial action on the hydroxylic substituents of the primary bile acids (6–8); and that some of these secondary bile acids are absorbed via the portal system and excreted in the bile. During their passage through the liver, secondary bile acids may be further metabolized either to the primary bile acids from which they originated or to still other cholanoic acids (9, 10). The important concept has thus emerged that the bile acids found in bile consist of a mixture of primary and secondary bile acids that reflects a complex series of hepatic and bacterial transformations.

Allodeoxycholate, the major bile acid constituent of the gallstones formed after the administration of choles-tanol (2), offered a clue to the pathogenesis of choles-tanol-induced cholelithiasis (Fig. 1). It was proposed that administered cholestanol is converted in the liver to the primary bile acid, allocholic acid, which is excreted in the bile as glycoallocholate. In the large intestine, bac-terial 7α-dehydroxylation and hydrolysis of the peptide bond produce allodeoxycholate, which is absorbed, conjugated with glycine, and excreted in the bile as glycoallodeoxycholate, which, in turn, is precipitated by calcium ions in the gallbladder to produce cholelithiasis. If this hypothesis were correct, bacterial dehydroxyla-tion of allocholate would be an essential step in the pathogenesis of cholelithiasis. Absence of dehydroxyla-tion in the germ-free animal or its suppression by treat-ment with antibiotic should cause a decreased rate of allodeoxycholate formation leading to lower concentra-tions of allodeoxycholate in bile and subsequent preven-tion of cholelithiasis. Since germ-free rabbits were not available, we elected to attempt to reduce bacterial activity by antibiotic treatment; initial experiments showed that neomycin prevented cholelithiasis (11). This paper describes the effect of neomycin on bile com-position and gallstone formation in cholestanol-fed rabbits.

METHODS

Experimental Design

Male and female rabbits under 1 yr old were housed in individual cages and fed Purina Rabbit Chow pellets.
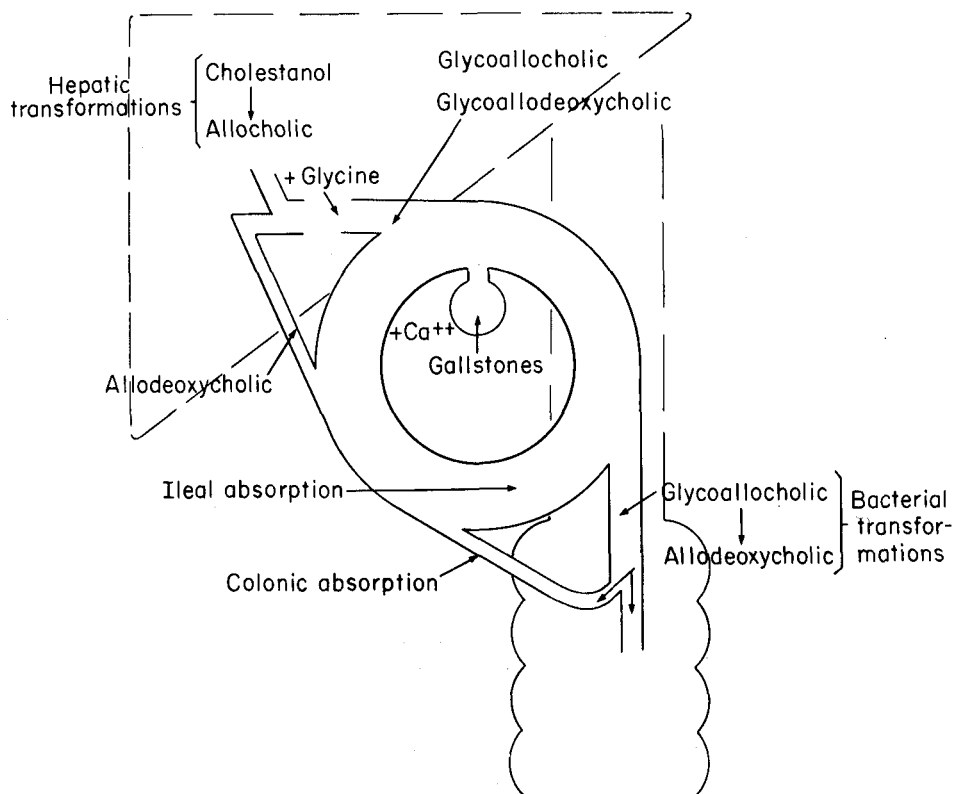

FIG. 1. The pathogenesis of cholestanol-induced cholelithiasis in the rabbit. 5β-bile acids, which pre-dominate in rabbit bile, undergo the analogous hepatic and bacterial transformations, but do not precipitate in the gallbladder unless a high allodeoxycholate concentration is present.

Six experimental groups (Table 1) were studied: I, control animals (stock diet; no antibiotic treatment), n (number of animals) = 7; II, animals receiving stock diet and given neomycin orally, 0.5 g/day, n = 8; III, animals receiving stock diet containing 1% by weight of cholestanol and no antibiotic, n = 18; and IV, animals receiving stock diet with 1% cholestanol and given 0.5 g/day of neomycin orally, n = 6. These groups are referred to, respectively, as control group (I), neomycin group (II), cholestanol group (III), and cholestanol plus neomycin group (IV). In group IV, four rabbits were pair-fed with animals in group III.

In a second set of experiments, two additional groups of cholestanol-fed rabbits were studied. In the first, the effect of neomycin dosage was examined; this group (V), which received 10–125 mg/day of neomycin in its drinking water, is termed the cholestanol plus neomycin dose-response group, n = 11. In the second group, neomycin was administered intramuscularly, 0.05 g/day. Assuming that the rabbit, like man, absorbs at most 3% of an oral dose of neomycin (12, 13), injection of 0.05 g would presumably result in higher plasma neomycin concentrations than the largest oral dose (0.5 g/day). This group is termed the cholestanol plus parenteral neomycin group (VI), n = 5.

Animals

Weights were recorded weekly, and blood samples were obtained at the beginning of the experiment and at the end of 1, 2, and 4 wk in randomly selected animals of the cholestanol group, cholestanol plus neomycin dose-response group, and the cholestanol plus parenteral neomycin group. Blood samples of the control group were obtained at the end of 4 wk. Bacteriological examinations were carried out on fecal specimens collected at the start of the experiment, at 2 wk, and at 4 wk. The

number of viable coliform organisms per g of dry feces was assayed by colony counts on eosin–methylene blue plates and by the five-tube lactose broth method (14). At the end of 4 wk the animals were killed by intravenous injection of sodium pentobarbital and autopsies were performed. Bile, gallstones, liver, and blood samples were taken for analysis.

Analytical Procedures

Bile was deproteinized with hot ethanol and filtered, and the filtrate was evaporated to dryness. The residue was hydrolyzed in 2 N NaOH and the liberated bile acids were extracted and esterified with methanol (2). The gallstones were hydrolyzed in 2 N NaOH, and then worked up like the bile samples. The trimethylsilyl (TMS) ethers of the methyl esters were prepared, and the bile acids were measured by GLC employing Hi-Eff 8B (Applied Science Laboratories Inc., State College, Pa.) as the liquid phase and a flame ionization detector (15). The TMS ethers had the following retention values relative to 5α-cholestane (mean $\pm$ sD): methyl allocholate, 1.60 $\pm$ 0.01, n (number of samples) = 43; methyl cholate, 1.95 $\pm$ 0.03 (n = 51); methyl allodeoxycholate, 2.28 $\pm$ 0.05 (n = 48); and methyl deoxycholate, 3.02 $\pm$ 0.07 (n = 53). The response of the flame detector was shown to be linear with respect to mass of sample applied (15).

Reference Compounds. Methyl allocholate was isolated from the hydrolyzed gallstones by adsorption chromatography of the methyl esters on columns of Woelm alumina, activity grade III (Alupharm Chemicals, New Orleans, La.) (2, 6). When the columns were eluted with a linearly increasing concentration of absolute ethanol in acetone, methyl cholate was eluted first, followed by methyl allocholate. The isolated methyl allocholate had a mobility on TLC and an IR spectrum identical with those of an authentic sample kindly furnished by Professor G. A. D. Haslewood (16). Methyl allodeoxycholate was prepared as described previously (2). Pure cholic and deoxycholic acids were gifts from the Ames Co., Inc., Elkhart, Ind.

Thin-Layer Chromatography. The identity of other bile acid methyl esters present in bile or gallstones was confirmed by TLC (17, 18). In some samples, especially from neomycin-treated animals, unidentified bile acids were observed. These unknown compounds had TLC and GLC characteristics corresponding to dihydroxy-monoketo-cholanoic acids. Since they consistently amounted to less than 10% of the known bile acids, they were not included in the calculations of bile and gallstone composition.

Sterols. Serum cholesterol was determined by the method of Abell, Levy, Brodie, and Kendall (19) and serum cholestanol, as described by Chattopadhyay and

TABLE 1 EXPERIMENTAL DESIGN

Group	No. of Rabbits	Dietary Addition	Dosage of Neomycin*
			g/day
I	7	None	None
II	8	None	0.5 (s.t.)
III	14	1% Cholestanol	None
IV	6	1% Cholestanol	0.5 (s.t.)
V	3	1% Cholestanol	0.01 (d.w.)
	4	1% Cholestanol	0.05 (d.w.)
	4	1% Cholestanol	0.125 (d.w.)
VI	5	1% Cholestanol	0.05 (i.m.)†

Animals were maintained on the experimental regimen for 4 weeks.

* Neomycin sulfate dissolved in drinking water (d.w.) for all doses except 0.5 g. Since most rabbits refused drinking water containing this amount of neomycin, the antibiotic was given in aqueous solution (0.5 g/ml) by stomach tube (s.t.) as indicated.

† Neomycin administered by intramuscular injection.

Mosbach (20). Liver total sterols were determined gravimetrically after formation of the digitonide (21). The digitonides were cleaved with pyridine and the liberated sterols (cholesterol and cholestanol) were assayed as described (20).

Calcium and Magnesium. Analyses were performed on the aqueous phases remaining after saponification, acidification, and solvent extraction of the hydrolyzed bile acids. The aqueous phase was evaporated to dryness and the residue was suspended in water, which was then adjusted to pH 8 with sodium hydroxide. The samples were placed in a boiling water bath to insure complete dissolution, and further diluted with 0.1 N NaOH to an appropriate calcium ion concentration. After centrifugation, the supernatant was analyzed for Ca^{++} content by a chelatometric method (22). To insure that no interfering substances were present, we determined the recovery of an added standard. The chelatometric method was also compared with a flame-spectrophotometric procedure (23) and good agreement between the two methods was obtained. Calcium contents were expressed as equivalents of Ca^{++} per equivalent of glycine-conjugated bile acid. Magnesium content was determined by flame spectrophotometry (24).

Statistical Analyses

Measures of dispersion and "*t*" tests of significance were calculated by conventional methods (25) and confirmed by nonparametric statistical procedures using the Wilcoxon rank sum test (26).

RESULTS

Bile Composition

The mean qualitative bile acid distribution in the four major groups is shown in Fig. 2, which also indicates that only the cholestanol group had cholelithiasis. Individual analyses of bile acid distribution, the percentage of 5α-bile acids, and the percentage of trihydroxy acids of all bile samples are given in Table 2.

Normal Group. The relative distribution of bile acids in bile of these animals was remarkably constant: all animals showed a predominance of deoxycholic acid, a secondary bile acid. There were smaller amounts of allodeoxycholic acid, and still smaller amounts of the primary bile acid, cholic acid.

Neomycin Group. Neomycin treatment altered the percentage of primary bile acids (allocholic and cholic) and secondary bile acids (allodeoxycholic and deoxycholic). The distribution of the 5β-bile acids did not change in a uniform manner: in two of the eight bile samples deoxycholate disappeared nearly completely and was replaced by cholate; in two samples deoxycholate was markedly decreased; and in four there was no change. The 5α-bile acids showed a greater change: seven of the eight samples contained allocholate, whereas in the normal group only one sample had shown a trace of allocholate. The effect of the antibiotic on bile composition was due in large part to the reduced dehydroxylation of bile acids.

Cholestanol Group. Cholelithiasis occurred in 13 out of 14 animals, and in most of these the gallbladder was completely impacted with concrements. As a result, only five bile samples could be obtained for analysis. These samples showed the expected rise in 5α-bile acids but the ratio of dihydroxy to tri-hydroxy acid (15:1) remained essentially the same as in the controls. This demonstrated that cholestanol feeding produced a shift in the 5β/5α ratio (to 2.5:1) but did not interfere with the dehydroxylation process. The animal with the lowest proportion of allodeoxycholate in its bile (19.2% allodeoxycholate) had no cholelithiasis. This animal ate poorly and consequently received less cholestanol; its bile had the lowest total 5α-bile acid content (21.3%) in this group.

FIG. 2. The dominant bile acids of bile in the four major groups of animals. The incidence of cholelithiasis is shown in the corner block.

TABLE 2   Distribution of Bile Acids in Bile*

Group	Dietary Addition	Dosage of Neomycin	Chole-lithiasis	Trihydroxy Allocholic 5α	Trihydroxy Cholic 5β	Dihydroxy Allodeoxy-cholic 5α	Dihydroxy Deoxycholic 5β	Total 5α	Total Trihydroxy
		g/day				% of total			
I	None	None	0	0	5.1	6.4	88.5	6.4	5.1
			0	0	3.1	5.7	91.2	5.7	3.1
			0	0	4.0	7.6	88.4	7.6	4.0
			0	0	3.3	6.0	90.1	6.0	3.3
			0	0	5.7	5.5	88.8	5.5	5.7
			0	0	4.4	6.7	89.0	6.7	4.3
			0	0.1	4.6	6.9	88.4	7.0	4.6
	Mean			0 ± 0	4.3 ± 0.3	6.4 ± 0.3	89.2 ± 0.4	6.4 ± 0.2	4.3 ± 0.4
II	None	0.5*	0	5.9	79.7	0	14.3	5.9	85.6
			0	1.6	98.4	0	0	1.6	100.0
			0	0	9.2	7.5	83.3	7.5	9.2
			0	0.4	1.2	4.1	94.3	4.5	1.2
			0	0.3	0.9	4.2	94.6	4.5	1.6
			0	1.4	17.4	1.9	79.3	3.3	18.8
			0	0.4	5.0	3.0	91.6	3.4	5.4
			0	0.2	0.6	7.6	91.6	7.8	0.8
	Mean			1.3 ± 0.7	26.6 ± 13.9	3.5 ± 1.0	68.6 ± 1.36	4.8 ± 0.8	27.8 ± 14.4
III	1% Cholestanol	None	+	3.3	3.8	24.5	68.4	27.8	7.1
			+	1.5	1.3	29.0	68.2	30.5	2.8
			+	2.7	4.9	24.7	67.8	27.4	7.3
			0	2.1	6.9	19.2	71.8	21.3	9.9
			+	4.1	2.4	28.6	64.9	32.7	6.5
	Mean			2.7 ± 0.4	3.9 ± 1.0	25.2 ± 1.8	68.2 ± 1.1	27.9 ± 1.9	6.5 ± 1.0
IV	1% Cholestanol	0.5†	0	31.0	46.0	0	23.0	31.0	77.0
		0.5†	0	20.9	56.3	0	22.8	20.9	77.2
		0.5*	0	30.0	41.0	0	29.0	30.0	71.0
		0.5*	0	22.3	26.6	0	46.9	26.5	48.9
		0.5*	0	4.5	4.7	21.8	69.0	26.3	9.2
		0.5*	0	3.4	5.3	16.9	74.4	20.3	8.7
	Mean			18.8 ± 5.0	30.0 ± 6.8	6.5 ± 4.1	44.2 ± 9.4	25.8 ± 1.8	48.6 ± 13.2
V	1% Cholestanol	0.01†	+	2.9	3.3	24.5	69.3	27.4	6.2
		0.05†	+	3.9	6.1	19.5	70.5	23.4	10.0
		0.05†	+	2.9	2.9	26.8	67.3	29.7	5.8
		0.125†	+	3.3	3.6	19.1	74.0	22.4	6.9
		0.125†	0	2.6	2.9	18.7	75.9	21.3	5.5
		0.125†	0	4.3	4.5	16.0	75.1	20.3	8.8
		0.125†	0	7.0	17.3	2.7	75.2	9.7	24.3
	Mean			3.8 ± 0.6	5.8 ± 2.0	18.2 ± 2.9	72.1 ± 1.2	22.0 ± 2.4	9.6 ± 2.5
VI	1% Cholestanol	0.05‡	+	4.3	4.2	22.3	69.1	26.6	8.5

Only animals whose gallbladder contained liquid bile at autopsy are listed in this table.   Mean values are followed by the SEM.
* Neomycin administered by stomach tube.
† Neomycin given in drinking water.
‡ Neomycin administered by intramuscular injection.

Cholestanol Plus Neomycin Group.   The three groups (III, IV, and V) that received cholestanol plus neomycin will be considered together. The control group for these three groups is the cholestanol group.

Neomycin at doses of 0.01 and 0.05 g/day did not influence bile composition of cholestanol-fed animals. Since all of the animals receiving the two lowest dosages of neomycin had gallstones, bile samples were obtained in only three animals. A higher dose of neomycin, 0.125 g/day, caused a marked reduction in allodeoxycholate in one out of four animals. The other three animals had a bile composition quite similar to that in the cholestanol group. In four out of six animals on the highest dose of neomycin (0.5 g/day), biliary allodeoxycholate was not detected; the other two animals showed a moderate reduction of allodeoxycholate. All six animals had increased allocholate levels. In the four most responsive animals, the 30-fold reduction in the allodeoxycholate/allo-

cholate ratio was accompanied by a 10-fold reduction of the deoxycholate/cholate ratio as compared with the cholestanol-fed controls.

Parenterally administered neomycin (Group VI) had no effect on bile composition of cholestanol-fed animals.

Cholelithiasis and Bile Composition

In the cholestanol groups and the three groups receiving both cholestanol and neomycin, cholelithiasis was associated with bile of high allodeoxycholate content. The animal in the cholestanol group that failed to develop gallstones showed low allodeoxycholate and allocholate concentrations in the bile. Similar low concentrations of allodeoxycholate were observed in the cholestanol plus neomycin group, but, in contrast, allocholate concentrations were high. Animals that did not receive cholestanol had bile of very low allodeoxycholate content and no gallstones.

Gallstone Composition

Gallstone composition was fairly constant (Table 3) regardless of the amount or route of neomycin administration. The major difference between gallstone composition and bile composition in the animals in which cholelithiasis occurred was the higher percentage of allodeoxycholate in the gallstones. In three animals both bile and stones were obtained for analysis. The gallstones were found to be suspended in bile that differed markedly in composition from the precipitated material (Fig. 3). The gallstones of animals possessing bile were identical in composition with those removed from gallbladders in which bile was no longer present.

Calcium Analyses

There was no significant difference in the ratio of calcium to bile acid in the four groups (I–IV) studied. The results obtained were (equivalents of Ca^{++}/equivalents of glycine-conjugated bile acid): normal group, 0.24, n = 5; neomycin group, 0.28, n = 1; cholestanol group, 0.20, n = 2; and cholestanol plus neomycin group, 0.22, n = 4. The stones also contained about 1 μg of magnesium per 10–20 μg of calcium.

Tissue Sterol Analyses

Cholestanol feeding caused an increase in the total sterol concentration in serum and liver (Table 4). Much of this increase was ascribable to absorbed cholestanol. Administration of neomycin (0.5 g/day) to rabbits on a cholestanol-free diet produced a pronounced increase of serum cholesterol concentration—in agreement with previous observations (27) and in contrast to the response in man (28). In cholestanol-fed animals, 0.5 g/day of neomycin inhibited the accumulation of stanol in liver and serum (44%) as compared with the cholestanol-fed controls (61%).

Bacterial Flora

The effect of different regimens on the coliform population in rabbit feces is shown in Table 5. The data indicate that the number of these organisms was unaffected by administration of cholestanol. In contrast, increasing suppression of coliforms was observed with increasing oral doses of neomycin. However, in two cholestanol-fed rabbits on 0.5 g/day of neomycin, gallstones were absent despite a marked increase in the number of coliform organisms. The isolated organisms from these two rabbits were resistant to neomycin.

DISCUSSION

Allodeoxycholic acid is a normal component of rabbit bile (Table 2). It does not induce cholelithiasis in normal

TABLE 3   DISTRIBUTION OF BILE ACIDS IN GALLSTONES*

| | | | | Bile Acids | | | | | |
| | | | | Trihydroxy | | Dihydroxy | | | |
Group	Dietary Addition	Dosage of Neomycin	Chole-lithiasis	Allocholic 5α	Cholic 5β	Allodeoxycholic 5α	Deoxycholic 5β	Total 5α	Total Trihydroxy
		g/day				*% of total*			
III	1% Cholestanol	None	+	6.6	3.7	46.3	43.4	52.9	10.3
			+	5.2	3.6	48.5	42.7	53.7	8.8
V	1% Cholestanol	0.01*	+	3.1	2.8	54.5	39.7	57.6	5.9
		0.01*	+	4.3	2.1	50.5	43.1	54.8	6.4
		0.01*	+	3.0	1.9	55.8	39.4	58.8	4.9
		0.05*	+	2.3	2.9	51.3	43.6	53.6	5.2
		0.05*	+	2.0	0.0	66.0	32.0	68.0	2.0
		0.05*	+	4.0	4.7	47.8	43.5	51.8	8.7
		0.125*	+	2.5	2.8	52.7	42.0	55.2	5.3
VI	1% Cholestanol	0.05†	+	3.8	3.2	49.5	43.5	53.3	7.0
Mean for all groups				3.7 ± 0.5‡	2.7 ± 0.6	52.3 ± 1.8	41.3 ± 1.1	56.5 ± 1.5	6.5 ± 0.8

* Neomycin administered in drinking water.
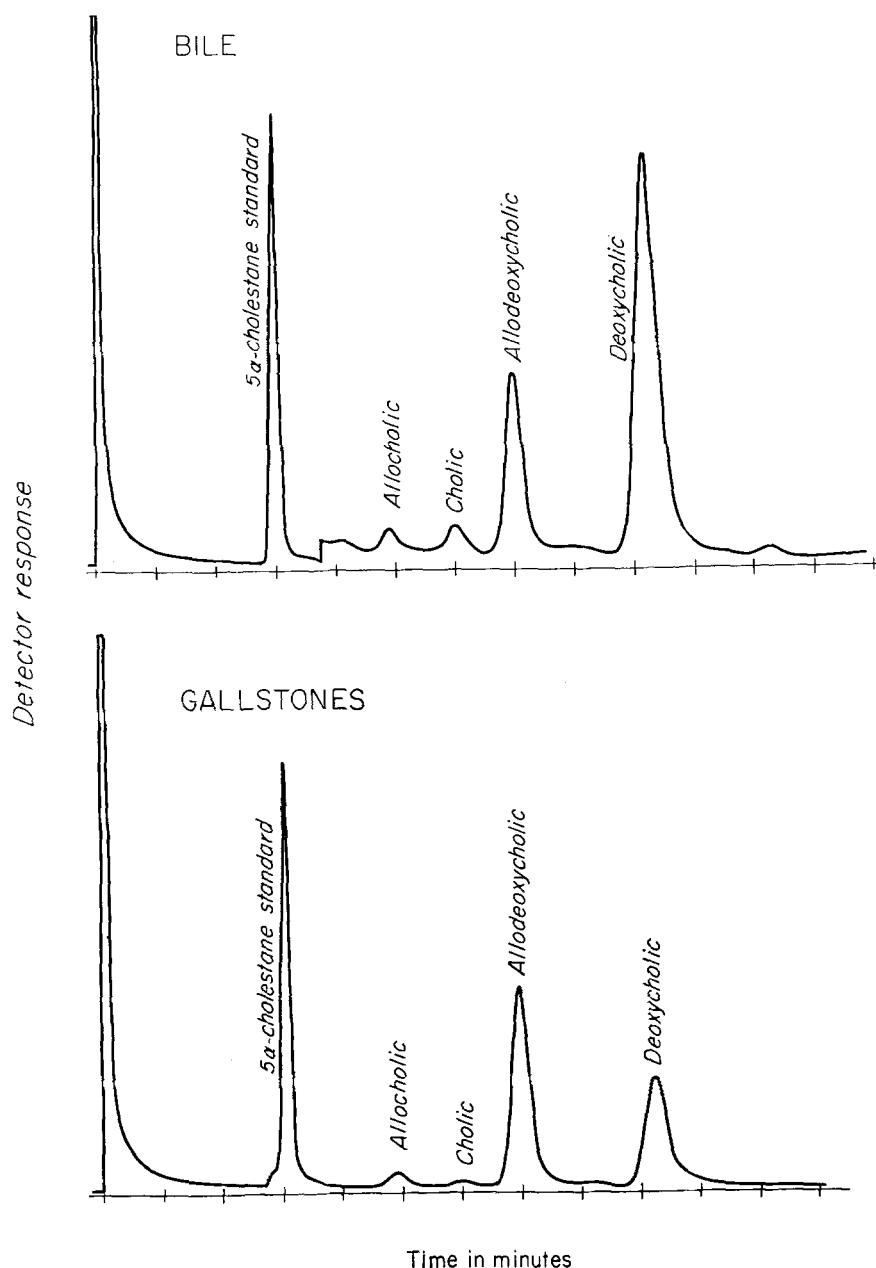† Neomycin injected intramuscularly.
‡ Mean ± SEM.

FIG. 3. Gas–liquid chromatographic separation of the bile acids of gallstones (below) and bile (above) obtained from the gallbladder of a rabbit from the cholestanol plus neomycin dose–response group. TMS ether derivatives of the methyl esters were chromatographed at 230°C with 1% Hi-Eff 8B in a 6 ft column.

rabbits, in which it constitutes less than 8.0% of the total bile acids. Cholestanol feeding increases allodeoxycholic acid levels of the bile. Animals in which allodeoxycholic acid made up more than 24% of total bile acids invariably had gallstones; in contrast, rabbits in which allodeoxycholic acid constituted less than 19% failed to produce gallstones. No clear-cut pattern was obtained in the 19–21% range. Thus, it would appear that the threshold of insolubility is reached when the concentration of allodeoxycholic acid in bile approaches 20% of total bile acids.

Allodeoxycholic acid is in all probability a secondary bile acid derived from the cholestanol-induced allocholic acid. Evidence for this may be obtained by analogy, as follows, with the transformation of cholic acid to deoxycholic acid. (a) In the rabbit with bile fistula, deoxycholic acid disappears and cholic acid becomes the predominant bile acid (29); (b) radioactive cholic acid administered to the rabbit is converted to deoxycholic acid after repeated enterohepatic cycles (5) whereas administered deoxycholic acid is not further metabolized (30); (c) cholic acid is the major bile acid of the germ-free rabbit, whereas

TABLE 4 TISSUE STEROLS AT END OF 4 WK FEEDING PERIOD

Group	Dietary Addition	Dosage of Neomycin	Average Wt. of Animals		Liver		Serum	
			Before	After	Total Sterol	Cholestanol	Total Sterol	Cholestanol
		g/day	kg		mg/g wet weight	% of total sterol	mg/ml	% of total sterol
I	—	—	*	*	3.9 ± 0.2†	7.2 ± 0.5	32 ± 11	5.3 ± 0.3
II	—	0.5	2.5	2.8	4.5 ± 0.3	7.4 ± 0.8	179 ± 26	6.2 ± 0.7
III	1% Cholestanol	0	2.8	2.8	7.2 ± 0.9	61.2 ± 0.8	127 ± 14	61.2 ± 0.4
IV	1% Cholestanol	0.5	2.4	2.3	4.2 ± 0.2	44.1 ± 2.1	47 ± 8	44.4 ± 2.9
V	1% Cholestanol	0.125	2.3	2.5	7.2 ± 0.1	63.4 ± 1.3	165 ± 36	59.8 ± 1.8
	" "	0.05	2.4	2.5	7.0 ± 0.3	60.6 ± 4.1	96 ± 20	57.1 ± 3.6
	" "	0.01	2.3	2.6	7.1 ± 0.1	61.5 ± 2.8	97 ± 12	57.8 ± 4.2

* Not determined.
† Average ± SD. Numbers of animals as in Table 1.

TABLE 5 CHANGES IN FECAL COLIFORM POPULATION DURING ADMINISTRATION OF CHOLESTANOL AND NEOMYCIN

Group	Dosage of Neomycin	Number of Animals	Coliform Organisms			
			On Stock Diet* (Control Period)		On Stock Diet with 1% Cholestanol for 4 Wk	
			Geom. Mean	Range	Geom. Mean	Range
	g/day		log_{10}/g dry feces			
III	0	15	3.7	7.2–1.9	3.5	8.0–1.8
V	0.01†	3	6.6	7.1–5.9	4.6	4.7–4.4
	0.05†	4	3.3	4.6–2.3	2.4	3.1–1.3
	0.125†	4	4.2	7.5–2.0	1.3	1.5–1.0
II	0.5‡	6	4.9	7.1–1.7	1.7	2.3–1.5

* Before administration of cholestanol or neomycin.
† Neomycin administered in drinking water.
‡ Neomycin administered by intramuscular injection.

deoxycholic acid is absent (unpublished observation); and (d) rabbit feces contain a bacterium which, in vitro, converts cholic acid into deoxycholic acid (31).

More direct evidence for allodeoxycholic acid being a secondary bile acid in normal rabbit bile has been obtained recently. Firstly, rabbits with permanent bile fistula cease to excrete secondary bile acids, leaving allocholic acid as the only 5α-bile acid; and secondly, in the germ-free rabbit allocholic acid is the only 5α-bile acid (unpublished observations). Allodeoxycholic acid is absent. Thus, it seems reasonable to assume that allodeoxycholic acid is a secondary bile acid formed by bacterial dehydroxylation of allocholic acid in the cholestanol-fed rabbit as well as in the normal rabbit. Consequently, both the hepatic transformation of cholestanol and the bacterial dehydroxylation of allocholic acid appear to be essential steps in the pathogenesis of gallstone formation in the cholestanol-fed rabbit. Fig. 1 indicates the anatomical sites and types of chemical transformations considered to occur in this induced disease of the enterohepatic circulation.

It follows that cholestanol-induced cholelithiasis should be preventable by any compound capable of reducing the relative concentration of allodeoxycholic acid in bile below 20%. Neomycin has this ability (Table 2). Since it is poorly absorbed from the intestinal tract (12, 13) and since parenterally administered neomycin did not alter biliary composition or prevent gallstones, the probable site of action of neomycin is the intestinal lumen. Three mechanisms for intraluminal effect of neomycin may be considered: (a) an antibiotic effect, i.e. suppression of bacterial dehydroxylation, (b) a sequestrant effect, i.e. interference with bile acid adsorption, and (c) an interference with cholestanol absorption, either directly by the neomycin molecule, or indirectly, as a result of bile acid sequestration.

Neomycin usually reduced the coliform flora of the intestinal tract. However, two cholestanol-fed rabbits, that received neomycin and failed to form gallstones, had increased numbers of coliform organisms. In view of this, coliform bacteria are unlikely to be involved in the dehydroxylation of bile acids. Indeed, Gustafsson, Midtvedt and Norman (32) recently isolated obligatory anaerobes from feces of rat and man capable of in vitro dehydroxylation of chenodeoxycholic acid, and Mosbach, Bokkenheuser, Hofmann, Hoshita, and Frost have isolated from rabbit feces similar anaerobes which 7-dehydroxylate cholic and allocholic acids (31).

The fact that neomycin is a polycation which precipitates bile salt conjugates from aqueous solutions (33, 34), suggests that it could act as a sequestrant. A comparison of groups III and IV (Table 2) shows that in cholestanol-fed animals on neomycin the proportion of primary bile acids is increased (allocholic acid from 2.7% to 18.8%, and cholic acid from 26.6% to 30.0%) while the proportion of secondary bile acids is decreased (allodeoxycholic acid from 25.2% to 6.4%, and deoxycholic acid from 68.6% to 44.2%). A selective sequestration by neomycin of secondary bile acids and, in particular, allodeoxycholic acid would afford a satisfactory explanation for the absence of gallstones in the neomycin-treated rabbit. However, our data do not permit a dissociation of the antibiotic and sequestrant effects of neomycin. A recently de-

scribed derivative of neomycin, N-methyl neomycin (34) might be of interest in this connection, since in experiments with chickens it seemed to be devoid of antibiotic activity and yet act as a bile acid sequestrant.

Reduced cholestanol absorption, whether brought about (*a*) by reduced food intake in neomycin-treated animals, (*b*) by a neomycin effect on the intestinal epithelium, or (*c*) indirectly as a result of bile acid sequestration, would tend to prevent cholelithiasis. (*a*) Reduced ingestion of cholestanol as the cause of failure to produce gallstones was eliminated by feeding experiments. Four animals in group III (1% cholestanol) were pair-fed with four animals in group IV (1% cholestanol plus 0.5 g/day of neomycin). All animals in the former and none in the latter group developed gallstones. (*b*) Administration of 0.5 g neomycin daily to cholestanol-fed animals had a suppressing effect on tissue sterol levels (Table 4). This, however, could not be the sole factor responsible for the prevention of cholelithiasis. For example, three of the four animals in group V (1% cholestanol plus 0.125 g/day neomycin) were free of gallstones (Table 2), yet the cholestanol contents of liver and serum were 63% and 60% respectively, i.e., of the same order of magnitude as the levels of the animals which uniformly had cholelithiasis. (*c*) On the assumption that cholestanol absorption, like cholesterol absorption, requires the presence of bile acids, total sequestration of bile acids would be effective in preventing cholestanol-induced cholelithiasis. Our data show that both cholestanol (Table 4) and bile acids (Table 2) are absorbed in the neomycin-treated animal.

Cholestanol and neomycin appear to affect the bile composition independently (Fig. 4). Cholestanol administration alters the 5β-/5α-bile acid ratio and thus influences the steroid *nucleus* present. Neomycin treatment changes the primary/secondary bile acid ratio, and modifies the number and position of nuclear substituents. In contrast, cholestanol feeding had no effect on the primary/secondary bile acid ratio, and neomycin did not alter the 5β-/5α-bile acid ratio.

These studies are in agreement with recent studies which suggest that a change in bile composition alone is sufficient to produce gallstones in experimental animals (35, 36) and even in man (37). There appear to be at least two major types of experimental cholelithiasis: (*a*) cholesterol and bile pigment cholelithiasis induced either by dietary manipulation (35, 36) or by the feeding of bile acid sequestrants (38); and (*b*) bile acid cholelithiasis, where insoluble calcium salts of conjugated bile acids are formed. They may be induced by the direct feeding of an appropriate secondary bile acid, e.g. lithocholic acid (39, 40), or by the feeding of its sterol precursor in large amounts, as in these experiments. The term *bile acid*
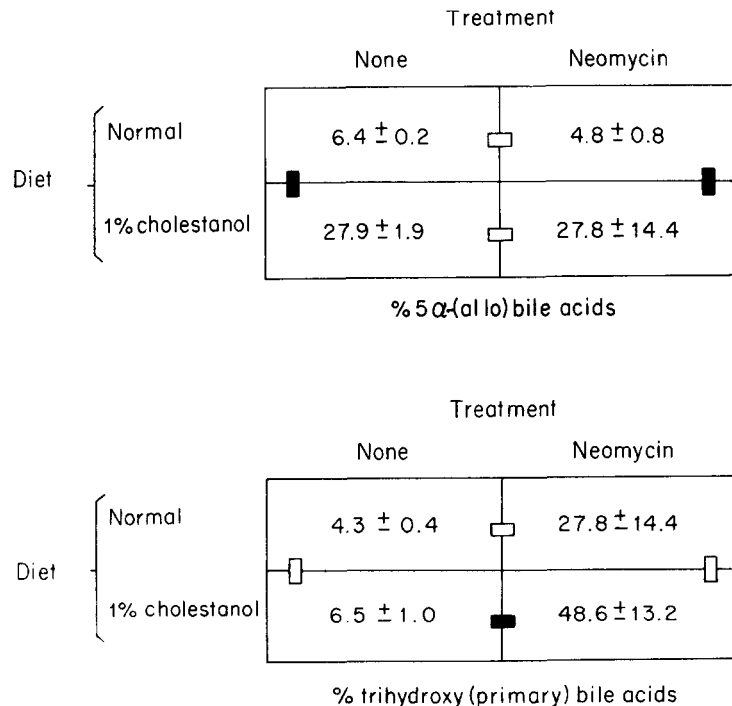

Fig. 4. The percentage of 5α-(allo) bile acids (above) and trihydroxy (primary) bile acids (below) in the bile of the four major groups of animals. The solid bar indicates differences that are significant at the $P < 0.01$ level. The effect of cholestanol feeding on the 5α/5β bile acid ratio is not influenced by neomycin treatment; the effect of neomycin treatment on the primary/secondary bile acid ratio is not influenced significantly by cholestanol feeding. Data are from Table 2 and show mean ±SEM.

cholelithiasis is proposed as a general term for the occurrence of gallstones composed of bile acids in association with an elevated concentration of certain bile acids in the enterohepatic circulation.

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