Bile acid excretion and cholesterol 7α-hydroxylase expression in hypercholesterolemia-resistant rabbits

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Abstract We have developed a partially inbred substrain of New Zealand white rabbits (CRT/mlo) that are resistant to the hypercholesterolemia that accompanies cholesterol feeding to normal rabbits. The plasma cholesterol concentration of normal rabbits increases dramatically from about 30 mg/dl to >300 mg/dl after they are fed a 0.1% cholesterol-enriched diet for 3–4 months. Cholesterol-fed CRT/mlo animals, however, maintain a cholesterol level of about 30 mg/dl during the entire cholesterol feeding period. In addition to the low plasma cholesterol level, measurements of cellular cholesterol indicate that the hepatic cholesterol content of the cholesterol-fed resistant rabbit remains markedly lower than it does in normal animals fed the same diet. The only mechanism for removal of significant quantities of cholesterol carbon from the body is via the fecal excretion of cholesterol, neutral sterol metabolites, and bile acids. In comparison to the basal, low-cholesterol diet, we observed that cholesterol-fed resistant rabbits had increased excretion of lithocholic acid, while excretion of this bile acid by cholesterol-fed normal rabbits remained similar to basal diet levels. Deoxycholic acid excretion, the other main bile acid excreted in the feces of rabbits, was decreased in response to cholesterol challenge in animals with either resistant or normal phenotypes, but the decrease was significantly less in the resistant rabbits. Thus, the resistant rabbits excreted relatively more lithocholic and deoxycholic acid than did the cholesterol-fed normal rabbit. The difference in bile acid excretion was also manifest by a higher than normal level of cholesterol 7α-hydroxylase activity and cholesterol 7α-hydroxylase mRNA in the livers from resistant versus normal rabbits. As cholesterol 7α-hydroxylase is the putative rate-limiting step of bile acid synthesis, we believe that the increased excretion of bile acids by resistant animals is due, at least in part, to increased levels of cholesterol 7α-hydroxylase expression.—**Poorman, J. A., R. A. Buck, S. A. Smith, M. L. Overturf, and D. S. Loose-Mitchell.** Bile acid excretion and cholesterol 7α-hydroxylase expression in hypercholesterolemia-resistant rabbits. *J. Lipid Res.* 1993. **34:** 1675–1685.

Supplementary key words cholesterol-resistant rabbits • hypocholesterolemia • cholesterol absorption • cholesterol 7α-hydroxylase • bile acids

We have developed and partially characterized a colony of partially inbred cholesterol-resistant New Zealand White rabbits (CRT/mlo) that are resistant to the hypercholesterolemia that typically accompanies cholesterol feeding (1–4). Normal animals, when fed a 0.1% (wt/wt) cholesterol-enriched rabbit chow, exhibited an increase in plasma cholesterol from the basal state of about 30 mg/dl to approximately 300 mg/dl within 8 weeks. At necropsy the cholesterol-fed normal rabbits had developed moderate to severe aortic atherosclerotic lesions that involved greater than 50% of the surface area of the vessel. In contrast, resistant rabbits maintained plasma cholesterol levels of approximately 30 mg/dl throughout the cholesterol feeding period and no lesions were found. Furthermore, when typical rabbits were fed a 0.25% cholesterol-enriched diet for 10 weeks, plasma cholesterol levels approaching 1,300 mg/dl were observed while the plasma cholesterol levels of resistant animals were typically <70 mg/dl (4), and the resistant rabbits remained free of vascular atherosclerotic lesions.

Variations in the response to dietary cholesterol feeding, which has been observed in several species, have been shown to be at least partially under genetic control (5). The phenotype most closely related to CRT/mlo hypercholesterolemia-resistant rabbit appears to be that of the IIIVO/JU and AX/JU JAX rabbit breed that are hypo- and hyperresponsive to dietary cholesterol, respectively (6). Sterol balance and absorption studies completed during periods of cholesterol feeding indicated that the hyporesponsive trait of the IIIVO/JU rabbits was due to an increase in bile acid excretion and a decrease in intestinal cholesterol absorption. The study of the JAX rabbits suggests that not only is cholesterol responsiveness

Abbreviations: NZW, New Zealand White; C7αH, cholesterol 7α-hydroxylase; HPLC, high performance liquid chromatography; GC, gas chromatography; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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under genetic control but that multiple genes are probably involved in determining the phenotype.

Previously we described cholesterol absorption, and neutral sterol and bile acid excretion in the CRT/mlo cholesterol-fed, hypercholesterolemia-resistant New Zealand White (NZW) rabbit (3). When fed a 0.1% cholesterol-enriched diet these animals exhibited absorption of cholesterol and excretion of fecal neutral sterols equal to that of typical NZW rabbits. However, the resistant animals excreted 2.5-fold more deoxycholic acid than their normal counterparts, leading to the conclusion that the increase in bile acid excretion is a major contributor to expression of the resistant phenotype.

The focus of the present study was to further assess bile acid excretion in the resistant animal by examining the regulation of cholesterol excretion and bile acid synthesis. In this study we observed no significant difference in total neutral sterols or bile acid excretion from normal and resistant animals fed a basal, low-cholesterol diet. An increase in dietary cholesterol consumption resulted in increased total neutral sterol excretion, and decreased total bile acid excretion in both phenotypes, but the regulation of the excretion of the individual bile acids by dietary cholesterol was complex. In response to cholesterol feeding, lithocholic acid excretion by normal rabbits remained unchanged from basal levels. In contrast, the resistant animals increased excretion of lithocholic acid from basal levels that were lower than in normal animals to levels equivalent to the normal rabbits. The excretion of deoxycholic acid was decreased in both phenotypes in response to cholesterol feeding, but the decrease of the resistant rabbits was only moderately attenuated in comparison to the response of normal rabbits. Thus, as previously reported (3), the resistant animals excreted 2.5-fold more fecal deoxycholic acid. In the resistant animals, both the activity and mRNA levels of cholesterol 7α-hydroxylase (C7αH), the putative rate-limiting enzyme of bile acid synthesis, were significantly higher than they were in normal rabbits. We have concluded, therefore, that increased excretion of bile acids is due at least partially to an increased level of C7αH expression.

MATERIALS AND METHODS

Materials

Plasma cholesterol assay kits, restriction enzymes, nucleotides, hexanucleotides, RNasin, and SP6 polymerase were purchased from Boehringer Mannheim (Indianapolis, IN). [^{3}H](1,2(n))cholesterol (47 Ci/mmol), [^{14}C]β-sitosterol (56 mCi/mmol), and [^{32}P]UTP (3000 Ci/mmol) were obtained from Amersham (Arlington Heights, IL). Steraloids (Wilton, NH) was the source of 7α-hydroxycholesterol, 7β-hydroxycholesterol, cholesterol, and sodium cholate. Taq polymerase, Duralon membranes, and the Stratalinker were purchased from Stratagene (La Jolla, CA). The Riboprobe transcription kit and pGEM-3Zf(+) bacterial host vector were obtained from Promega (Madison, WI). Sequenase was purchased from U.S. Biochemicals (Cleveland, OH). Other reagents were of the highest purity available and were obtained from Sigma Chemical Co. (St. Louis, MO). The Hewlett-Packard 5890 gas chromatograph was fitted with a thermal conductivity detector. A DB-1701 megabore fused silica column [ID 0.5 mm × 15 m, polymethyl (50% phenyl)siloxane, 0.25 μm film thickness] was purchased from J&W Scientific (Folsom, CA). The HPLC system consisted of a Beckman 100A solvent delivery system, a Waters 712 WISP sample processor, an Altex 420 microprocessor controller, a Hitachi 100-10 spectrophotometer, a Shimdadzu C-R1A chromatographic data processor, and a Supelco LC-18 (Bellefonte, PA) reverse phase column (ID 4.6 mm × 25 cm, pore size 100 Å). HPLC grade solvents were purchased from J. T. Baker (Phillipsburg, NJ). Oligonucleotides were synthesized in a Cyclone DNA synthesizer and were then purified over Oligopak oligonucleotide columns purchased from Milligen (Novato, CA).

Animals, housing, and diets

Breeding methods and procedures and criteria for phenotypic characterization of normal and resistant rabbits have been described (1). All animals were caged individually and maintained on normal 12-h light/dark cycles. The 0.1% and 0.25% cholesterol-enriched diets were prepared by supplementing standard Purina laboratory rabbit chow, which contained about 60 μg cholesterol/g chow, with 0.10 or 0.25 g cholesterol/100 g chow, respectively. The cholesterol and 2 ml corn oil/100 g chow were dissolved in a large excess of chloroform, mixed with intact chow pellets, and allowed to dry in a fume hood until the odor of chloroform was no longer detected. The cholesterol-enriched diets were prepared at least twice monthly to reduce the accumulation of oxidized lipids. At monthly intervals during the experimental period and at necropsy, blood specimens were drawn from the central ear artery of unanesthetized rabbits. Plasma total cholesterol levels were determined by an enzymatic assay kit (Boehringer Mannheim). The animals in these studies were age- and weight-matched and consisted of seven male and five female normal rabbits, and eight male and four female CRT/mlo rabbits. Normal rabbits had basal cholesterol levels of 44.8 ± 6 mg/dl which increased to 178 ± 22 mg/dl when fed the 0.1% cholesterol-enriched diet for 8 weeks. Corresponding values for the CRT/mlo rabbits were 34 ± 4 and 40 ± 4 mg/dl.

Cholesterol absorption, and sterol and bile acid excretion

The methods used to evaluate cholesterol absorption have been detailed (3). Briefly, six pairs of age- and

weight-matched normal and resistant rabbits were given a single test meal containing 1.1×10^7 dpm of both [^{3}H]cholesterol and [^{14}C]β-sitosterol. Total stool samples were collected during the next 9 days at 24-h intervals. Duplicate 0.5-g samples of fecal material were saponified with 1 N NaOH in 95% ethanol for 1 h at 95°C. The neutral sterols were extracted repetitively into hexane, and dried with nitrogen. The radioactivity in the samples was determined by scintillation spectrometry. The lower aqueous phases, containing conjugated and free bile acids, were extracted repetitively with chloroform–methanol 2:1 (v/v). The samples were dried with nitrogen and the radioactivity was measured by scintillation counting. Recovery of the nonabsorbable [^{14}C]β-sitosterol allowed for correction of possible losses of cholesterol-associated radioactivity due to degradation, and provided an estimate of intestinal transit time. Cholesterol absorption was calculated from the formula:

$$\% \text{ cholesterol absorbed} = 1 - \left[\frac{^3\text{H activity in fecal sterols}}{^{14}\text{C activity in fecal sterols}} \times \frac{^3\text{H activity in test oral meal}}{^{14}\text{C activity in test oral meal}}\right] \times 100$$

The cumulative fecal ^{3}H radioactivity in the neutral sterol fraction from Day 1 through Day 6 was used to calculate cholesterol absorption.

The methods used to measure sterol and bile acid excretion have been described in detail (3). Briefly, after the addition of 0.25 mg nor-deoxycholic acid as an internal standard to determine recovery, duplicate 100-mg fecal samples were saponified in 2.5 N NaOH–95% methanol. The samples were acidified to pH 1.0, extracted with chloroform–methanol 2:1, filtered, and dried with nitrogen. The dried samples were butylated in 1 ml 6% sulfuric acid in n-butanol at 40°C for 2 h, washed with water, and dried. Acetylation of the samples was done at 40°C overnight in 1 ml of chloroform, 30 mg dimethylaminopyridine, and 0.5 ml acetic anhydride. The derivatized samples were extracted with chloroform, back-extracted with water, passed through magnesium sulfate–potassium carbonate 1:1, and taken to dryness under nitrogen. The residue was resuspended in chloroform and analyzed by gas chromatography. The butyl ester-acetate derivatives of fatty acid, sterols, and bile acids were separated on a megabore fused silica column. No conjugated bile acids were detectable by GC in the fecal samples. Peak area correction factors were determined by analyzing quantitative mixtures of standard compounds.

C7αH activity assay

After an overnight fast, age- and weight-matched pairs of normal and resistant rabbits were anesthetized and killed at 9:00 AM. The medial lobe of the liver was perfused with cold phosphate-buffered saline (PBS), and homogenized on ice in four volumes 0.05 M Tris-acetate, 1 mM EDTA, 1.15% KCl, 50 mM NaF, pH 7.5. All subsequent procedures were performed at 4°C. Cellular debris was pelleted by centrifugation at 10,000 *g* for 20 min. Microsomes were pelleted by centrifugation of the 10,000-*g* supernatant at 100,000 *g* for 60 min. The microsomal pellet was resuspended and washed in a volume of 1.15% KCl, 1 mM EDTA, 50 mM NaF equal to that of the supernatant and collected by centrifugation at 100,000 *g* for 60 min. The final pellet was resuspended in a volume of 100 mM potassium phosphate, 20% glycerol, 0.10 mM EDTA, 5 mM DTT, 50 mM NaF, pH 7.4, equal to 0.5 ml/g liver homogenized (final concentration was about 30 mg microsomal protein/ml). The microsomes were stored at −20°C and enzyme activity was measured within 2 weeks. Microsomal protein concentration was determined by a modified Lowry procedure as described by Peterson (7) and the absorbance of the samples was measured spectrophotometrically at 750 nm. The concentration of microsomal protein was determined by comparison to a bovine serum albumin (BSA) standard curve.

C7αH activity was measured in liver microsomes according to modifications of the method of Chiang, Miller, and Lin (8). Liver microsomes (2 mg) were incubated in 1 ml of buffer containing 0.10 M potassium phosphate, 50 mM NaF, 5 mM DTT, 0.105% CHAPS, pH 7.4. Ten μl 10 mM cholesterol in 3% Triton X-100 was then added and the mixture was pre-incubated for 5 min at 37°C. The reaction was initiated by the addition of 100 μl of 12.5 mg/ml NADPH. The samples were incubated at 37°C for 30 min and the reaction was terminated by adding 30 ml 20% sodium cholate. 7β-Hydroxycholesterol, 0.5 g dissolved in ethanol, was added as an internal standard to estimate recovery as described (8). The samples were then derivatized by addition of cholesterol oxidase (1 unit) in 10 mM potassium phosphate, 1 mM DTT followed by incubation at 37°C for 10 min. The reaction was terminated by the addition of 2 ml ethanol and the samples were extracted 3 times with 6 ml petroleum ether. The combined organic phases were dried under nitrogen, and the residue was resuspended in 50 ml of acetonitrile–methanol 85:15. A 10-μl aliquot was separated in acetonitrile–methanol 85:15 with a C_{18} reverse phase HPLC column. The absorbance of the samples was measured at 240 nm. At a flow rate of 1.0 ml/min, the 7α- and 7β-hydroxy-4-cholesten-3-one derivatives eluted between 10 and 13 min. Peak areas were converted to product mass by comparison to a standard curve. The endogenous mass of 7α- and 7β-hydroxycholesterol in the microsomal preparations was determined in aliquots of microsomes that were boiled for 10 min before experimental assay for C7αH activity.

Cloning rabbit C7αH cDNA

Oligonucleotide primers were used to clone rabbit C7αH using the polymerase chain reaction. Primers were

synthesized based on published rat C7αH cDNA sequence (see Fig. 5) (9). The sequence of these primers was:
primer 7: 5'-GCATGGATCCCCCAAATGATGGAAA TACC-3',
primer 1: 5'-GCATGGATCCGGATGTTATAGGAACCG TCC-3'. The 10 bases on the 5'-end of each oligonucleotide were added to provide a Bam HI restriction site for subcloning.

Total cellular RNA was isolated by the guanidine isothiocyanate/CsCl method (10) from a normal and a resistant rabbit fed a 0.25% cholesterol-enriched diet for 8 weeks. The cDNA synthesis and PCR amplification were carried out using random primers (Boehringer Mannheim) as described by Gibbs et al. (11). Samples consisting of 10% of the cDNA synthesis, dNTPs, and 5 U of Taq polymerase were incubated at 65°C in an Ericomp thermal cycler for 5 min and then subjected to 30 cycles of denaturation (94°C, 25 sec), annealing (50°C, 45 sec), and polymerization (72°C, 2 min 10 sec). The PCR products were purified, digested with Bam HI, and subcloned into a bacterial host vector pGEM-3fZ(+) by standard techniques. Multiple clones were isolated and sequenced with dideoxy nucleotides and Sequenase according the manufacturer's specifications (U.S. Biochemicals).

Quantitation of C7αH mRNA

A 329 nucleotide rabbit antisense C7αH probe was labeled with [^{32}P]-UTP (3000 Ci/mmol) by in vitro transcription according to the manufacturer's specifications (Promega).

A 142 bp clone corresponding to the rabbit glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was provided by Dr. P. Schimmel (12). This fragment was subcloned into pGEM3, digested at an internal HinfI restriction site, and transcribed from the SP6 promoter (Riboprobe transcription kit) as described below to produce a 132 bp probe.

Total cellular RNA was isolated from the livers of normal and resistant rabbits fed a basal or 0.25% cholesterol-enriched diet for 8 weeks by the guanidine isothiocyanate/CsCl method (10). Ten μg total RNA was denatured in 15 mM methyl-mercuric hydroxide (Alfa) and fractionated on a 0.9% agarose gel in 20% formaldehyde. The RNA was transferred to Duralon membranes (Stratagene, La Jolla, CA) and crosslinked to the membrane with UV-light (Stratalinker 1800, Stratagene).

Membranes were hybridized for 18 h at 60°C in 50% formamide, 0.8 M NaCl, 50 mM PIPES, 1 mM EDTA with 1 × 10^6 dpm/ml of the cRNA probe. Membranes were washed in 2 × SSC, 0.1% SDS for 10 min at 60°C. Filters were exposed to Hyperfine-MP film (Amersham) with intensifying screens for 2 weeks at −80°C. Autoradiograms were quantitated using a BioSearch video densitometer.

Statistical analyses

Statistical comparisons were made using Student's *t*-test for unpaired data. The differences in comparisons were judged significant at the $P < 0.05$ level. The data expressed in the figures and tables are mean and standard error of the mean.

RESULTS

Six normal and six CRT/mlo animals that were age- and weight-matched were fed the 0.1% cholesterol-enriched diet; plasma cholesterol levels were allowed to return to baseline, and then the animals were restudied when fed a low-cholesterol diet. Cholesterol absorption and sterol and bile acid excretion were measured during the consumption of each diet. Previously we reported the measurement of cholesterol absorption, and fecal neutral sterol and bile acid excretion of the animals fed the 0.1% cholesterol-enriched diet (3). Some data from that study are also shown in this report for comparison between basal diet and cholesterol-fed states.

Fecal samples from the normal and resistant rabbits contained only two major neutral sterols, cholesterol and coprostanol. Total neutral sterol excretion from normal and resistant rabbits fed either the basal, low-cholesterol or the 0.1% cholesterol-enriched diets is shown in **Fig. 1.** Cholesterol feeding resulted in a significant increase in total neutral sterol excretion in the resistant rabbits ($P < 0.05$). While cholesterol feeding to normal animals also increased cholesterol excretion, this did not attain statistical significance. There was no statistically

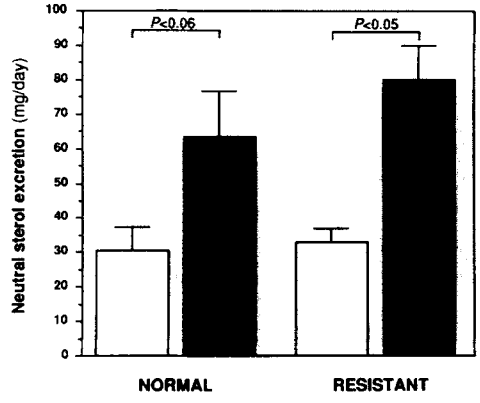

Fig. 1. Total neutral sterol excretion by normal and resistant rabbits. Six normal and six resistant rabbits that were age- and weight-matched were fed 100 g/day of the basal (open bars) or a 0.1% cholesterol-enriched diet (filled bars) for 6–8 months. At the end of the feeding period total fecal specimens were collected daily for 9 days. The masses of the fecal neutral sterols were determined by gas chromatographic separation of the butyl ester-acetate derivatives. The data represent the mean sums of daily fecal masses (mg/day) of cholesterol plus coprostanol determined in samples combined from Day 8 and Day 9 (mean ± SEM).

TABLE 1. Excretion of coprostanol and cholesterol by normal and resistant rabbits

	Coprostanol		Cholesterol	
Diet	Basal	0.1% CED	Basal	0.1% CED
Normal	(A) 12.8 ± 3.0	(B) 11.4 ± 4.3	(E) 17.9 ± 4.0	(G) 51.9 ± 11.5
Resistant	(C) 10.7 ± 2.0	(D) 16.3 ± 5.0	(F) 22.3 ± 3.7	(H) 63.7 ± 11.1

Fecal samples were collected for 9 days and the masses of fecal coprostanol and cholesterol were determined by gas chromatographic separation of the butyl ester-acetate derivatives. The data represent the average masses in mg/day (± SEM) of the individual neutral sterols in samples collected on Day 8 and Day 9 (n = 6). All inter- and intra-group statistical comparisons were performed and the differences between means that reached significant levels ($P < 0.05$) were E versus G and F versus H.

significant difference in total neutral sterol excretion between normoresponsive and resistant animals during consumption of either the basal or the cholesterol-enriched diet.

The amounts of the individual fecal neutral sterols excreted by normal and resistant rabbits fed the basal and the 0.1% cholesterol-enriched diets are shown in **Table 1.** Cholesterol excretion was greater in both groups during the consumption of the cholesterol-enriched diet ($P < 0.05$), however there was no difference in cholesterol excretion between normal and resistant groups. Coprostanol excretion was not significantly affected by diet and was not different between normal and resistant rabbits. Since coprostanol excretion was not increased with increased cholesterol intake, it is suggested that during consumption of the basal diet the conversion rate of cholesterol to coprostanol by intestinal bacteria was maximal.

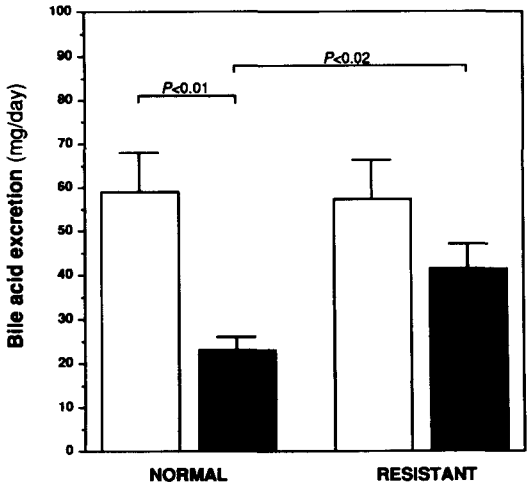

Fig. 2. Total bile acid excretion by normal and resistant rabbits. Six normal and six resistant rabbits that were age- and weight-matched were fed 100 g/day of the basal (open bars) or the 0.1% (filled bars) cholesterol-enriched diet for 6–8 months. Total fecal specimens were collected for 9 days. The mass of the individual fecal bile acids was determined by gas chromatographic separation of the butyl ester-acetate derivatives. The data represent the mean sums of daily fecal lithocholic and deoxycholic acids (mg/day) determined in samples from Day 8 and Day 9 (mean ± SEM).

Total bile acid excretion rates by normal and resistant rabbits fed the basal low-cholesterol diet were not significantly different (**Fig. 2**). However, when the animals were fed the 0.1% cholesterol-enriched diet, total bile acid excretion was twofold greater in the resistant rabbits compared to normal rabbits ($P < 0.02$). Cholesterol feeding resulted in decreased total bile acid excretion by both phenotypes. Significantly, normal rabbits had a 60% decrease in the rate of bile acid excretion in response to increased dietary cholesterol load ($P < 0.01$), whereas the resistant animals had only an insignificant ($P > 0.05$) decrease in bile acid excretion. Thus, in the cholesterol-fed state the resistant rabbits excreted twofold more total bile acids than normal rabbits did.

The two major bile acids in the feces of normal and resistant rabbits were lithocholic acid and deoxycholic acid. The daily excretion rates of each of these acids in the feces of normal and resistant animals are shown in **Table 2.** During consumption of the basal diet there was no difference between resistant rabbits and normal rabbits in the amount of lithocholic acid excreted ($P > 0.05$). Previous measurement of fecal lithocholic acid excreted by cholesterol-fed animals also showed that the excretion rates were not significantly different between resistant and normal rabbits (3). However, cholesterol feeding did increase the rate of lithocholate excretion in resistant rabbits from 9.3 to 16.7 mg/day ($P < 0.01$) but this increase was not observed in the normal rabbits.

When fed the basal low-cholesterol diet there was no significant difference in the amount of deoxycholic acid excretion by the resistant and normal rabbits ($P > 0.05$) (Table 2). However, during consumption of the 0.1% cholesterol-enriched diet deoxycholic acid excretion was decreased in both groups of rabbits ($P < 0.05$), nonetheless the resistant rabbits, in comparison to the normal rabbits, excreted nearly threefold more deoxycholic acid ($P < 0.01$). In response to the 0.1% cholesterol-enriched diet, normal animals had an 80% decrease in the excretion rate of deoxycholic acid ($P < 0.01$). Since lithocholic acid excretion was equivalent in normal rabbits fed either the basal or 0.1% cholesterol-enriched diet, the decrease in total bile acid excretion in response to dietary

TABLE 2. Excretion of lithocholic and deoxycholic acids by normal and resistant rabbits

	Lithocholic Acid		Deoxycholic Acid	
Diet	Basal	0.1% CED	Basal	0.1% CED
Normal	(A) 14.4 ± 2.0	(B) 14.5 ± 1.5	(E) 44.7 ± 6.8	(F) 8.4 ± 1.6
Resistant	(C) 9.3 ± 1.5	(D) 16.7 ± 1.8	(G) 48.0 ± 7.4	(H) 24.5 ± 3.9

Fecal samples were collected daily for 9 days and masses of fecal lithocholic and deoxycholic acids were determined by gas chromatographic separation of the butyl ester-acetate derivatives. The data represent the average masses in mg/day (± SEM) of the individual bile acids in samples collected on Day 8 and Day 9 (n = 6). Differences in the mean that reached statistical significance were C versus D, $P < 0.01$; E versus F, $P < 0.01$; G versus H, $P < 0.01$; F versus H, $P < 0.01$.

cholesterol load was due largely to a reduction in the excretion of deoxycholic acid. Cholesterol-fed resistant animals, in contrast, exhibited a decrease in fecal deoxycholic acid excretion of only 50% ($P < 0.01$) compared to basal diet levels.

Sterol balance calculations were done to compare the input–output of cholesterol in normal and resistant rabbits. The sums of the mass of fecal neutral sterols (Table 1) and bile acids (Table 2) were subtracted from the daily dietary cholesterol intake. When fed the basal diets, (6 mg cholesterol intake/day), the normal and resistant rabbits excreted more cholesterol as fecal sterols and bile acids than was consumed, being −83.8 mg/day and −84.3 mg/day, respectively. There was no significant difference in absorption of cholesterol between normal and resistant animals fed the basal diet. Normal rabbits absorbed 79.8% ± 3.4 and resistant rabbits absorbed 75.1% ± 2.4 of the total dietary cholesterol (3). In contrast, there was a significant difference in the sterol balance of normal and resistant rabbits fed the 0.1% cholesterol-enriched diet. On a daily basis, the normal rabbits excreted less cholesterol as fecal sterols and bile acids than was consumed, which resulted in an accumulation of 13.8 mg of dietary cholesterol/day. The resistant rabbits fed the 0.1% diet had a negative dietary cholesterol balance and excreted 21.2 mg more cholesterol/day as fecal sterols and bile acids than were consumed. Thus the total difference in cholesterol accumulation between the normal and resistant animals fed the 0.1% cholesterol-enriched diet was 35 mg/day. As demonstrated previously, when fed the 0.1% cholesterol-enriched diet, the resistant rabbits absorbed only slightly less cholesterol than normal rabbits (3), but this difference of about 10 mg/day was not sufficient to account for the net difference of 33.1 mg/day in sterol balance between cholesterol-fed normal and resistant rabbits.

The increased rate of bile acid excretion, yet similar levels of cholesterol absorption, suggested that the resistant animals had an increased rate of hepatic bile acid synthesis. The first, and putative rate-limiting step in the major pathway of bile acid synthesis is the conversion of cholesterol to 7α-hydroxycholesterol by hepatic microsomal enzyme cholesterol 7α-hydroxylase. Accordingly, our next objective was to determine whether the increase in bile acid excretion was accompanied by an increase in C7αH activity.

Matched pairs of normal and resistant animal were fed a basal or 0.25% cholesterol-enriched diet for 10–14 weeks. Liver microsomes were prepared and assayed for C7αH activity (**Fig. 3**). Due to the expected low C7αH activity in rabbit microsomes (2–10 pmol/min per mg; J. Y. L. Chiang, personal communication), we used a sensitive reverse-phase HPLC method to measure C7αH activity in the liver microsomes (8). When fed either the basal or the 0.25% cholesterol-enriched diet, the resistant rabbits had significantly higher C7αH activity than normal rabbits did ($P < 0.05$). There was no change in C7αH activity in either the normal or CRT rabbits as a result of increased dietary cholesterol intake.

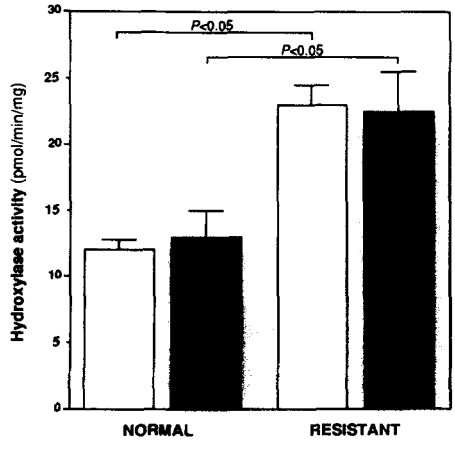

Fig. 3. C7αH activity in normal and resistant rabbit liver microsomes. Normal and resistant rabbits were fed a basal (open bars) or a 0.25% cholesterol-enriched (filled bars) diet for 10–14 weeks. Liver microsomes were prepared and assayed for C7αH activity by measuring the conversion of cholesterol to 7α-hydroxycholesterol. The hydroxylated cholesterol was derivatized to 7-hydroxy-4-cholesten-3-one by cholesterol oxidase and the mass was quantitated by HPLC. The data represent triplicate determinations for two to four animals per group (mean ± SEM). Intrasample variability was 14.7%.

Due to the likely importance of this enzyme in contributing to the expression of the resistant phenotype, we examined the structure and regulation of this enzyme. Specifically, we cloned a partial cDNA of the rabbit C7αH using the polymerase chain reaction (PCR). Oligonucleotide primers (underlined in Fig. 5), corresponding to unique regions of the rat C7αH cDNA sequence (9), were synthesized and used to amplify normal and resistant rabbit liver cDNAs (11). Primers 1 and 7 produced the expected 800 bp PCR product from both normal and resistant rabbit liver cDNAs. Primer sets consisting of oligonucleotides 5 and 1, or 6 and 1, produced the expected PCR product from the amplification of rat liver cDNAs, but no product was produced from either normal or resistant rabbit liver cDNAs.

The 800-bp products obtained from amplification of normal and resistant rabbit liver cDNAs were cloned into a bacterial host vector by standard techniques and sequenced. Independent clones were isolated from three normal and two CRT/mlo animals. The alignment of all the five clones and the positions of base mismatches between the five rabbit cDNA clones are shown in **Fig. 4.** Differences between normal and resistant PCR products (e.g., at nucleotide 841) were likely due to misincorporation of a guanine nucleotide by the Taq polymerase and thus do not represent a true sequence polymorphism.

The alignment of one rabbit C7αH clone, RAB800-55, and a portion of the rat cDNA sequence (9) are shown in **Fig. 5.** At the nucleotide level the rabbit and rat sequences are 77% identical. The predicted peptide sequences of RAB800-55 and the rat C7αH cDNA (**Fig. 6**) are also 77% identical and, based on conservative amino acid substitutions, 81% homologous. Within the 800 bp sequenced thus far, the rabbit C7αH has a nine basepair in-frame deletion relative to the rat sequence. As the functional domains of this enzyme are not well characterized, we cannot speculate as to the effect this three amino-acid deletion might have on the structure or function of the rabbit C7αH.

RAT	677 T	841 A	1026 A	1061 G	(bp)
NORMAL					
RAB800-55	T	G	A	A	
RAB800-56	T	A	A	A	
RAB800-57	C	A	A	A	
RESISTANT					
RAB800-P	T	A	G	T	
RAB800-13	T	A	A	A	

Fig. 4. Comparison of rabbit C7αH PCR clones. The PCR products from amplification of normal and resistant rabbit liver cDNAs with C7αH primers 1 and 7 were digested with the restriction enzyme BamHI and subcloned into pGEM-3Zf(+). Five independent subclones, three normal and two resistant, were sequenced by the dideoxy method (U.S. Biochemicals). The positions of the nucleotide differences among the five clones, as well as the corresponding rat cDNA sequence (9), are indicated.

Expression of the C7αH mRNA in normal and resistant animals was assessed in normal and resistant rabbits fed a basal or 0.25% cholesterol-enriched diet for 8–14 weeks (Fig. 6). As illustrated in Fig. 6A, there is one predominant transcript of 3.9 kb in both the normal and resistant rabbits. After normalization to GAPDH mRNA levels, we found that when fed either the basal or 0.25% cholesterol-enriched diets, the resistant rabbits had 7-fold higher levels of C7αH mRNA in the liver as compared to normal rabbits (Fig. 6B) ($P < 0.02$). This suggests that the increase in C7αH activity in resistant liver microsomes may be due to an increase in synthesis and not an increase in the intrinsic activity of the enzyme. Similar to the regulation of enzyme activity by dietary cholesterol, we found no evidence for the regulation of C7αH mRNA levels by increased cholesterol consumption. As diet produced no significant difference in C7αH mRNA levels in either of the rabbit phenotypes, we pooled the data from all of the animals investigated and the data are shown in Fig. 6B; overall the resistant rabbits had significantly ($P < 0.005$) higher steady-state levels of C7αH mRNA.

DISCUSSION

Little difference existed in the excretion of neutral sterols by normal and resistant rabbits. When fed the basal diet, normal and resistant rabbits excreted similar quantities of both neutral sterols and bile acids. When normal and resistant rabbits were fed a 0.1% cholesterol-enriched diet, a twofold increase in the excretion of neutral sterols was observed that was consistent with other reports (6, 13). Extraction and analysis of the mass of the individual fecal neutral sterols showed that the increased excretion by both phenotypes was due entirely to an increase in cholesterol, not coprostanol, excretion.

No difference was observed in bile acid excretion by normal and resistant rabbits fed the basal, low cholesterol diet. During consumption of the 0.1% cholesterol-enriched diet, however, the resistant animals excreted significantly greater amounts of bile acids than normal animals did. Surprisingly, bile acid excretion decreased in response to cholesterol feeding in both normal and resistant rabbits. Normal animals showed a 60% decrease in total bile acid excretion due to increase dietary cholesterol load, but the resistant animals had only a 28% decrease in total bile acid excretion. Thus, the resistant animals were refractory to the dietary cholesterol-induced decrease in bile acid excretion that was seen in the normal animals. We believe that this reduction in cholesterol ex-

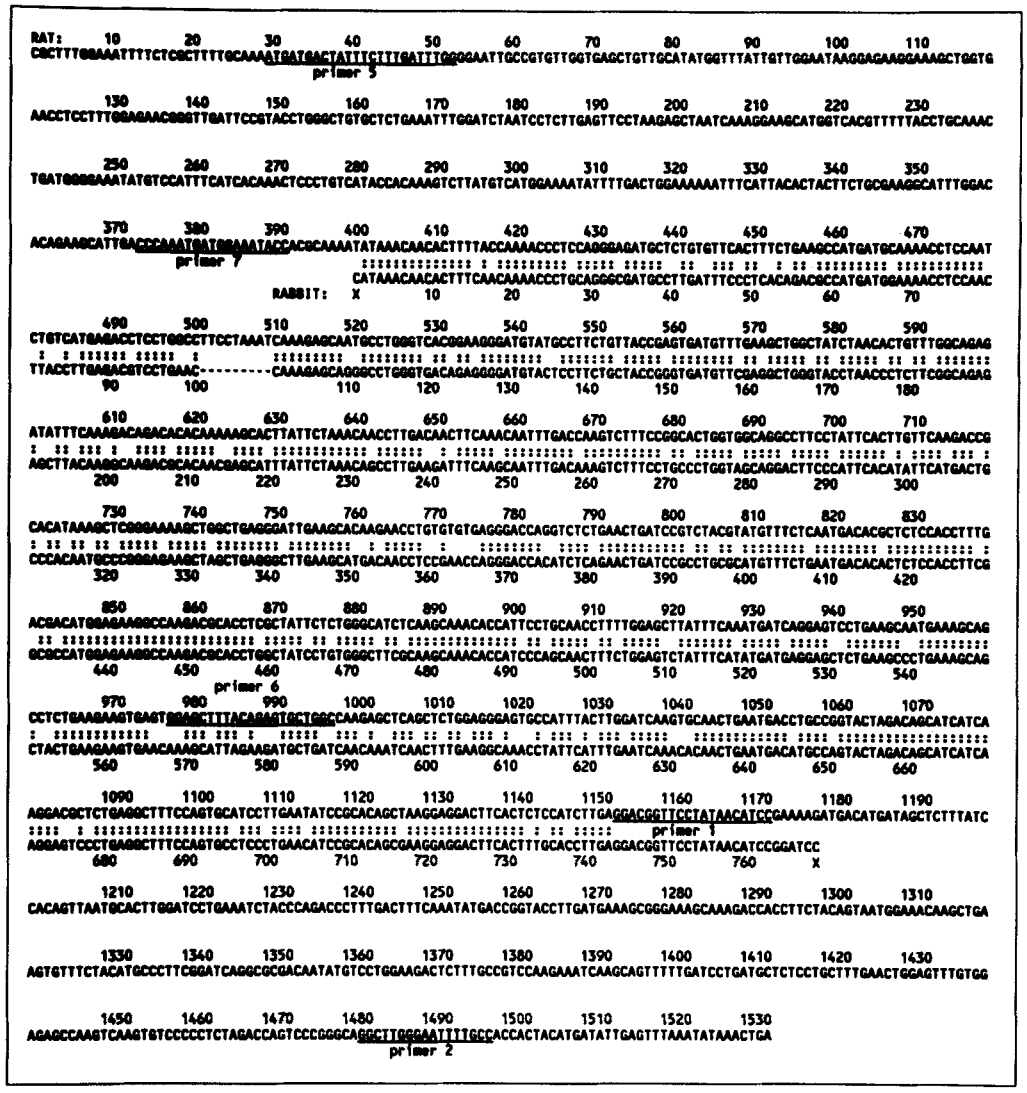

Fig. 5. Alignment of the rat C7αH cDNA sequence and a rabbit C7αH PCR clone. Nucleotide sequence alignment between a portion of the rat C7αH cDNA and one of the rabbit clones (RAB800-55) is indicated. The locations of the primers used for PCR amplification of normal and resistant rabbit liver cDNAs are indicated as single underlines.

cretion as bile acids contributes significantly to the susceptibility of the normal rabbits to hypercholesterolemia, since as more cholesterol is consumed less is excreted. This is in contrast to species such as the rat (14) and monkey (15, 16) that increase bile acid excretion when transferred from basal diets to cholesterol-enriched diets. Notably, while cholesterol feeding has resulted, in some cases, in an increase in bile acid excretion by humans, the response is variable (17–20). We have not yet determined whether there are differences in the total bile acid pool between normal and resistant rabbits or the effect that cholesterol feeding has on this parameter.

The regulation of the synthesis and excretion of bile acids in the rabbit is complex. During consumption of the basal diet, resistant rabbits excreted 34% less lithocholic acid than normal rabbits. When the animals were then fed the 0.1% cholesterol-enriched diet, lithocholic acid excretion by normal rabbits remained essentially unchanged. The resistant animals exhibited a marked increase in lithocholic acid excretion during cholesterol feeding as compared to the basal diet, attaining levels that were similar to those of cholesterol-fed normal rabbits. Deoxycholic acid excretion decreased in both phenotypes in response to dietary cholesterol but while it decreased 80% in normal rabbits, the decrease was only 50% in resistant rabbits. Therefore, under conditions of increased dietary cholesterol load, the resistant rabbits excreted threefold more deoxycholic acid. When fed the cholesterol-enriched diet, the resistant animals thus increased lithocholic acid excretion as compared to the

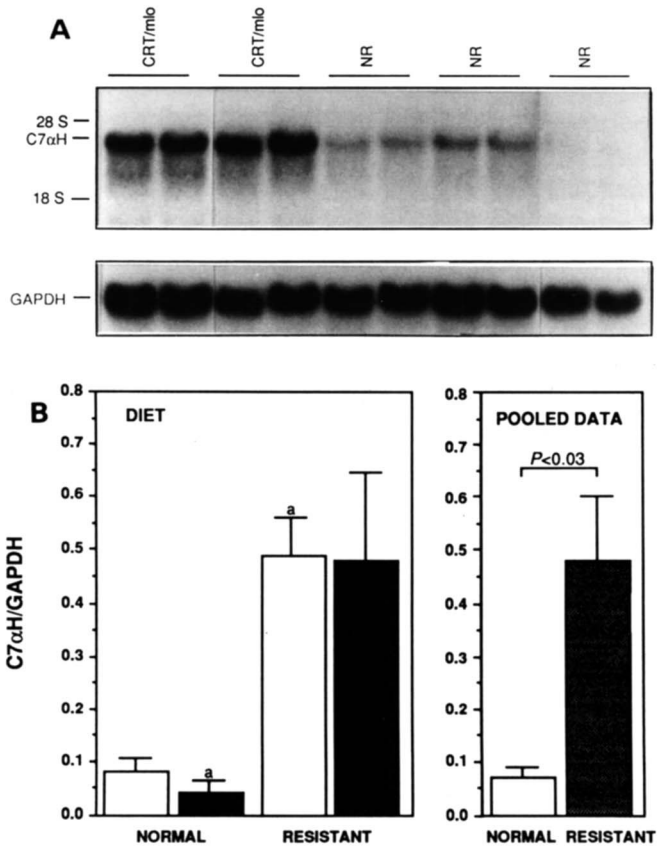

Fig. 6. A:Hepatic C7αH mRNA levels in normal and resistant rabbits. Age- and weight-matched normal and resistant rabbits were fed either the basal (open bars) or the 0.25% cholesterol-enriched diet (filled bars) for 8–14 weeks. Total cellular RNA was isolated from the livers of resistant (CRT/mlo) and normal (NR) rabbits and hybridized with probes specific for C7αH and GAPDH mRNA. The migration of 18S and 28 S rRNAS is indicated along with the specific C7αH band. B: after hybridization and autoradiography, the levels of these two mRNAs were quantitated by video densitometry. The data represent the average C7αH mRNA levels (± SEM), normalized to GAPDH, in four to six animals per group. The right panel combines all of the data (pooled) from NR and CRT animals fed either basal or cholesterol-enriched diets.

basal state, and had higher levels of deoxycholic acid excretion than cholesterol-fed normal rabbits.

The increase in fecal bile acid excretion in the resistant animals upon dietary cholesterol challenge was also reflected in the gallbladder bile. Twenty-four h following ingestion of [^{14}C]cholesterol along with the 0.1% cholesterol-enriched diet, the levels of radioactivity in the gallbladder bile of resistant animals were twofold higher than normal. Furthermore, upon necropsy, the resistant animals fed either the basal, or 0.10% or 0.25% cholesterol-enriched diets, had volumes of gallbladder bile that were about 1.5-fold greater than their respective normal counterparts (3).

Two mechanisms could account for the increase in bile acid excretion by the resistant animals: *1)* malabsorption of bile acids and/or, *2)* increased synthesis of bile acids. Reabsorption of bile acids by the intestine consists of two simultaneously operating systems: *1)* passive uptake via non-ionic, ionic, and micellar diffusion of conjugated and unconjugated bile acids and, *2)* active, Na^+-dependent saturable absorption of conjugated bile acids against a concentration gradient. Several species, including rabbits, have been shown to possess both of these processes of bile acid reabsorption (21–23). Early studies of bile acid reabsorption concluded that the terminal ileum, due to its active transport of bile acids and increased lumenal osmolarity, was the primary site of bile acid absorption. However, McClintock and Shiau (24), using in vivo rat preparations and physiologic concentrations of bile acids in the presence of a meal, have concluded that under normal dietary conditions, reabsorption of the bulk of the bile acid load occurs by passive diffusion in the jejunum, not the terminal ileum. Thus bile acid reabsorption would be dependent on the concentration of the individual bile acids, the physical form of the bile acids (free versus micellar), the time of exposure to the mucosal surface (transit time), and concentrations of the phospholipids, fats, and cholesterol which also comprise the micelles.

Several observations, in addition to the non-discriminating, diffusional uptake of bile acids, argue against a bile acid malabsorption in the resistant animals. A general absorption defect would most likely be reflected as an increased loss of cholesterol in the feces and a decrease in cholesterol absorption (25). Sterol balance studies in this and a previous report demonstrated similar levels of cholesterol absorption in normal and resistant animals fed the basal or 0.1% cholesterol-enriched diets (3). The observation that the relative fraction of cholesterol absorbed, about 75% of the dietary load, is not different in normal and resistant animals and does not change with increased dietary cholesterol load also suggests that resistant animals do not possess an absorption defect. These results are in contrast to the hyporesponsive IIIVO/JU rabbits which, along with an increase in bile acid excretion, exhibited a significant decrease in cholesterol absorption (6). In addition, decreases in bile acid absorption have been associated with increased intestinal transit time, diarrhea due to the increased osmolarity of the intestinal contents, increased risk of gallstone formation, and increased VLDL secretion (25), none of which were exhibited by the resistant animals. Finally, preliminary studies of the absorption of orally administered radiolabeled cholic acid in normal and resistant animals suggests that the increase in deoxycholic acid excretion in cholesterol-fed resistant animals is not due to malabsorption (M. L. Overturf, unpublished observation).

The increased bile acid excretion in the absence of an indication of bile acid malabsorption, suggested that bile acid synthesis was likely increased in the resistant animal. The rate-limiting step in the conversion of cholesterol to bile acids is the hydroxylation of cholesterol by C7αH. We

observed that, independent of dietary cholesterol load, liver microsomal C7αH activity was twofold higher in resistant rabbits than normal rabbits. The activity of C7αH could be increased as a result of several different mechanisms including increased enzyme protein levels, increased substrate affinity, and/or increased conversion between active and inactive forms. To begin to address these options we cloned a partial cDNA of the rabbit C7αH. We found higher levels of C7αH mRNA in the livers of resistant animals compared to normal animals during consumption of both the basal and 0.25% cholesterol-enriched diets. However, differences in bile acid excretion were only observed after consumption of the cholesterol-enriched diet. This suggests that regulation of excretion involves much more than simply the level of hepatic cholesterol C7αH expression; other metabolic steps may be rate-limiting in the rabbit. However, due to the increased levels of this mRNA, we believe that the increased C7αH activity in resistant rabbits is due to increased synthesis of C7αH protein, although this must be evaluated experimentally. Cholesterol feeding to either of the rabbit phenotypes had no effect on C7αH activity or mRNA levels in contrast to the increase that has been observed in the rat (26, 27).

Previously, we reported the characterization of several proteins involved in cellular cholesterol homeostasis in the resistant animals: the LDL receptor; HMG-CoA reductase; and acyl coenzyme A:cholesterol acyltransferase (ACAT) (4). LDL receptor binding, as assessed by the binding of ^{125}I-labeled β-VLDL, was significantly higher in resistant animals. Resistant rabbits fed the basal diet had 1.5-fold higher hepatic LDL receptor binding capacity than normal rabbits. When the dietary cholesterol load was increased to 0.25%, the difference in binding capacity increased to 2.4-fold. The resistant animals were, in each case, capable of removing more cholesterol, as β-VLDL, from the plasma compartment. Cholesterol synthesis in the resistant rabbit liver was also increased relative to the synthesis in the normal rabbit liver. After chronic consumption of the basal diet, the resistant animals exhibited a twofold higher HMG-CoA reductase activity than normal animals. During consumption of the 0.25% cholesterol-enriched diet, the difference between normal and resistant animals was striking. The resistant animals had 30-fold higher HMG-CoA reductase activity than normal animals. As indicated by the HMG-CoA reductase levels, the cholesterol-fed normal rabbits essentially halted cholesterol synthesis, while the cholesterol-fed resistant rabbits showed little change from basal HMG-CoA reductase activity levels. Measurement of ACAT activity revealed that, not only were the resistant animals binding more cholesterol as β-VLDL and synthesizing more cholesterol, they were not storing that cholesterol as cellular cholesteryl esters. That is, ACAT activity in the resistant rabbits was significantly lower than in normal rabbits during consumption of either the basal or the 0.25% cholesterol-enriched diet.

Based on our observation of increased activities of the LDL receptor and HMG-CoA reductase and the decrease in ACAT activity (4), we have constructed the following scenario for cholesterol routing in the resistant animal. We propose that the increase in the synthesis, via C7αH, and excretion of bile acids in the resistant rabbits clears cholesterol from the plasma and intracellular compartment more rapidly than in normal rabbits. This increased clearance rate, which decreases the effective concentration of intracellular cholesterol, effectively removes the negative feedback inhibition that cholesterol exerts on the expression of the LDL receptor. Accordingly the relatively up-regulated LDL receptor expression allows rapid clearance of the lipoproteins from the plasma, effectively lowering or maintaining a low plasma cholesterol level. The low intracellular cholesterol level also relieves negative feedback inhibition of HMG-CoA reductase. The synthesis of cholesterol from acetate, via HMG-CoA reductase, is increased to provide substrate cholesterol for cellular homeostasis and bile acid synthesis. In contrast, ACAT activity, which is known to be induced by increases in intracellular cholesterol levels, is decreased due to the rapid flux of cholesterol out of the intracellular compartment.

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